Agrobacterium-mediated transformation of Syrian maize with anti-stress genes

Ayman Almerei

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Agrobacterium-mediated transformation
of Syrian maize with anti-stress genes

by

Ayman Almerei

A thesis submitted to Plymouth University
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences
Faculty of Science and Engineering

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Agrobacterium-mediated transformation of Syrian maize with anti-stress genes

Ayman Almerei

Abstract

Agrobacterium is widely considered, when suitably modified, to be the most effective vector for gene transfer into plant cells. For a long time, many cereals crops (monocotyledonous plants) were recalcitrant species to genetic modification, mainly as a result of their recalcitrance to in-vitro regeneration and their resistance to Agrobacterium infection. However, recently Agrobacterium-mediated transformation has been used to transform monocot crops such as maize (Zea mays) but with severe restrictions on genotype suitability.

This study was carried out to evaluate the transformation amenability of 2 Syrian maize varieties and 2 hybrids in comparison with the hybrid line Hi II by the Agrobacterium tumefaciens-mediated transformation technique using a callus induction based system from immature zygotic embryos IZEs. A. tumefaciens strains EHA101, harbouring the standard binary vector pTF102, and the EHA105 containing the pBINPLUS/ARS:PpCBF1 vector were used.

The effects of genotypes and the size of IZEs explants on callus induction and development were investigated. Results showed that callus induction and subsequent callus growth were significantly affected by the initial explant size. Calli induction from IZEs explants sized 1.5-2.00mm was 76%. Callus weight however decreased to 8.2g, compared with 11.7g of callus derived from IZEs >2.00mm. Callus induction ranged between 73.6-78.9% for varieties and hybrids respectively. Calli derived from varieties weighed significantly more than those initiated from the hybrids.

Results demonstrated that Syrian maize genotypes were efficiently transformed via the A. tumefaciens strains but there was variation in transformation frequency. A transformation frequency of 3.7-4.2% was achieved for hybrids and varieties respectively confirming that the transformation frequency was genotype-dependent. The transformation frequency averaged between 3.2-5.6% for the EHA105 and EHA101 respectively. Fertile transgenic plants were regenerated from mature somatic embryos with an average regeneration frequency of 59.2 and 17% respectively for varieties and hybrids. Transgenic seeds of R₀ and R₁ progenies were produced from 74% of the outcrosses attempted and more than 98% of transgenic plants were normal in morphology. Fertile transgenic maize plants carrying the transferred gene CBF were produced using the Agrobacterium EHA105/PpCBF1 and these plants were shown to be more salt tolerant. Transient expression of the GUS gene was confirmed in transgenic calli, shoots, leaves, roots and floral parts of transgenic R₀ and R₁ progenies using histochemical GUS assays. The presence of the introduced bar and CBF genes in the genomic DNA of the transformants was confirmed by the PCR amplification. Further, the stable expression of the CBF and bar transgenes in the maize genome of transgenic R₁ progeny was confirmed by qRT-PCR.

The transformation protocol developed using an A. tumefaciens standard binary vector system was an effective and reproducible method to transform Syrian maize with an anti-stress gene in which fertile salt-resistant transgenic plants were routinely produced. This approach has great potential for development of Syrian maize breeding programmes for abiotic stress resistance for application in many areas in Syrian maize production.
Dedication

I would like to dedicate my work to my:

Beloved parents;

Dear wife who has shared me happiness and sadness in every stage of my life,

Beloved children, Jana, Hala, Amgad and Abdulkader who make my life full of love, and to:

My outstanding supervisory team.

Thank you for your great love and support.

Ayman Abdulkader Almerei
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Author’s declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance is acknowledged.

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Relevant scientific seminars and conferences were regularly attended and work presented either in platform presentation or poster form.

Ayman Almerei
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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA:</td>
<td>Analysis of variances</td>
</tr>
<tr>
<td>LSD:</td>
<td>Least Significant Difference</td>
</tr>
<tr>
<td>BLAST:</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CBF:</td>
<td>C-repeat binding factor</td>
</tr>
<tr>
<td>bar:</td>
<td>Phosphinothricin acetyl transferase gene</td>
</tr>
<tr>
<td>gus-int:</td>
<td>β-glucuronidase gene containing a portable intron</td>
</tr>
<tr>
<td>PpCBF1-OX:</td>
<td>over expression Prunus persica CBF vector</td>
</tr>
<tr>
<td>DREB:</td>
<td>dehydration responsive element binding</td>
</tr>
<tr>
<td>APX:</td>
<td>Ascorbate peroxidase</td>
</tr>
<tr>
<td>SOD:</td>
<td>Superoxide dismutase soluble</td>
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<tr>
<td>COR15:</td>
<td>Cold- regulated genes</td>
</tr>
<tr>
<td>cDNA:</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ddH2O:</td>
<td>Double-distilled water</td>
</tr>
<tr>
<td>MW:</td>
<td>Molecular water</td>
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<tr>
<td>dNTPs:</td>
<td>Deoxynucleoside Triphosphates</td>
</tr>
<tr>
<td>DREB:</td>
<td>Dehydration Responsive Element Binding Factor</td>
</tr>
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<td>EDTA:</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>HCl:</td>
<td>Hydrogen chloride</td>
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<tr>
<td>K3Fe(CN)6:</td>
<td>Potassium ferricyanide</td>
</tr>
<tr>
<td>K4Fe(CN)6:</td>
<td>Potassium ferrocyanide</td>
</tr>
<tr>
<td>2,4-D:</td>
<td>2,4-dichlorophenoxyacetic acid</td>
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<tr>
<td>AS:</td>
<td>Acetosyringone</td>
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</table>
Km: Kanamycin
YEP: Yeast Extract Peptone
LB: Luria-Bertani medium
MS: Murashige and Skoog
IM: Infection Medium
CCM: Co-cultivation Medium
RM: Resting Medium
SM_1: Selection Medium I
SM_II: Selection Medium II
RM_1: Regeneration Medium I
RM_II: Regeneration Medium II
IZEs: Immature zygotic embryos
Na_2HPO_4: Sodium Hydrogen Phosphate
NaCl: Sodium chloride
NaH_2PO_4: Sodium Dihydrogen Phosphate
NaOH: Sodium Hydroxide
NCBI: National Centre for Biotechnology Information
DNA: Deoxyribonucleic acid
RNA: Ribonucleic acid
PCR: Polymerase Chain Reaction
qRT-PCR: Quantitative Real-Time PCR
UV: Ultra Violet
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>kbp</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>cM</td>
<td>centimorgan</td>
</tr>
<tr>
<td>KJ</td>
<td>Kilojoules</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole butric acid</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>Ck</td>
<td>Cytokinins</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
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<tr>
<td>GAs</td>
<td>Gibberellins</td>
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Chapter 1:

General introduction and literature review
1.1 Introduction

Maize (*Zea mays* L.) is one of the most important agronomic crops in the world. It is considered a basic source of energy and protein for half the world's population (CIMMYT 2002). The kernel provides feed, food and a resource for many unique industrial and commercial products such as: starch, fiber, protein and oil. On a worldwide scale, it occupies the first place in terms of production and shifted from the third place after wheat and rice in terms of farming area during the period 2004-2006 to second place after wheat in 2013 (FAO 2013). In Syria it is in third place after wheat and barley, and the area planted with maize reached 70.86 thousand hectares in 2008. The crop yielded 281.34 thousand tonnes giving an average of 3.97 t ha\(^{-1}\) (Anonymous 2008). But, the yield dropped from 4.38 t ha\(^{-1}\) (2009) to 3.65 t ha\(^{-1}\) in 2013 according to the Food and Agriculture Organization of the United Nations (FAO) statistics (FAO 2013).

The importance of maize in Syria has increased steadily because of the demand for fodder to meet the needs of animal production which in turn has witnessed development in recent years especially in respect to poultry and cattle. Maize grains represent 70% of poultry fodder, and it has the richest energy reserves compared to other grains (1kg maize grains has 3200-3300 Calories) (Alfares and Alsaleh 1991). The Syrian demand has reached nearly 1 million tonnes of grain annually; however the local output does not meet 25% of the need. The maize area planted in Syria has increased from 5 thousand hectares in 1966 to 60 thousand hectares in 1990 until it reached nearly 71 thousand hectares in 2008 (Anonymous 2009).

The yield of maize in the Syrian region is still low (4.0 t ha\(^{-1}\)) compared with other Arab countries that are advancing in growing maize such as Egypt, 8.53 t ha\(^{-1}\), or
other advancing countries around the world such as USA 10.99 t ha\(^{-1}\) (FAO 2013). Local Syrian research efforts have been trying for more than 25 years to find varieties, lines and hybrids, which can meet the increasing need for maize. Social-economic circumstances play an important role in this matter as well as the adaptability of genotypes to the environmental circumstances in the planted areas (Anonymous 2009). Furthermore, the climate conditions experienced by the Syrian agro-climatic region especially the scarcity of water and high temperatures are major production constraints in large areas in Syria irrespective of agronomic practices during its growth. The wrong agricultural practices and the lack of agricultural awareness among farmers who needed training programmes are considered two of the most important challenges of maize breeding facing the local farmers in many areas in Syria. Farmer training programmes should be conducted by researchers and extension workers to provide local farmers necessary information needed to increase yield while conserving soil and water resources and germplasm. These constraints have stepped up local Syrian research efforts to find varieties, lines and hybrids, which can meet maize needs for the far future. Since maize adapts easily to a wide range of production environments, it is anticipated that it will fit well into the cropping system in many locations of Syria.

Most maize in Syria is cultivated as an autumn crop after the main winter cereal or sorghum crop and suffers from a high frequency of drought stress during silking, a stage which is very sensitive to water deficits. Since the region suffers from drought and due to the insufficient availability of irrigation waters, it is inevitable for plant breeders to look for genotypes of maize that can withstand drought, especially since the effect of drought is genotype dependent (Zarco-Perello et al. 2005). Drought limits agricultural production by preventing crop plants from expressing their full
genetic potential (Mitra 2001). The importance of heritable alteration made possible by new emergent plant breeding technologies to produce genotypes of yellow maize tolerating stressful conditions are crucial to this success. Genetic transformation using direct or indirect methods offers an effective means to integrate the beneficial genes from wild relatives into crop plants for production of genetically altered plants.

*Agrobacterium tumefaciens* (*A. tumefaciens*) mediated transformation is considered as an efficient and indirect method to transfer recombinant DNA into plant genomes through the interaction between bacterial and host plant cells (Ke et al. 2001; Cardoza and Stewart 2004; Bourras et al. 2015). This transformation can be highly efficient and is used to transform most cultivated plant species (Cardoza and Stewart 2004). It is a unique model system as well as a major biotechnological tool for genetic manipulation of plant cells (Tzfira et al. 2002). However “*Agrobacterium*- mediated genetic transformation of cereals has been largely confined to particular genotypes that combine the amenability to gene transfer by *Agrobacterium* with adequate regeneration potential” (Hensel et al. 2009).

Although many plant species can be transformed and regenerated using *Agrobacterium tumefaciens*, there are many important species or genotypes that remain highly recalcitrant to *Agrobacterium*-mediated transformation. Monocotyledonous crops are generally considered recalcitrant to *in-vitro* regeneration and show resistance to *Agrobacterium* infection and as a consequence have been comparatively difficult to transform by *Agrobacterium*-mediated transformation (Sahrawat et al. 2003; Trigiano and Gray 2011; Mehrotra and Goyal 2012; Cho et al. 2015). Most of these species are strategically important crops and grown increasingly in developed countries. In Syria, a high percentage of the acreage of such economically important crops including wheat, barley, cotton and
corn is susceptible to abiotic stress. Thereby, most of these varieties required to be transformed with anti-stress genes to resist the abiotic stress and to become promising crops in sustainable agricultural systems in these regions. Transgenic varieties can be generated by Agrobacterium-mediated transformation but, the T-DNA integration and regeneration of transformed plants in many instances may remain the limiting step to the genotype to be transformed. However, Agrobacterium can transiently transform a number of agronomically important species such as maize and soybean (Ke et al. 2001; Mamontova et al. 2010; Ombori et al. 2013; Vaghchhipawala and Mysore 2014; Benzle et al. 2015) and perhaps the major challenge is to transform efficiently with stable, durable traits.

There still remain many challenges facing the transformation of many crops depend on the genotype-independent transformation. This restricted genotype limitation severely limits the wide use of this technique. At the beginning of this project, it was not known whether or not Syrian maize varieties have suitable transformation ability or which genetic transformation methods are the most appropriate for local genotypes to be transformed with anti-stress genes and thereby acquire improved stress resistance. The important initial question therefore is, can Syrian maize genotypes be transformed via Agrobacterium tumefaciens-mediated transformation? If this can be overcome, then secondly, can important anti-stress genes be transferred and expressed in Syrian maize genotypes and confer a degree of improved stress resistance.
1.2 Literature review

1.2.1 Origin of maize; emergence and development

Maize has historically been an important focus of scientific research—how it originated, what its ancestors were, how different landraces are related to one another and what was the origin of this economically important food crop have attracted a lot of attention (Smith 2001; Staller et al. 2006).

Staller (2010) mentioned that Mangelsdorf and Reeves (1931, 1939) initially asserted that domesticated maize was the result of a hybridization between an unknown pre-Columbian “wild” maize, and a species of *Tripsacum*, a related genus. Alternatively, the “Tripartite Hypothesis” later supposed that *Zea mays* L. evolved from a hybridization of *Z. diploperennis* by *Tripsacum dactyloides* (Iltis et al. 1979; Eubanks 1997). Others, however, believe that maize developed from Teosinte, *Euchlaena mexicana* (Schrad), an annual crop that is possibly the closest relative to *Zea mays* as both have ten chromosomes which are homologous or partially homologous (FAO 1992).

“Maize”, the American Indian word for corn, means literally “that which sustains life” (FAO 1992). It is believed that the origin of diversity for maize is Mexico, where its closest wild relative, teosinte (*Zea mays* ssp. *parviglumis*), also grows. Its cultivation spread out of central Mexico rapidly to many areas in the context of regional trade and exchange networks, and subsequently farmers selected and adapted maize populations to thrive in new environments. One of the greatest accomplishments of plant breeding is the domestication and diversification of maize by Mesoamerica indigenous farmers (John et al. 2010). At the end of the fifteenth century, when the American continent was discovered, maize was introduced into
Europe through Spain (FAO 1992). It then spread to the Mediterranean where the warmer climates prevailed and later to northern Europe. Maize is now grown over a wide latitudinal range from 58° N in Canada to 40° S in the Southern Hemisphere. Moreover, it is harvested in regions at altitudes of more than 4,000 m in the Peruvian Andes and in other regions below sea-level in the Caspian Plain (FAO 1992). Thereby, there are genotypes of maize can be grown in most agricultural regions of the world.

1.2.2 Maize Classification

Maize is the best characterized species in the plant family Gramineae which contains about 10,000 species, including many of agronomic important crops (e.g., wheat, oats, rice, sugarcane and maize) (Arumuganathan and Earle 1991). Maize is an outcrossing and presumably ancient polyploid (2n=20), and has 10 chromosomes and the combined length of the chromosomes is 1500 cM (Gottlieb 1982). Maize is an annual tall plant with an extensive fibrous root system (FAO 1992) and is distinguished by the separation of the male reproductive organs (tassels) from the female flowers (ears) on the same plant. This helps to maintain cross-pollination. Each maize plant often produces one cob (ear) where the grains develop and each ear has about 300 to 1000 kernels, weighing between 190 and 300 g per 1000 kernels, in a variable number of rows (12 to 16) depending on the genotype (FAO 1992). Grain weight depends on genetic diversity and on environmental and cultural practices such as plant density (Sangoi 2001) and growth model (Boote et al. 1996; Mahajan and Chauhan 2011). The chemical compounds deposited or stored in the kernel vary between genotypes and the colour of kernels is often white or yellow, although there are a number of grain types which are black, red and a mixture of
colours (FAO 1992). Even the distribution of plant dry weight components may change, but typically the dry weight components of the plant consist of 1.8% dried flowers, 14.7 to 27.8% stalks, 7.4 to 15.9% leaves, 11.7 to 13% husks, and 9.7 to 11.5% cobs (FAO 1992). However, the grain makes about half of the dry weight of the plant and the other half is made up of plant residues (excluding roots) according (Barber, 1979 cited by FAO 1992).

Maize has an ability to adapt and reproduce in a wide variety of environmental circumstances (Matsuoka et al. 2002). Its great adaptability and flexibility are evidenced by the fact that today it represents the second most important food plant on earth and its current distribution is worldwide (John 2010). Moreover, there are many forms of maize, used for food, sometimes these have been classified as various subspecies related to endosperm traits and the amount of starch each has:

- Flour corn (Zea mays var. amylacea).
- Popcorn (Zea mays var. everta).
- Dent corn (Zea mays var. indentata).
- Flint corn (Zea mays var. indurate).
- Sweet corn (Zea mays var. saccharata and Zea mays var. rugosa).
- Waxy corn (Zea mays var. certain).
- Amylo maize (Zea mays ssp. amylo- saccharata).
- Pod corn (Zea mays var. tunicata).
- Striped maize (Zea mays var. japonica).

Maize is an important model organism for genetics and developmental biology (Brown 2009).
1.2.3 Environmental requirements of maize plant

1.2.3.1 Light

The amount of intercepted solar radiation, water and nitrogen supply, temperature and radiation intensity, that limit physiological processes are essential factors for the yield potential of maize (Birch et al. 2003). The production, retention and function of green leaf area are fundamental to radiation interception and capture, and thus plant growth.

On the other hand, the radiation use efficiency (RUE) affects maize grain yield through the variations in ear growth rates (EGR) at stage of post-silking, which depend on variations in RUE post-silking (Cicchino et al. 2010).

1.2.3.2 Heat

Temperature affects both growth and development processes. However, the optimum atmospheric temperature differs among growth processes. Both low and high temperatures lead to decreases in the rate of growth and development processes and final leaf size. Moreover, temperatures below 8°C (or 0°C after silking) or above 40°C usually cause a ceasing of crop development (Birch et al. 2003).

Cicchino et al. (2010) reported that heat stress (>35°C around noon) around flowering has negative effects on maize grain yield. High temperatures during pre-silking have caused (i) a larger delay in silking date than in anthesis date, (ii) an increase in male and female sterility, and (iii) a reduction in plant and ear growth rates (EGR) and leaf area index. In addition, heat stress represents a physiological determinant of grain yield through a decrease in the radiation use efficiency (RUE) around silking, and depression of harvest index (HI).
1.2.3.3 Water supply

The water requirement of any crop is very much dependent on the prevailing environment (temperature and humidity) in which it is grown. Maize requires 500-800 mm of water during its life cycle of 80 to 110 days (Critchley and Siegert 1991). As well as this, water requirement of maize at the time of tasselling is 135 mm/month (4.5 mm/day) and this requirement may increase up to 195 mm/month (6.5 mm/day) during hot windy conditions (Jamieson et al. 1995). On the other hand, over-irrigation close to tasselling and silking causes waterlogging which has damaging effects leading to reduced yield (Anonymous 2010). However, it is important for irrigated maize to have adequate water during this time and also during grain filling to achieve its full potential yield. Maize is neither drought adapted nor waterlogging tolerant, although, it is sometimes grown under rainwater harvesting (Critchley and Siegert 1991).

Rainfall limitations are expressed through seasonality and variability in both time and amount of rainfall received. It is one of the most important limiting factors for dryland maize production in areas which have winter dominant rainfall where water supply depends on rainfall stored in the soil during a fallow period and rainfall during winter. This is of particular importance to maize production, since maize has a large total water requirement and is more susceptible (sensitive) to water stress, especially during flowering and grain filling period than some crops such as beans, sorghum and soybean (Critchley and Siegert 1991). Jayasree et al. (2008) state that there is a significant positive relationship between Water Requirement Satisfaction Index (WRSI) and yield. Often the yield decreases when irrigation is ceased during the reproductive phase.
Development of high yielding maize varieties under drought conditions could be a suitable method to cope with limitations of water shortage.

1.2.4 Maize agronomy

Planting time affects crop yield principally through the effect of environmental conditions (especially temperature and day length) on canopy production and crop development processes. Itabari et al. (1993) have referred that soil water content, depth of planting and their interaction have significant effects on final germination and emergence. Furthermore, increasing depth of planting by 1 cm, or decreasing soil water content by 1% increases the thermal time required for emergence by (2.8 and 3.2) degree days- °Cd respectively.

Maize is sown in Syria for grain yield production from the middle of April to the start of May as the main crop, or in June after principal winter crops for fodder or for fresh consumption. It is grown at plant distances of 75 cm between rows and 20-30 cm between plants, at 45 to 75 thousand plants per hectare (Anonymous 2010). Sometimes, it is grown in combination with other crops such as beans (Phaseolus spp.), squash (Cucurbita spp.), and chilli peppers (Capsicum spp.).

1.2.5 Plant density

Plant density is one of the most important cultural practices determining grain yield, and other important agronomic attributes. But, there is no standard recommendation for all conditions because optimum density is dependent on environmental factors as well as on controlled factors such as soil fertility, genotype, water supply, planting date and planting pattern.
Maize is more sensitive to variations in plant density than other agronomic crops of the grass family. Maize population for economic grain yield varies from 30,000 to over 90,000 plants per hectare depending on water availability, soil fertility, maturity rating, planting date and row spacing (Itabari et al. 1993; Sangoi 2001). When the number of plants per planted area is increased beyond the optimum plant density, the grain yield is affected through the phenomenon of detrimental barrenness. Similarly, ear differentiation is delayed in relation to tassel differentiation due to high plant density. “Functional florets also extrude silks slowly, decreasing the number of fertilized spikelets due to the lack of synchrony between anthesis and silking” (Sangoi 2001). In addition, there are fewer spikelet primordia transformed into functional florets by the time of flowering leading to reductions in the ear shoots growth rate.

Liang et al. (2010) mentioned that there were significant differences in yield between different plant densities for three tested maize hybrid combinations and the optimum plant density was 63 thousand plants per hectare (6.3 plants \( m^{-2} \)).

1.2.6 Nutrition

Maize needs large amounts of nutrients, especially when it is planted for silage; it exhausts soil nutrient reserves excessively. However, it is a C4 cereal crop, which is high yielding for total dry matter.

1.2.6.1 Nitrogen

For high yielding maize crops, adequate nitrogen supply is needed as one of the three key resources needed. Typically, 22-27kg nitrogen is required per tonne of grain produced, approximately 12-16kg, of which is in the grain, and the remainder in the residue (Birch et al. 2003). Vos et al. (2005) studied the nitrogen supply effect on
leaf area production and photosynthetic capacity in maize and their results have confirmed that the canopy, and thus the radiation use efficiency were affected greatly although, leaf appearance rate and the duration of leaf expansion were unaffected. However, the nitrogen limitation has an effect on leaf N content, photosynthetic capacity, and ultimately radiation use efficiency more than leaf area expansion and solar interception. Maize cultivation after dual-purpose legumes is recommended for improving productivity, profitability, nitrogen use efficiency and soil fertility leading to sustainability of maize cropping systems (Sharma and Behera 2009). Sharma and Behera (2009) have mentioned that growth and production of maize following summer legumes was improved compared with that after fallow. Also, there was a saving of N to the extent of 37-49 kg ha\(^{-1}\) with cowpea and green gram, since the response of maize to N fertilizer rates was increased.

The relationship between yield and fertilizer levels is linear up to a point and the application of fertilization at 250 kg nitrogen and 15 kg zinc per hectare at a plant density of 99900 plants ha\(^{-1}\) has shown the best results for yield according to (Muhammad et al. 2010). These results clarified that the application of zinc accelerated tasselling and silking of maize, but the application of nitrogen had no significant effect on either.

Increasing plant density leads to reduced numbers of ears per plant and the length and girth of the ear, while nitrogen application improves them (Saha et al. 1994). The highest grain yield was recorded by Saha et al. (1994) at a density of 67,000 plants per hectare and with 60 kg N ha\(^{-1}\) applied. Christopher et al. (2009) have clarified that the tolerance of high plant densities is dependent on nitrogen application. So, receiving 165 kg nitrogen per hectare, the maize plants planted at
optimal and supra-optimal plant densities (79,000-104,000 plants ha\(^{-1}\)) respectively showed a strong N response, high N use efficiency and evident density tolerance.

1.2.6.2 Phosphorus

Soil phosphorus (P) availability commonly limits crop growth, grain yield and forage production. Furthermore, P deficiency has been shown to lead to a significant decrease in grain yield (-11%) and above-ground biomass production (-60%) for yellow maize. This was because of the reduced leaf area index, which led to decreased amount of photosynthetically active radiation (PAR) absorbed by the canopy. P deficiency affects plant growth, especially leaf growth, and photosynthesis (Plenet et al. 2000).

1.2.6.3 Potassium

Potassium (K) and iron (Fe) fertilization have positive effects on dry weight of maize, leaves, shoots and roots. Fe uptake has been increased by increasing Fe and K fertilization levels to a certain extent, but elevated K and Fe doses have also led to a decrease in the uptake of micro elements (Mn, Zn, Cu and Na) in both roots and shoots. In addition, increasing levels of K decreased the macro element concentrations of P, Mg, and Ca in both leaves and roots of maize. Furthermore, too high a concentration of K may cause competition with Fe (Çelik et al. 2010).

1.2.7 Maize uses

Most developed countries have witnessed increasing population densities which depend on the agricultural economy to sustain them. As a consequence, there has been intense focus on crops that can be intensively cultivated such as maize (John 2010). Indeed, globally, maize is extensively grown as grain for humans and fodder for livestock consumption and it has a role as one of the most important economic
staples in the world recently (FAO 2010). Its important economic role in social development is at the basis of its development from its wild progenitor *Zea mays* ssp. *parviglumis* to domesticated corn (Matsuoka *et al.* 2002).

The demand of maize crop has been continually increasing and is now a source for various products obtained from industrial processes, such as starch, protein, corn oil, glucose and food sweeteners. Correspondingly, there are studies at the molecular level which have identified the existence of various alleles responsible for characteristics such as starch production and sugar content, which are necessary to improve the manufacture of maize into flour for human consumption (Whitt *et al.* 2002; Jaenicke-Despres *et al.* 2003). Maize grain has inherently high nutritive value as it contains about 72% starch, 10% protein, 4.8% oil, 5.8% fibre, 3.0% sugar and 1.7% ash (Chaudhary 1983, from Sharar *et al.* (2003)). Other studies have demonstrated that maize grain is rich in its relative nutritional content and it was found that 100 g portion contains 1528 KJ energy, 9.4 g protein, 74 g Carbohydrates, 7.3 g fibre and 4.74g fat from Nutrient data laboratory, United States Department of Agriculture (USDA 1992).

As indicated above, maize can be used as human food, as feed for livestock and as raw material for industry. As a foodstuff, there are special varieties of maize widely used for sweet corn and popcorn which provide either mature or immature whole grains to be consumed by humans. Dent, starchy or floury and flint maize are also widely used in foods by dry milling techniques to give intermediary products, such as maize flour, maize grits of different particle size, maize meal and flaking grits. Immature cobs of corn are widely consumed either boiled or roasted in many countries.
Maize is used, even in developing countries in which maize is a staple human food, as an animal feed ingredient (FAO 2012). In developed countries more than 95% of the production is used as feed for livestock (FAO 2012). The green plants of maize are used to make silage which is frequently used as a feed in the dairy and beef industries. Although the maize plants residues, following harvest of the cobs for human consumption, are important as soil conditioners, they have also been used by small farmers in developing countries for livestock feed (FAO 1992). The plant residues such as the dried leaves, flowers and stalks are used to provide relatively good forage to feed ruminant animals owned by many small farmers in developing countries.

More recently maize is being increasing used to produce biofuels such as bioethanol (Torney et al. 2007; Hertel et al. 2010) or as a feedstock for bio-digesters producing methane to drive electric generators.

1.2.8 Transforming maize for abiotic-stress (drought resistance)

Due to long-term trends in global climate change and the expansion of maize production in drought-prone regions, the development of drought-tolerant maize varieties is of high importance, particularly for maize producers in developing nations where local plant breeding improvements are more easily adopted than high-input agronomic practices.

As well as the production under the drought stress being lower than the output under the irrigated conditions the quality of droughted maize is also of a lower level. Indeed, soil water deficits, especially when accompanied by excessively high temperatures, are probably the most common yield limiting factors in maize production. Drought severely affects the agricultural production by limiting the
genetic potential of crop plants. The integration of beneficial genes from wild relatives into the genome of crop plants by genetic engineering is an effective mean to produce genetically modified plants, in which genetic recombination events occur between the two genomes.

Since the mid-1990s the genetic engineering of cereals has provided a novel field of opportunities for faster and more directed modification or introduction of agronomically useful traits (Repellin et al. 2001). On a worldwide level, there is great interest in such heritable altered plants resistant to biotic-stresses (insects and diseases) and abiotic- stresses (drought, saltiness and frost).

Molecular techniques are used to develop strategies to identify anti-stress genes and to understand gene expression in responses to drought (Newton et al. 1991). Importantly, Newton et al. (1991) indicated that there is a great need to understand how genes that respond to drought are integrated into cells and organs. Recent advances in molecular biology provide technologies that can help researchers understand gene expression and physiological responses in both "model" plants such as Arabidopsis, and in crop plants such as maize, wheat, rice and barley.

Several genes have been identified which can increase the durability of a plant against drought and saltiness such as DREB 1B/1A/2A, CBF/DREB1, APX and SOD and these genes have been isolated from several plant species, sequenced and homologues created which are available to be inserted into desirable plants by transformation technologies. Genetic transformation can take place by use of the gene gun (Mitra 2001; Harwood and Smedley 2009), or by using Agrobacterium (Quan et al. 2004b; Wang et al. 2005; He et al. 2010), and by use of other technologies including the small interfering RNAs (siRNAs) system (Bart et al. 2006;
Liu and Zhu 2014) and genome engineering technology via the CRISPR/Cas-based gene editing (Shan et al. 2013) and gene replacement (Li et al. 2013; Schaeffer and Nakata 2015; Bortesi and Fischer 2015) to obtain altered plants which can be used in breeding programs to produce potentially high yielding, drought resistant genotypes. These technologies have very recently become the gene editing technique of choice due to advantages in efficiency in genome editing and RNA interference (RNAi) and RNA antisense which reduce gene transcript abundance and ability to cleave methylated loci (Hsu et al. 2013; Belhaj et al. 2013). There are various types of siRNAs which involve non-coding RNAs (ncRNAs) and their final product function leads to gene silencing (Jin and Zhu 2010). Castel and Martienssen (2013) have recently demonstrated the functional roles of nuclear small RNAs in abiotic and biotic stress responses and in plant development.

Production of maize varieties that can withstand or are resistant to the scarcity of available water is important to increase the yield under stress conditions. Sangoi (2001) emphasized that the mean yield is the main criterion for advancement, through the hybrid yield test programs, and the important component is the stability of yield across a range of environments. It is necessary therefore that these altered plants should be grown in different areas, and the mean yield calculated, as "Selection based on performance in multi-environment trials (MET) has increased grain yield under drought through increased yield potential and kernel set, rapid silk extension, and reduced barrenness, though at a lower rate than under optimal conditions" (Campos et al. 2004).
1.2. 9 Water deficit and drought effects on yield

Severe water deficit is considered critical especially if it occurs at a sensitive stage of plant life such as the flowering stage, not only if the plant is in the height of growth, but also if the roots are unable to secure sufficient water from great depths. Drought stress affects directly the kernel water status, and drought during grain filling decreases final kernel mass in maize. Westgate (1994) showed that drought stress, after final kernel number was established, decreased endosperm and embryo mass by 16% compared with controls. Also high water deficit after anthesis shortened the duration of grain filling and limited kernel development by causing premature desiccation of the endosperm and by limiting embryo volume. As a result, drought stress or an unfavourable water status within the embryo or endosperm may be an important determinant of kernel development.

Maize is often grown in drought-prone environments or goes through grain fill at times of maximum soil moisture deficit and, thus, drought resistance is an important trait in most agricultural systems. In order to minimize production losses, plants need to respond and adapt early and fast to excessive moisture loss in the root zone. Knowledge of root distribution is very important for characterization and modelling of water and nutrient uptake, biomass, and yield, due to the heterogeneous distribution of roots in soil layers. Since the scarcity of available water has various negative effects on plant growth, the upper soil layers, in which the water use is highest, are a focus of attention for irrigation especially as maize consumes more water from the top soil (0-45 cm) than other species such as sorghum and pearl millet (Singh and Singh 1995).
Liedgens *et al.* (2000) indicated that maximum root density did not occur at the same time as pollen shed, in contrast to maximum leaf area. In fact, maximum root density occurred 2 weeks after pollen shed, at a depth of 100 cm. Wiesler and Horst (1994) emphasized that the relationship between the vegetative yield and root length density (RLD) was positive in the 60-90 cm soil layer and RLD typically decreases exponentially with depth.

The effects of water stress on maize growth and development vary with severity and timing of stress (Çakir 2004). Pollination can be greatly affected by water stress when ovaries fail to expose their silks (stigmas) synchronously with pollen shedding, and the number of developed kernels per ear decreases leading to reduction of grain yield (Otegui 1997; Cicchino *et al.* 2010). Water deficit also causes a reduction in the rate of tissue expansion and cell division, resulting in delayed silk emergence and thereby the grain yield is affected under drought conditions (Fuad-Hassan *et al.* 2008). The ability to predict the effects of water stress on maize production is vital to improve risk assessment in water-limited conditions.

1.2.10 Genetic diversity and drought resistance

Introducing maize germplasm into a particular environment or agricultural system can increase existing genetic diversity, but it can cause phenological and morphological changes as a consequence of its inability to adapt agronomically and this may be undesirable.

The utilization of existing genetic variation and the ability of plant breeders to identify and manipulate important genes will open new avenues for development of highly drought resistant plants of yellow maize adapted to local conditions. This will provide the main basis for the development of the next generation of maize and of
new products to meet future needs (Balconi et al. 2007). Mitra (2001) described that the main constraints for genetic improvement of drought resistance are the lack of a multidisciplinary approach and precise screening techniques, incomplete knowledge about the genetic basis of drought resistance, also the difficulty of obtaining available appropriate genes required for genetic transformation, and a negative correlation of drought resistance with productivity traits. So, both definition of genes responsible for drought tolerance and the development of high efficiency genetic transformation methods are important.

There are three main strategies contributing to drought resistance, namely drought escape, drought avoidance and drought tolerance (Mitra 2001). However, breeding for drought tolerance is further complicated because crop plants are simultaneously exposed to several types of abiotic stress, such as, high temperature, high radiation and nutrient toxicities or deficiencies (Fleury et al. 2010). Furthermore, drought tolerance is a quantitative trait, with a complex phenotype (Fleury et al. 2010).

Genetic modification of plants to allow growth and yield under unfavourable conditions which severely limit plant growth and crop productivity, is an important component to solve problems of environmental stress such as drought, salinity and low temperature (Zhang et al. 1999). Haake et al. (2002) reported that the plant’s genetic resistance to cold and drought has evolved mostly from the overexpression of CBF4 which is up-regulated by drought stress. CBF is an abiotic stress transcription factor and primarily responsible for switching on adaptation genes which acclimate the plant to the stress. However, transgenic CBF/DREB1 Arabidopsis plants have also shown more tolerance to drought and freezing stress.
through gene duplication and promoter evolution (Haake et al. 2002). Enhanced resistance of transgenic wheat plants to drought stress has been achieved through integration of the betA gene, encoding glycine betaine GB, into common wheat (Triticum aestivum L.) via Agrobacterium-mediated transformation, and the transgenic wheat lines exhibited greater root length and better growth compared with the wild-type (WT) (He et al. 2011).

1.2.11 Gene transfer and genetic transformation

A major goal of genetic modification is to generate new stress-tolerant plant varieties that are not only suitable for but also desired by the local farmer and consumer communities.

Transgenesis is an important adjunct to classical plant breeding. It allows the production of specific characters by targeted manipulation using genes of interest from a range of sources. Over the past centuries, improvement of cereals was achieved mostly by conventional selective breeding techniques. However, due to the ever-growing world population, limited availability of water and the changing climatic conditions, new technologies, such as transformation and the production of genetically modified plants, are urgently required to cope with future challenges.

In recent years, genes from different sources have become available to be introduced asexually into plants through the introduction of new tools of biotechnology such as using of Agrobacterium tumefaciens mediated transformation (Jenesl et al. 2012). The results of Agrobacterium-mediated transformation of cereals recently confirmed that this technique is a reliable and a repeatable method for monocotyledonous plants, especially cereals. Agrobacterium tumefaciens is also a very useful vector to transfer foreign genes into dicotyledonous species (Nadolska-
Orczyk et al. 2000; Karthikeyan et al. 2012). Transformation of foreign genes into explants, for example, immature embryos (Shrawat and Lörz 2006), embryogenic pollen cultures (Kumlehn et al. 2006), in vitro cultured ovules (Holme et al. 2006, 2012) and somatic embryos (Lenis-Manzano et al. 2010) have proven useful in cereals which are not naturally susceptible to Agrobacterium infection.

Hiei et al. (1994) reported that efficient transformation of rice mediated by Agrobacterium tumefaciens to efficiently produce transgenic plants requires the choice of starting materials, tissue culture conditions, bacterial strains and vectors to efficiently ensure gene transfer. Generally, successful transformation requires:

1. Identification and isolation of the target gene which could provide to the target plant a desirable trait.

2. A plant cell source as a host to the recombinant DNA fragment. And these cells must have ability for differentiation and division be able to regenerate to produce a new integral plant.

3. A mediator for introducing the DNA fragment into the target cell.

4. Selection of the modified cells and confirmation of expression of the transgene with physiological effectiveness.
1.2.12 Global production of Genetically Modified Organisms (GMO)

Agricultural biotechnology, especially the generation of transgenic crop plants, is currently one of the most controversial and emotive agrotechnology issues. The general public are presently mostly against the introduction of this technology on a commercial scale especially in Europe. This is principally due to perceived food safety issues, the potential environmental impact of these crops and concerns that a few agricultural biotechnology companies may establish unparalleled control of the global seed supply. Therefore, the socio-economic dimension of improved maize production and the analysis of factors influencing the acceptance of novel altered varieties will be of equal or more importance than the scientific and technological optimisation.

When looking at the status of approved transgenic lines grown worldwide, it cannot be denied that more and more transgenic plants are entering agricultural systems and are subsequently being used in food and animal feed. There has been a year on year increasing interest in the cultivation of genetically modified (GM) crops (Figure 1.1). GM crops have been cultivated on a commercial scale since 1996 (1.7 million hectares) and the planted area in 2012 was 170.3 million hectares. During the seventeen-year period from 1996 to 2012, there was a steady and continual growth resulting in about 100-fold increase of the global GM crops growth rate (International Service for the Acquisition of Agri-Biotech Applications (ISAAA 2012). James (2012) reported that 17.3 million farmers, in 28 countries, planted 170.3 million hectares of the GM crops in 2012 with an unprecedented sustained increase of 6% or 10.3 million hectares over 2011 (Figure 1.1), and forecasts are that this will continue to increase (Figure 1.2).
Genetically modified maize was the second most widely grown biotech crop in 2008, after soybean, occupying 37.3 million hectares or 30% of global biotech area (James 2008). Area harvested of biotech maize increased from 51 million hectares in 2011 to 55.1 million hectares, with an increase of 8%, in 2012 (ISAAA 2013). Modified maize crops remained the second most dominant biotech crop after soybean in 2012 (39.9 million hectares) with an increase up to 2.6 million hectares of biotech maize planted in 2011. The biotech maize crop area planted in 2012 was occupied 23% of the global biotech area (ISAAA 2013). Moreover, the percentage of areas planted with biotech maize of the total area of maize increased in the last few years. 35% (55.1 million hectares) of the global hectares (159 million hectares) of maize crop grown in 17 countries in 2012 were biotech maize; this compares with 32% (51 million hectares) grown in 16 biotech crop countries worldwide in 2011 (FAO 2009). There were 17 countries around the world which grew biotech maize in 2012. The biotech maize grown within EU countries also increased in 2012 compared with 2011 (ISAAA 2013). The adoption of biotech maize growing benefits farmers economically and farmers growing biotech maize enjoyed increases in their income during the period 1996 to 2011, and this increase was US$30 billion and US$8.6 billion for 2011 alone (Brookes and Barfoot 2013). The expansion of biotech maize to date has been based on just three traits, DT drought-tolerant, Bt insect resistance to the corn borer and herbicide resistance (glufosinate and glyphosate). By 2015, the yield in the dry regions of the USA was expected to be increased by using the biotech drought-tolerant maize according to annual report of International Service for the Acquisition of Agri-Biotech Applications (ISAAA 2012).

The first biotech drought tolerant hybrids of maize were commercialized in 2013 in the USA and estimated 50,000 hectares and this planted area increased over 5
fold to 275,000 hectares in 2014 reflecting high acceptance by US farmers of the drought-tolerant maize technology (ISAAA 2014). The first tropical biotech drought tolerant maize and stacked biotech insect resistant/drought tolerant (Bt/DT) maize hybrids are expected to be deployed in sub-Saharan Africa. The Water Efficient Maize for Africa (WEMA) project is expected to deliver the first biotech drought tolerant maize to South Africa as early as 2017 (ISAAA 2014).

Figure 1.1: Increase of the worldwide genetically modified plants cultivation areas, in millions of hectares, from 1996 to 2014 (adapted from James 2014). Trait hectares: areas, in million hectares, where GM crops with two or three “stacked traits” (containing two or three genes in a single GM crop) are cultivated. Total hectares: the total area (in million hectares) of cultivated GM crops in industrial countries (blue squares) and developing countries (red rectangles).
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Figure 1. 2: Global map of countries growing GM plant crops and biotech crops mega-countries in 2014. adapted from James (2014). Has: hectares.

*Biotech mega-countries growing 50,000 hectares, or more, of biotech crops.

There are 19 biotech mega-countries such as USA, Brazil, Argentina, India, Canada and China which approved and planted biotech crops in 2014 such as Maize, soybean, cotton, sugar beet, alfalfa, canola, squash, papaya and tomato (ISAAA 2014).
1.2.13 Transformation Systems

The established genetic transformation methods often used by genetic engineers are many and varied, but generally fall under one of three categories: the plasmid method, the vector method, and the biolistic method.

Some of these methods are direct methods which do not need to use a mediator vector to introduce the transferred DNA fragment and are Biolistic or Microprojectile bombardment (Nadolska-Orczyk et al. 2000; Harwood and Smedley 2009), Electroporation of protoplasts (Nadolska-Orczyk et al. 2000) and use of Polyethylene glycol (PEG). *Agrobacterium tumefaciens*-mediated transformation is considered as a method of indirectly introducing the recombinant DNA to the plant genome during the interaction between bacterial and host plant cells. The use of *A. tumefaciens* to create transgenic plants has become routine for many dicotyledonous (dicots) as well as for some monocots (Zhu et al. 2000; Coutu et al. 2007; Hensel et al. 2009). Today, *Agrobacterium* is still the tool of choice for plant genetic engineering with an ever expanding host range that includes many commercially important crops.

The biolistic method, also known as the gene-gun method, is a technique that is most commonly used in engineering monocot plants. This technique relies on the acceleration of micro-sized pellets of metal (usually tungsten or gold particles) coated with the desirable DNA into plant cells as a method of directly introducing the DNA (Harwood and Smedley 2009). Those cells that take up the DNA (confirmed with a marker gene) are then allowed to grow into new plants, and may also be cloned to produce genetically identical clones (Scandalios 2007).
Drought-resistant transgenic rice plants have been transformed with the barley *hval* gene, which is responsible for late embryogenesis abundant (LEA) proteins, through a biolistic approach (Mitra 2001).

1.2. 14 *Agrobacterium* mediated plant transformation

*Agrobacterium tumefaciens* is classified as a Gram-negative bacteria and is a soil-borne, rod-shaped bacterium which is commonly associated with the roots of plants (Kado and Hooykaas 1991). These bacteria parasitize on many types of plants causing a neoplastic tumour disease known as Crown gall (Figure 1.3) which is an economically important disease (Kado and Hooykaas 1991; Kelly and Kado 2002).

Nester *et al.* (1984) showed that the tumour is based on the transfer of a DNA fragment from the bacterial Ti (for tumour-inducing) plasmid, called "transferred DNA", or T-DNA, to the host plant cell chromosomes, and it is integrated into the plant cell genome (Kelly and Kado 2002) where it expresses onco-genes contained on the T-DNA (Scandalios 2007). Later studies revealed that the T-DNA is well defined within the Ti plasmid by the presence of two 25 bp flanking borders as direct repeat, characterized by a high homology in their sequences and referred to as the left and right T-DNA borders (Yadav *et al.* 1982). These findings were the starting point of plant genome engineering. Indeed, any foreign DNA placed between the T-DNA borders can be transferred to plant cells and the first vector systems for plant transformation were constructed on this early model (Hamilton 1997). The Ti plasmid also carries the transfer (tra), the opine catabolism and the virulence (vir) genes (Zhu *et al.* 2000) which are also important in the transformation process.
Figure 1.3: Schematic diagram for mechanism of T-DNA transfer from *Agrobacterium* to plant cell causing crown gall disease, adapted from Kado (2002).

The border repeats that delimit the T-DNA, the Virulence genes (*vir*) that code for the *in trans*-acting type IV secretion system, and various bacterial chromosomal genes are three necessary genetic elements for the transfer and the integration of the T-DNA in the plant genome (Lee and Gelvin 2008).

*Agrobacterium*-mediated plant transformation frequency is influenced by several bacterial and plant and environmental factors (Tzfira *et al.* 2002). At the plant level, it has been reported that the type of plant tissue used was the critical factor of successful *Agrobacterium*-mediated transformation of cereals (Nadolska-Orczyk *et al.* 2000). On the bacterial side, the density of the bacterial culture (Cheng *et al.* 2004; Opabode 2006) and the strain ability to attach and transfer its T-DNA to the host cells (Cheng *et al.* 2004) were described to influence the transformation
frequency. Different Agrobacterium strains are commonly reported to influence the transformation frequency depending on the transformed plant or crop.

1.2. 15 Agrobacterium tumefaciens-mediated gene transfer to cereal crop plants

Agrobacterium tumefaciens is used to transform most plants with high efficiency (Cardoza and Stewart 2004). Agrobacterium-mediated transformation system is the most widely applied method for genetic transformation of many plants such as cabbage (Brassica oleracea subsp. capitata) (Cardoza and Stewart 2004). However, for a long time, most of the important cereals crops (as monocotyledonous plants) were difficult to genetically engineer, mainly as a result of their recalcitrance to in vitro regeneration and their resistance to Agrobacterium infection (Ke et al. 2001; Repellin et al. 2001; Sahrawat et al. 2003).

The efficiency of A. tumefaciens in infection of maize was first indicated in the studies of “agro-infection” by Grimsley (1987), in which cDNA of maize streak virus was delivered to maize plants by A. tumefaciens and the plants became systemically infected. Ishida and colleagues (1996) were then the first to publish a protocol for the generation of transgenic maize, which also relied on A. tumefaciens that carried "super-binary" vectors. In the following years, similar protocols for all major cereal crops including barley (Tingay et al. 1997) and wheat (Cheng et al. 1997) were published. These breakthroughs were based on the final empirical optimisation of protocols often using cereal reproductive organs as the explants for transformation.

Transformation of maize mediated by Agrobacterium, as a high efficiency method of gene delivery, has many advantages, such as the transfer of relatively large segments of DNA with little rearrangement and integration of low numbers of
gene copies into plant chromosomes (Gelvin 2003). Ishida et al. (1996) succeeded in integration of one to three copies of the transgenes into host plant chromosomes with little rearrangement through transformants of maize using immature embryos co-cultivated with *Agrobacterium tumefaciens* that carried "super-binary" vectors. Accordingly, the availability of an effective binary vector system is now considered a precondition in maize *Agrobacterium*-mediated transformation techniques. So, a binary vector, which is small in size, containing suitable bacterial and plant selectable marker genes with one of the most widely used reporter genes is the most applicable starting material that offers a high copy number in *Escherichia coli* and *Agrobacterium* (Slater et al. 2003).

Optimization of the *in vitro* system for maize involved the regeneration from complete shoot meristems (3-4 mm) explants. Also transgenic plants were produced through callus induction in five days of incubation on an auxin-modified Murashige and Skoog (MS) medium, by using super-virulent strains of *Agrobacterium* (Sairam et al. 2003). Nadolska-Orczyk and Przetakiewicz (2000) have reported that the most important advantages of Agro-based system include “relatively high transformation efficiency, integration of defined piece of DNA (transgene) frequently as a single copy, Mendelian transmission to the next generation, simple transformation procedure and lower cost of equipment than biolistic methods.

In most of the published material on the development of the maize transformation protocols, a single maize hybrid is used; Hi II and many attempts to transform other germplasms are known to fail to produce any transformants. This can be an extreme limitation to the lateral application of transformation technologies between laboratories and in breeding programmes.
1.2. 16 Factors influencing *Agrobacterium*-mediated transformation- an efficient protocol

Identification and optimisation of the factors affecting T-DNA delivery and plant regeneration is necessary to develop an efficient *Agrobacterium*-mediated transformation protocol for a recalcitrant species like maize, barley and wheat. Research has shown that embryo size, duration of pre-culture, inoculation and co-cultivation, and the presence of acetylsyringone and Silwet-L77 in the media are significant factors that influence the successful *Agrobacterium*-mediated genetic transformation of wheat and barley (Wu et al. 2003; Shrawat et al. 2007). On the other hand, Nadolska-Orczyk and Przetakiewicz (2000) have stated that the crucial factors of successful *Agrobacterium*-mediated transformation of cereals are: “type of plant tissue used, *A. tumefaciens* strains and plasmids (vectors), activation of bacterial virulence system and promoter, reporter as well as selectable genes applied for transgene construction”.

The establishment of a highly efficient genetic transformation system for maize mediated by *A. tumefaciens* includes the pre-treatment of explants with centrifugation (Hiei et al. 2006), osmotic treatment of explants (Jin et al. 2000), inoculation conditions such as bacterial concentration (Zhao et al. 2002) and immersion time (Xing et al. 2007), co-cultivation condition such as period of co-cultivation (Vasudevan et al. 2007), and temperature conditions (Salas et al. 2001). An essential requirement is a competent regeneration system after co-cultivation of explants (Lawrence and Koundal 2001). Also, Wei, (2009) results showed that the genotype had a highly significant impact on both induction of embryogenic callus and callus differentiation and the concentration of 6-BA, AgNO3, ABA, 2,4-D, and the
medium are the significant factors for induction of embryonic callus with the concentration of 6-BA having a strong effect on callus differentiation.

Molecular 'markers' are used to position important genes, which are responsible for stress resistance, on a donor plant chromosome and to ensure that they can be 'crossed in' to another plant chromosome. There are several ways of obtaining molecular markers like using the expressed sequence-tags (ESTs) which help establish partial sequences of messenger RNA's extracted from tissues of interest (e. g. beans (Phaseolus spp.) developing pods) (Broughton et al. 2003).

Through monitoring beta-glucuronidase (GUS) expression in a transient transfer assay, high-efficiency of gene transfer to recalcitrant plants (rice and soybean) by the double mutant of Agrobacterium tumefaciens was observed (Ke et al. 2001). Also, Shen et al. (1993) observed expression of the GUS gene delivered with high efficiency to maize shoots by A. tumefaciens. It has been emphasised through polymerase chain reaction (PCR) analysis of total isolated DNA that integration of T-DNA carrying the marker gene nptII in the genomes of diploid and haploid maize plants could be achieved by the treatment of pistil filaments with a suspension of Agrobacterium during artificial pollination (Mamontova et al. 2010).

1.2. 17 Abiotic stress

Most crops growing in dry areas are often exposed to various environmental abiotic stresses. Abiotic stresses include water deficits, waterlogging, high temperatures, cold and freezing, deficiency of essential nutrients from the soil, nutrient imbalances, salinity (the accumulation of toxic ions during salt stress), restriction of photosynthesis and restriction of root growth caused by increased hardness of the soil, high light intensity, UV-radiation, metal toxicities and climate
change (Verslues et al. 2006; Roy et al. 2011a). These stress conditions cause extensive losses to crop production worldwide (Mittler and Blumwald 2010). The most important factors causing loss of production during the life cycle are drought, salinity, extreme temperatures (heat and freezing) and mineral deficiency or toxicity stress (Carena 2009; Wani et al. 2013). Several distinct abiotic stresses may occur in the field concurrently, or a plant may experience these abiotic stresses at different times through the growing season (Tester and Bacic 2005).

Global climate change with its complex implications to the field environment is an undefined challenge facing modern agriculture (Mittler and Blumwald 2010). The fast changes in global climate with a global scarcity of water resources and the increased salinization of soil are leading to an increase in aridity for the semiarid regions and will increasingly affect crop growth and crop yield potential in these areas. Water deficit is identified to be the most common abiotic stress in limiting the crop growth worldwide (Araus et al. 2002), and leads to increasing constraints on realizing the potential yield of crops (Passioura 2007), but salinity is increasingly a major limiting factor in plant growth and crop productivity (Munns and Tester 2008). Furthermore, drought and salinity frequently occur together in the field situation (Morison et al. 2008; Cramer 2010). Drought and salinity stresses are already widespread in many regions around the world, and recently became a serious problem in many regions of maize agriculture in Syria.

Severe abiotic stresses need to be overcome by the implementation of biotechnologies and employing physiological, biochemical, molecular and breeding tools aimed to develop plant tolerance to abiotic stresses (Vinocur and Altman 2005; Roy et al. 2011a) with due consideration to the genetic background and physiology of different germplasms (Mittler and Blumwald 2010). Development of transgenic
plant production using plant genetic engineering techniques is still considered an effective technology to improve abiotic stress tolerance (Roy et al. 2011a) and there is great interest in developing varieties of Syrian maize that are abiotic stress-resistant in order to help increase yield. It is based on this recognition that a scholarship was awarded to the author to develop the skills necessary to transfer this technology to Syria’s maize breeding programme.

1.2. 18 The effect of abiotic stress on plants, crop production

Plant growth and yield are all limited by abiotic stress (Araus et al. 2002). The abiotic stresses could be defined in plants as any external abiotic (salinity, water deficit or drought and heat) constraint that reduce the plant’s ability to convert sunlight energy to biomass by photosynthesis. Environmental conditions that reduce plant growth and yield below optimum levels are considered as abiotic stresses. However, the concept of stress is complex and confusing, so it is useful to define the stress as an external factor that leads to changes in growth conditions through its unfavourable influences on the plant (de Oliveira et al. 2013). In some cases of stresses, plants trend to achieve a new state of homeostasis that requires an adjustment of metabolic pathways by the acclimation process (Mittler 2006). The plant acclimation to an unfavourable condition is associated with the plants tolerance or a plant resistance to stress. The plant’s ability to respond to abiotic stress (stress tolerance or stress resistance) is affected by the inherent physical, morphological and molecular limitations (Cramer et al. 2011). Moreover, the response of plants to a combination of different stresses (such as a combination of drought and salinity, drought and heat, salinity and heat or any of the major abiotic stresses combined with biotic stresses) is complex and largely controlled by different, and sometimes
opposing, signalling pathways that are integrated and impact negatively on plant growth and physiological traits (Suzuki et al. 2014).

Bioprocesses such as the synthesis, metabolism, concentration, transport and storage of sugars are directly or indirectly affected by abiotic stresses. Soil environment factors, water deficiency, salinity, sodicity, structure, temperature, pH, nutrients, and mineral toxicities can all interact to limit plant growth. Abiotic stress like drought and salinity affects the regular metabolism of plant cells and leads to the production of toxic molecules and reduced photosystem II (PSII) activity (Chaitanya et al. 2003). Moreover, it has been reported that the reduction in the growth of cell, root and shoot was combined with the decrease in the water potential that caused inhibition of cell expansion and reduction in cell wall synthesis.

Abiotic stresses impose injury on cellular physiology and result in growth inhibition by metabolic dysfunction via similar pathways of damage (Mahajan and Tuteja 2005). For example, salinity and drought stresses have similar effects on plant growth by a same pathway or related pathways to inhibit the growth, as the ability of plants to take up water under salt stress was reduced, and that quickly cause reduction in growth rate induced metabolic changes identical to those caused by drought stress (Munns 2002). However, salinity stress has a specific impact on plant growth, when sodium is accumulated to toxic levels especially in older transpiring leaves, which causes salt-specific effects including the reduction of photosynthetic activity of the plant causing premature senescence (Munns 2002).
1.2. 19 Salt stress damages on plant growth and development

The accumulation of salt in soil layers and high water tables caused by surface irrigation carrying salt to the soil surface leads to a sharp decrease in plant fertility. Due to accumulation of salts in soils in arid and semi-arid regions of the world, agricultural productivity is often very low (Ashraf and Sarwar 2002; Munns 2002). Salinity in the soil or water causes a stress condition to crop plants, and it is of increasing importance to crop agriculture in the dry areas. Maize, a summer crop requires a large amount of irrigation water and secondary salinization is one of the most serious production constraints and is becoming increasingly limiting for crop production (Akbar and Ponnampерuma 1982; Witcombe et al. 2008; Tester and Langridge 2010).

The negative effects of salt on plant growth is associated with the osmotic stress component caused by drought and decreases in soil water potential that lead restriction of water uptake by roots (de Oliveira et al. 2013). Osmotic stress is caused by the high salt concentrations and ions in the cytosol affect the whole plant because ions move to the plant shoot through the transpiration stream (Munns and Tester 2008). Osmotic stress caused by salinity treatment damaged the epidermal cell wall of maize plant leading to loose β-expansin proteins of cell walls and reducing the growth of maize leaves (Zörb et al. 2014).

Salinity stress affects both vegetative and reproductive plant development. The negative implications of salinity stress varies depending on the harvested organ of plant (leaf, shoot and root biomass) (Wang et al. 2013). In general, shoot growth can be affected by salinity stress more than root growth, and salinity can reduce flowering and increase sterility. Many researchers have reported that the salinity of
irrigation water is one of the main factors to have a growth limiting effect in large parts of semiarid and arid areas.

The decrease in the water potential that occurs in both drought and salinity stress affects the regular metabolism of the cell such as the carbon-reduction cycle, light reactions, energy charge and results in reduction of photosynthetic rate, cell growth, root growth and shoot growth (Chaitanya et al. 2003). Salinity stress can affect the plant growth in similar aspects to water stress (Munns 2002), except for the additional effect of ion cytotoxicity, which caused by high salt concentrations in soil (Taiz 2010). Evelin et al. (2009) reported that the effects of salt stress on plant growth are associated with low osmotic potential of the plant, imbalance of nutrition in plants caused by an increase in the Na+/K+ ratio and excessive toxicity due to Cl- and/or Na+ ions in the cell, disruption of cell organelles and their metabolism, or a combination of these factors leading to a decrease in the plant yield (Ashraf and Harris 2004; Díaz-López et al. 2012). Salt-stressed plants are affected by toxic damage resulting from the accumulation of high salt levels in a plant that lead to nutritional disequilibrium. In summary, salinity affects plant growth as a result of both osmotic and ionic effects. Azevedo Neto et al. (2004) evaluated the NaCl differential tolerance of different maize genotypes and reported that the accumulation of Na+ and soluble organic solutes in roots due to salinity appeared to play an important role in the acclimation of maize genotypes to salinity stresses.

1.2. 20 Tolerance/resistance to abiotic stress

The plant response to abiotic stress, involving many genes actions and biochemical-molecular mechanisms, is highly complex and involves adaptive changes and/or detrimental effects. Plant responses involve changes at
transcription, cellular, and physiological levels. In addition, the sensitivity of crop plants to a particular abiotic stress varies depending on their developmental stage (Flowers and Yeo 1981; Lutts et al. 1995). The stress tolerance mechanisms by which crops maintain yield under abiotic stress are controlled by a variety of genes, which are expressed at different times during stress stages (Witcombe et al. 2008; Fleury et al. 2010). There are more than 30 thousand genes in a typical plant cell which encode many numbers of proteins that can response to abiotic stresses through more than 200 known post-translational modifications (PTMs) in a very complex molecular pathway (Cramer et al. 2011). Many plants can be acclimated to the particular stress by using a variety of mechanisms and combinations of mechanisms (Reynolds and Tuberosa 2008) and there is a range of traits that combine to contribute to a plant’s tolerance of abiotic stresses through plant adaptation (Roy et al. 2011b).

The plant responses to stress can be tissue or cell specific in the organ affected by the stress and can be different depending on the stress involved (Dinneny et al. 2008). The salt-tolerant maize hybrid, which maintained leaf growth under salinity stress, responded to stress by modification the cell wall to be more extensible under salt stress. But the epidermal cell walls of a sensitive hybrid of maize that displayed a clear reduction of leaf growth were stiffer under stress (Zörb et al. 2014). Plant response to salinity stress was associated with an alteration of the epidermal apoplastic pH to be more suitable for growth in acid solutions.

Plants response to abiotic stress to develop tolerance or resistance involves the use of transcriptional factors to activate specific operons or genes whose upregulation lead to re-establishment of cellular homeostasis and functional and structural protection of proteins and membranes (Roy et al. 2011a). Moreover, some
genes expressed during abiotic stress promote or enhance cellular tolerance to stress through encoding proteins that enhance the protective functions in the cytoplasm, cell membrane or control ion accumulation and further regulation of other gene(s). In response to both salinity and cold stresses, specific genes of calcium-signalling and nucleic acid pathways were up-regulated with the increase of gene expression (Mahajan and Tuteja 2005).

Whereas herbicide-tolerant plant or insect resistance requires the insertion of single gene that have highly specific mechanisms with clearly defined roles, tolerance to abiotic stresses can be achieved by the interaction of numerous genes and regulatory pathways (Howles and Smith 2012). “Breeding for resistance/tolerance to abiotic stresses is generally more challenging than most other stresses due to their complex, inconsistent and elusive nature” (Carena 2009). There are two loci encoding transcription factors associated with abiotic stress in cereals: the Frost Resistance (FR1) locus containing VERNALIZATION1 (VRN1), and the (FR2) locus containing CBF (C-Repeat Binding Factor)/DREB (Dehydration Response Element Binding) (CBF/DREB) genes (Stockinger et al. 2007; Dhillon et al. 2010; Knox et al. 2010; Zhu et al. 2014). Specific genetic loci exist within the germplasm of crops which encode transcription factors and are often associated with distinct regulation or function, duplication and/ or neo-functionalization of genes such as CBF and VRN1 that maintain plant homeostasis. There is a variety of genes that are involved in tolerance to abiotic stress and interactions and crosstalk with many molecular pathways contribute to the plant molecular responses to abiotic stresses (Takahashi et al. 2004; Mahajan and Tuteja 2005). Specific genes encode proteins with known enzyme activities that are involved in stress tolerance however, FR2 defines a region containing numerous duplicated CBF genes (Skinner et al. 2005; Badawi et al. 2007;
Knox et al. 2010) that regulate cold acclimation and freezing tolerance in annual or perennial monocots and dicots (Thomashow 2010; Miura and Furumoto 2013). Transcriptomic studies have indicated that overexpression of a peach (Prunus persica) CBF gene (PpCBF1) in transgenic plants under field conditions and improved cold hardiness but also reduced growth rates and short-day induced dormancy of non-acclimated plants of ‘M.26’ apple (Malus × domestica) under low temperatures (Artlip et al. 2014). In a similar study, Hinchee et al. (2011) have reported that a CBF2 gene as a transcription factor enhanced winter growth of Eucalyptus grandis × E. urophylla under low temperatures. It appears that overexpression of CBF in plants has a significant role on the plant response to environmental stresses as it has significant long-term effects on development of transgenic plant physiology and increases freezing tolerance (Wisniewski 2014). Similarly it has been reported that DREB increased tolerance of cold, drought and salinity (Kasuga et al. 1999). According to similar studies, it was confirmed that DREB1A increased drought tolerance of wheat (Triticum aestivum) (Pellegrineschi et al. 2004; Morran et al. 2011) and CBF1 increased cold tolerance of Arabidopsis (Jaglo-Ottosen et al. 1998; Chow et al. 2014) and induces the expression of many COR genes with roles in stress protection (Thomashow 2010). Genomic studies have revealed that cold acclimation is controlled by many cold-regulated genes such as COR15, lipid transfer protein and β-amylase (Steponkus et al. 1998; Kreps et al. 2002; Seki et al. 2002; Lee and Lee 2003; Thalhammer and Hincha 2014).

Plant hormones that are known to be associated with plant defence such as: Jasmonates (JAs), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA), or that are associated with plant development such as: gibberellins (GAs), auxins (IAAs and IBA), brassinosteroids (BRs), and cytokinins (CK), play an important role in
regulating responses to environmental stresses such as salinity (Zörb et al. 2013; Kazan 2015). Furthermore plant hormones can collaborate or interact during plant responses to abiotic stresses and can have direct and/or indirect effects on multiple plant functions (Kazan 2013; Santino et al. 2013; Colebrook et al. 2014).

Chen and Murata (2008) reported (i) protection of the photosynthetic system mediated by the high levels of glycine betaine (GB) that accumulate in plant organs such as leaves and reproductive organs; (ii) induction of specific genes in transgenic plants to enhance stress tolerance; (iii) protection of the plasma membrane; and (iv) reductions in levels of reactive oxygen species (ROS) as metabolic responses to stress. This illustrates that various metabolic pathways under stress can be possible mechanisms for tolerance to a variety of abiotic stresses. Glycinebetaine is one of a number of compatible solutes that enable plants to tolerate abiotic stress and accumulate in response to abiotic stress (Kishitani et al. 1994; Allard et al. 1998; Chen and Murata 2011; Fan et al. 2012; Wani et al. 2013). The accumulation of GB in plants effectively maintains the highly ordered state of membranes and stabilizes the quaternary structures of enzymes and complex proteins as osmotic stress removes water from cells (Papageorgiou and Murata 1995). The enzyme involved in the production of GB has been isolated and moved into transgenic plants (Schmitz and Schütte 2000). Enhanced tolerance to abiotic stresses was observed in transgenic plants accumulating GB within its organs, in particular, the reproductive organs of transgenic plants (Chen and Murata 2008). The tolerance to abiotic stress was increased by the introduction of the GB-biosynthetic pathway into transgenic maize plants engineered with the transgene betA that enhanced tolerance to drought (Quan et al. 2004a), and to chilling (Quan et al. 2004b). Thus, the accumulation of
GB in transgenic GB-accumulating plants increased tolerance of transgenic plants to various abiotic stresses at all stages of their life cycle (Chen and Murata 2011).

1.2. 21 Chlorophyll Fluorescence monitoring Fv/Fm

When light energy is absorbed by the chlorophyll complex within a plant leaf, electrons are excited and raised to a higher energy state. This electron configuration is inherently unstable and its duration is less than $10^{-8}$ sec. The absorbed energy can subsequently be dissipated by either photochemical or non-photochemical processes. Photochemical processes utilise the absorbed energy in photosystem II (PSII), where the energy can be converted to chemical energy to drive photosynthesis (photochemical reactions). However, energy that is excess to photosynthesis can be re-emitted via non-photochemical processes in the form of heat (non-photochemical quenching; NPQ) which does not drive photosynthesis, or emitted as chlorophyll fluorescence. The efficiency of photochemical and non-photochemical quenching can be assessed by measuring the yield of chlorophyll fluorescence. The efficiency of light used for photochemistry can be predicted by measuring changes in the extent of fluorescence emission (Maxwell and Johnson 2000; Logan et al. 2014). These processes are in competition for absorbed energy, so a reduction in the dissipation of energy by non-photochemical processes (heat production and chlorophyll fluorescence) will be associated with a corresponding increase in the energy dissipated by photochemical processes. Likewise, the increase in dissipation of the excitation energy to heat leads to a decrease of quantum yield of chlorophyll fluorescence below its maximum yield. Fluorescence yield is highest when less energy is emitted as heat or used in photochemistry and vice versa.
Abiotic stresses have an effect on the photosynthetic performance of the plant. Stress factors such as drought, cold, salt, high temperatures, herbicide damage and nutrient deficiency limit the photosynthetic capabilities of the plant.

The maximum quantum yield of PSII, Fv/Fm is a fluorescence parameter used to measure of the intrinsic or maximum efficiency of PSII. This parameter is calculated by the equation:

\[ \text{Fv/Fm} = (\text{Fm} - \text{Fo}) / \text{Fm} \]

Where: \( \text{Fm} \) is the maximum fluorescence yield. \( \text{Fo} \), the yield of fluorescence in the absence of an actinic (photosynthetic) light; minimum fluorescence yield (Maxwell and Johnson 2000). \( \text{Fv} \) is the variable fluorescence, calculated as \( \text{Fv} = \text{Fm} - \text{Fo} \).

Changes in Fv/Fm values relate to a change in the efficiency of non-photochemical quenching. Therefore, these changes give important information concerning the effect of abiotic stress on the plant and by extension, the performance of PSII.

Dark-adapted values of Fv/Fm reflect the potential quantum efficiency of PSII and can vary between plant species. The optimal value of Fv/Fm is almost constant for many different plant species under non-stressed conditions and typically has a value of 0.83 (Björkman and Demmig 1987; Johnson et al. 1993; Roháček et al. 2008). This parameter is used as a sensitive indicator of plant photosynthetic performance. When the plant has been exposed to stress, Fv/Fm values will be lower than the optimal value indicating in particular the phenomenon of photoinhibition, or other kinds of injury caused to the PS II complexes (Roháček et al.)
Moreover, the Fv/Fm value might also be reduced due to fluorescence emission from PS I contributing to the F0 level (Papageorgiou 2004; Schreiber 2004).

Fluorescence measurements have the ability to give information about the plant's ability to tolerate environmental stresses and it can give insights into the extent the photosynthetic apparatus can affected by stresses (Maxwell and Johnson 2000). Fv/Fm measurements are widely used to indicate the occurrence of photo-inhibitory damage in response to abiotic stresses (Gamon and Pearcy 1990; Groom and Baker 1992; Epron et al. 1992; Akram et al. 2011; de Souza et al. 2013) and are also used as an indicator of how plants respond to environmental stresses (Liu et al. 2012; Murchie and Lawson 2013). These indicators are still accepted as reliable diagnostic indicators of photo-inhibition (He et al. 1996; Valladares and Pearcy 1997; Molina-Bravo et al. 2011; Suebma et al. 2012).
1.3 Conclusion

Climate change is predicted to have a huge global impact on agricultural production through its effect on the development and utilization water resources of many regions. Drought is a major agricultural threat reducing crop productivity and limiting the use of land throughout the world and this is particularly acute in the Middle East region.

Maize is a versatile crop that adapts easily to a wide range of production environments, and fits well in the existing cropping systems in the eastern-northern areas of Syria. Success, though, will depend on developing appropriate agronomic practices and using varieties that are adapted to the environmental conditions prevailing in these areas. Therefore, maize breeding with improved abiotic stress-tolerance via genetic manipulation is of huge potential importance.

Genetic transformation using direct or indirect methods is an effective means of rapid genetic improvement of crops, consuming less time compared to other methods of genetic improvement such as the production of haploid or doubled-haploid (DH) plants and conventional breeding. Future improvement of current cereal cultivars is expected to benefit greatly from information emerging from genomics and bioinformatics. And yet, new insights about fundamental aspects of Agrobacterium-plant interactions will lead to improved technologies in maize plant transformation.

The added value in these innovative approaches to local maize production in Syria lies in the combination of existing and novel genetic approaches with conventional breeding in association with socio-economic criteria that will effectively target the farmer and consumer. By considering the socio-economic implications to improve the local maize, this project should lead to sustainable development, and to
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move firstly towards self-sufficiency but then to greater production so that Syria can enter into international grain-trade with maize. Whilst the agronomy of maize has been reasonably well developed in Syria for the continuation of the development in production in currently planted areas refinements will be needed to meet changing economic conditions and environmental expectations. For new areas into which agronomy of maize may be expanded in the future, new and innovative solutions to meet environmental limitations to maize production will be required. The production of abiotic stress-tolerant maize plants by Agrobacterium-mediated transformation is one of the most important opportunities to develop new germplasm to cope with the environmental conditions. As has been demonstrated in many previous studies Agrobacterium tumefaciens has been used with great efficiency to transform most plants, by extension, if Syrian maize genotypes have suitable transformation ability using Agrobacterium, this approach has the potential to significantly improve stress resistance of Syrian maize varieties.
1.4 Aims and Objectives

This study aims to:

1- Study the production of genetically altered plants of yellow maize with improved abiotic-stress (drought, saltiness and frost) resistance.

2- Estimate of the expression and productivity of altered plants under stress circumstances within controllable circumstances.

The objectives of this report are summarised as follows:

a) Develop procedures for the maintenance of *Agrobacterium tumefaciens* *in-vitro*, and refine co-cultivation techniques of *A. tumefaciens* and maize immature embryos tissue cultures.

b) Improve the understanding of maize transformation techniques and whether this could be used by further scientific research in this field.

c) Determine whether Syrian maize varieties have suitable transformation ability, if so, can Syrian maize genotypes be transformed via *Agrobacterium tumefaciens* -mediated transformation?
Chapter 2:

General materials and methods
Materials and Methods

2.1 Maize germplasm

Seven distinct sources of maize germplasm were used in this study. Six germplasms were Syrian genotypes, originating from the General Commission for Scientific Agricultural Research, Syria (GCSAR), and were used in this research project to test their response to *A. tumefaciens*-mediated transformation in comparison with Hi II hybrid originating from the Maize Genetics Coop (Table 2.1).

Table 2.1: Maize genotypes used for *Agrobacterium tumefaciens*-mediated transformation.

<table>
<thead>
<tr>
<th>Germplasm</th>
<th>Name of Genotypes</th>
<th>Generation &amp; type of germplasm</th>
<th>Origination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varieties</td>
<td>Ghota.1 (GH.1)</td>
<td>F2. Synthetic varieties</td>
<td>GCSAR- Syria</td>
</tr>
<tr>
<td></td>
<td>Ghota.82 (GH.82)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrids</td>
<td>Basil.1 (B.1)</td>
<td>F2. Single hybrid</td>
<td>GCSAR- Syria</td>
</tr>
<tr>
<td></td>
<td>Basil.2 (B.2)</td>
<td>F2. Double hybrid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hi II</td>
<td>F2. Hybrid (A188xB73)</td>
<td>Maize Genetics Cooperation- Stock Center, USA</td>
</tr>
<tr>
<td>Inbred-Lines</td>
<td>IL.3: 565- 06</td>
<td>F1. Inbred lines*</td>
<td>GCSAR- Syria</td>
</tr>
<tr>
<td></td>
<td>IL.4: 792- 06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*data of Syrian inbred lines (IL.3 & IL.4) transformation not presented in this study.

2.2 *In-vitro* plant materials

F2 immature zygotic embryos (1.5 to 2.0 mm) of the studied maize genotypes were initially used for maize transformation. Immature zygotic embryos were aseptically dissected in a laminar flow hood from greenhouse-grown ears harvested after 20 to 22 days post pollination depending on germplasm type and glasshouses conditions. Ears were stored up to three days at 4°C before dissection.
2.3 *Agrobacterium tumefaciens* strains and DNA plasmid constructs used for plant transformation in this study

2.3.1 pTF102 plasmid:

*Agrobacterium tumefaciens* strain EHA101 (Hood *et al*. 1986), which has been used in the current study, harbours the standard binary vector pTF102 (11.6 kb). pTF102 (Figure 2. 1) contains a broad host range origin of replication (pVS1) (Hajdukiewicz *et al*. 1994) and a spectinomycin-resistant marker gene (*aadA*) in order to maintain bacterial selection and to avert reversion to WT. The cauliflower mosaic virus (CaMV) 35S promoter (P35S) was used to drive both the *bar* gene selectable marker cassette\(^1\) and the *gus* reporter gene. A tobacco etch virus (TEV) translational enhancer (Carrington and Freed 1990) was included at the 5’ end of the *bar* gene. The soybean vegetative storage protein terminator (Mason *et al*. 1993) was cloned to the 3’ end of the *bar* gene. The *gus* gene contained a portable intron in its codon region (Vancanneyt *et al*. 1990) to prevent GUS activity in *Agrobacterium* cells.

The *Agrobacterium* strain EHA101 harbouring the binary vector pTF102 was kindly provided by Professor Kan Wang (Iowa State University, Department of Agronomy (College of Agriculture and Life Sciences), Plant Science Institute, USA.

2.3.1.1 DNA sequence components for the pTF102 construct

*aadA*: Aminoglycoside 3’-adenylyltransferase gene of *Shigella flexneris* 2a that confers resistance to antibiotic spectinomycin (Chinault *et al*. 1986).


Tnos: 3’ terminator from nopaline synthase gene of *Agrobacterium tumefaciens* (Depicker *et al.* 1982).


T35S: 3’ terminator from 35S transcripts of cauliflower mosaic virus (Condit and Meagher 1983).


RB: The T-DNA right border fragment from nopaline strain of *Agrobacterium tumefaciens* (Zambryski *et al.* 1982).

LB: The T-DNA left border fragment from nopaline strain of *Agrobacterium tumefaciens* (Zambryski *et al.* 1982).

pVS1: A broad host range plasmid from *Pseudomonas* (Itoh and Haas 1985; Hajdukiewicz *et al.* 1994).
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Figure 2.1: A map of the pTF102 construction (11.6 kb) including the right (RB) and left (LB) T-DNA border fragments from a nopaline strain of A. tumefaciens with bar, encoding phosphinothricin acetyltransferase gene; gus-int, β-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator; Hind III, is one of the unique restriction enzyme sites. And aadA, a spectinomycin resistant marker gene for bacterial selection; pVS1, origin of replication for Agrobacterium; and pBR322, origin of replication for E.coli. Adapted from Frame et al. (2002).
The complete sequence of the pTF102 plasmid which obtained from the Plant Transformation Facility, Department of Agronomy (College of Agriculture and Life Sciences), Plant Sciences Institute, Iowa State University, USA is presented in (Appendix 4).
2.3.2 PpCBF1-OX construct (CBF)

The PpCBF1-OX construct includes the pRTL2 (Figure 2. 2) and pBINPLUSARS (Figure 2. 3) plasmids along with the final construct (Figure 2. 4). An expressed sequence tag (EST) library from peach (Prunus persica ‘cv. Loring’) bark collected in December was screened via PCR using degenerate primers as per standard methodology to obtain a full-length coding sequence for a CBF transcription factor. The resulting clone, Prunus persica CBF1 (PpCBF1) (GenBank accession HM992943), was sequence verified, then mobilized into pRTL2 which contains an enhanced 35S promoter to form 35S::PpCBF1. The enhanced 35S promoter -PpCBF1 portion of the plasmid was then digested with Hind III and ligated into an appropriately digested pBINPLUS/ARS vector (Belknap et al. 2008).

Agrobacterium tumefaciens strain EHA105 (pCH32) was transformed with the pBINPLUS/ARS vector containing the enhanced 35S-PpCBF1 fragment via electroporation and verified via PCR using the following gene-specific primers: CBF85F, ATGGTCATGGACATGATCTTCG and CBF557R, AGCTAAGCATTGGGGTGGAGAAAG (Wisniewski et al. 2011).
Figure 2. 2: Map of the pRTL2 vector (3.9 kb) containing a dual CaMV 35S enhancer as well as a TEV leader and CMV terminator. Adapted from Michael Wisniewski and Timothy S. Artlip, USDA-ARS, USA.

Figure 2. 3: Map of the pBINPLUSARS vector (12.46 Kb). CaMV 35S, enhancer; NOS, terminator; ubi3-UQ, promoter; NptII, the neomycin phosphotransferase gene; Hind III, restriction enzyme sites. (Adapted from Michael Wisniewski and Timothy S. Artlip, USDA-ARS, USA).
Figure 2. 4: Map of the PpCBF1-OX vector (14.58 Kb) over expression construct with the interest of gene CBF; a dual CaMV 35S, enhancer; NOS, terminator; ubi3-UQ, promoter; NptII, the neomycin phosphotransferase gene; Hind III, restriction enzyme sites. Adapted from Belknap et al. (2008).

The CBF gene construct was kindly provided by Professor Michael Wisniewski and Dr Timothy S. Artlip, Appalachian Fruit Research Station- United States Department of Agriculture / Agricultural Research Service (USDA-ARS), USA.

The binary vector pBINPLUS/ARS, complete sequence is presented in (Appendix 4).
2.4 Tissue culture stock solutions

1. **N6 vitamin stock**: 1 g of N6 was dissolved in 100 mL of double-distilled water (ddH2O). This stock solution (1,000×) was filter sterilized, and stored at -20°C in 25 mL aliquots, which were thawed and used over a period of weeks as needed.

2. **2,4-D**: 15 mg of powdered 2,4-dichlorophenoxyacetic acid (2,4-D) was dissolved in 10 mL of ethanol. The stock solution (1.5 mgmL⁻¹) was stored at 4°C.

3. **Acetosyringone (AS)**: stock solution 200 mM of AS was prepared by dissolving 392 mg of AS in 10 mL of dimethyl sulfoxide (DMSO). This solution is diluted 1:1 with ddH2O and filter-sterilized. Aliquots (0.5 mL) of stock solution (100 mM) were stored at -20°C for up to 6 months.

4. **Bialaphos**: 150 mg of Bialaphos was dissolved in 100 mL of ddH₂O. The stock solution (1.5 mgmL⁻¹) was filter sterilized and stored at 4°C for up to 6 months.

5. **Silver nitrate**: 0.085 g of silver nitrate was dissolved in 100 mL of sterile distilled water ddH₂O. The stock solution (0.85 mgL⁻¹) was filter sterilized and stored at 4°C.

6. **Kanamycin**: 0.5 g of kanamycin was dissolved in 10 mL ddH₂O. Then the stock solution (50 mgmL⁻¹) was filtered sterilized and divided into aliquots 0.5 mL in Eppendorf tubes, 1.5 ml. The stock solution was stored at -20°C for up to 1 month.

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2 AS will sometimes precipitate after freezer storage and is re-dissolved by vortexing for 15 min
7. **Spectinomycin**: 1.0 g of Spectinomycin was dissolved in 10 mL ddH₂O. The stock solution (100 mg ml⁻¹) was filter sterilized, aliquoted (0.5 mL), and stored at -20°C for up to 1 month.

8. **Tetracycline**: A stock of Tetracycline (10 mg ml⁻¹) was prepared by dissolving 0.1 g of Tetracycline HCl in 10 ml distilled water. This stock was filter sterilized and aliquots of 0.5 ml were stored at -20°C for up to 1 month.

9. **Cefotaxime**: 1.0 g and 2.5 g of cefotaxime was dissolved in 10 mL ddH₂O separately to form stock solutions (100, 250 mg ml⁻¹) respectively. These stock solutions were filter sterilized, aliquoted (0.5 mL) and stored at -20°C for up to 1 month.

10. **Vancomycin**: 1.0 g of vancomycin hydrochloride was dissolved in 10 mL ddH₂O. The stock solution (100 mg mL⁻¹) was filter sterilized, aliquoted (0.250 mL), and stored at -20°C for up to 1 month.

### 2.5 Tissue culture media

Infection, resting, and selection media as described by Zhao *et al.* (2002) were used for this *A. tumefaciens* protocol of maize transformation. Co-cultivation media was modified from Zhao *et al.* (1999) and Olhoft and Somers (2001) to contain 300 mgL⁻¹ cysteine; resting and selection media contained a combination of cefotaxime (100 mgL⁻¹) and vancomycin (100 mgL⁻¹) for counter selection of *Agrobacterium* after co-cultivation.

Regeneration medium was based on Armstrong and Green (1985) and prepared as described in (Frame *et al.* 2002) and supplemented with 3 mgL⁻¹ bialaphos. Bialaphos-resistant events were sub-cultured to MS salts media (Murashige and
Skoog, 1962) and modified MS vitamins (Frame et al. 2006a), 6% sucrose, 100mgL⁻¹ myo-inositol, no hormones (Armstrong and Green 1985), 0.3% gelrite, pH; 5.8.

Over all the tissue culture steps, for preparation of solid media, 100 x 20mm Petri-plates were used except for YEP (Yeast extract peptone) and LB (Luria-Bertani) media which were in 90 x 15mm Petri-dishes.

2.6 Media for Agrobacterium maintenance

- **YEP Medium:**

  5 gL⁻¹ yeast extract, 10 gL⁻¹ peptone, 5 gL⁻¹ NaCl, 15 gL⁻¹ Bacto-agar. pH was adjusted to 6.8 with NaOH and autoclaved at 120°C for 15 minutes (Wang 2006). Appropriate antibiotics were added to the medium when it had cooled to 50°C.

- **LB Medium:**

  5 gL⁻¹ yeast extract, 10 gL⁻¹ tryptone, 5 gL⁻¹ NaCl, 2 gL⁻¹ glucose, and 15 gL⁻¹ Bacto-agar. pH was adjusted to 6.8 using NaOH. Appropriate antibiotics were added to the medium when it had cooled to 50°C after autoclaving.

2.7 Media for Agrobacterium-mediated transformation

- **Infection Medium (IM):**

  N6 salts and vitamins (Chu et al. 1975), 1.5 mgL⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 gL⁻¹ L-proline, 68.4 gL⁻¹ sucrose, and 36 gL⁻¹ glucose (pH 5.2). This medium was filter-sterilized and stored at 4°C. Filter-sterilized acetosyringone (AS, 100 mM) was added prior to use. 100mM AS stocks (dissolved in Dimethyl Sulfoxide DMSO to 200mM then diluted 1:1 with distilled water) and stored at -20°C for use as needed.
Co-cultivation Medium (CCM) (made fresh – use within 4 days):

N6 salts and vitamins, 1.5 mgL⁻¹ 2,4-D, 0.7 gL⁻¹ L-proline, 30 gL⁻¹ sucrose, and 3 gL⁻¹ gelrite (pH 5.8). Filter sterilized silver nitrate (0.85 mgL⁻¹), AS (100 mM), Cysteine (300 mgL⁻¹), and N6 Vitamins were added to this medium after autoclaving.

Resting Medium (RM):

N6 salts and vitamins, 1.5 mgL⁻¹ 2,4-D, 0.7 gL⁻¹ L-proline, 30 gL⁻¹ sucrose, 0.5 gL⁻¹ 2- (4-morpholino)-ethane sulfonic acid (MES), and 8 gL⁻¹ purified agar (pH 5.8). Filter sterilized N6 vitamins, cefotaxime (100 mgL⁻¹) and vancomycin (100 mgL⁻¹), and silver nitrate (0.85 mgL⁻¹) were added to this medium after autoclaving.

Selection Medium I (SM₁):

N6 salts and vitamins, 1.5 mgL⁻¹ 2,4-D, 0.7 gL⁻¹ L-proline, 30 gL⁻¹ sucrose, 0.5 gL⁻¹ MES, and 8 gL⁻¹ purified agar (pH 5.8). Filter sterilized N6 vitamins, cefotaxime (100 mgL⁻¹) and vancomycin (100 mgL⁻¹), silver nitrate (0.85 mgL⁻¹), and Bialaphos (1.5 mgL⁻¹, Shinyo Sangyo, Japan) were added to this medium after autoclaving.

Selection Medium II (SM₂):

N6 salts and vitamins, 1.5 mgL⁻¹ 2,4-D, 0.7 gL⁻¹ L-proline, 30 gL⁻¹ sucrose, 0.5 gL⁻¹ MES, and 8 gL⁻¹ purified agar (pH 5.8). Filter sterilized N6 vitamins, cefotaxime (100 mgL⁻¹) and vancomycin (100 mgL⁻¹), silver nitrate (0.85 mgL⁻¹), and Bialaphos (3 mgL⁻¹) were added to this medium after autoclaving.
2.8 Culture media for regeneration of transformed materials

- **Regeneration Medium I (RM_I):**
  MS salts (Murashige and Skoog, 1962) and modified MS vitamins, 60 gL\(^{-1}\) sucrose, 100 mgL\(^{-1}\) myo-inositol, no hormones and 3 gL\(^{-1}\) gelrite (pH; 5.8). Filter sterilized cefotaxime (250 mgL\(^{-1}\)) and Bialaphos (3 mgL\(^{-1}\)) or Glufosinate Ammonia (4 mgL\(^{-1}\)) were added to this medium after autoclaving.

- **Regeneration Medium II (RM_I):**
  MS Salts and modied MS vitamins, 100 mgL\(^{-1}\) myo-inositol, 30 gL\(^{-1}\) sucrose, 3 gL\(^{-1}\) gelrite, (pH; 5.8). Infection medium was filter sterilized, whereas all other solid media were autoclaved at 120 °C for 15 minutes according (Wang 2006).
2.9 Methodology

Agrobacterium-mediated transformation of immature zygotic embryos (IZEs) protocol

2.9.1 Agrobacterium strain culture and preparation

The vector system, in Agrobacterium strain EHA101 or in EHA105, used in these experiments was maintained on Yeast Extract Peptone (YEP) and Luria-Bertani (LB) medium (An et al. 1989) containing the appropriate antibiotics. Bacteria cultures for weekly experiments were initiated from stock plates that were refreshed from long-term -80°C glycerol stocks.

Every 4 weeks, a “mother” plate was re-initiated from this long-term glycerol stock by streaking the bacteria to YEP or LB (with antibiotics) and growing for 2 days at 28°C. The “mother” plate was then kept in the refrigerator (4°C) and used as a source plate for Agrobacterium cultures. For weekly transformation experiments, the mother resource plate was used to prepare the Agrobacterium inoculation by culturing and plating Agrobacterium cells for 3 days at 19°C for pTF102 or at 28°C for 2 days (CBF).

In all transformation experiments, bacteria cell densities were adjusted immediately before embryo infection to a spectrophotometer optical density (OD$_{550 \text{ nm}}$) between 0.35 to 0.45 for pTF102 and lower than 0.7 for the CBF construct.


2.9.2 Explant preparation

2.9.2.1 Embryo dissection

_in-vitro_ tissue culture techniques require not only an aseptic work environment, but also contaminant-free starting materials.

A well-established protocol was adopted for the immature embryos sterilization (IES) as an initial material used for _Agrobacterium_-mediated transformation (Frame _et al._ 2000). Accordingly the steps described below were followed:

1. The top 1 cm of the ear was cut off and discarded. Then the tip of a sterilized straight netting needle was inserted into this end of the ear. This “handle” facilitated aseptic handling of the cob during embryo dissection, and made the dissection easier.

2. In a sterilized container in laminar flow bench, the impaled ear was immersed into a sterilizing solution [700 mL of 50% commercial bleach (5.25% sodium hypochlorite) in water + 1 drop of surfactant Tween 20]. During the 20-minute disinfection, occasionally the container containing the ears was tapped on the surface of the flow bench to dislodge air bubbles for thorough surface sterilization of the ear. Also, the ears were stirred every 5 minutes to remove the air bubbles and make the ears fully sterilized. After that, the bleach solution was poured off and the ears were rinsed three times in generous amounts of sterilized distilled water. The final rinse was drained off and the ears were ready for embryo dissection.

3. In a laminar flow bench, the kernel crowns (the top 1-2 mm) of the handling sterilized ear were cut off with a sterile, sharp scalpel blade.
4. The final step was to excise the embryos by inserting the narrow end of a sharpened spatula between the endosperm and pericarp at the basipetal side of the kernel (towards the bottom of the cob) popping the endosperm out of the seed coat. This exposed the untouched embryo, which sat at the topside of the kernel, close to the kernel base. The embryo was gently coaxed onto the spatula tip and submerged in infection medium. A Bead Sterilizer was used for intermittent re-sterilizing of utensils throughout this protocol.

Inoculation with Agrobacterium and co-culture of the embryo clusters

2.9.3 Agrobacterium infection

Four days prior to inoculation, appropriate A. tumefaciens cultures were streaked in a petri dish containing an appropriate solid medium (LB or YEP) amended with antibiotics. The medium contained the antibiotics to select for the plasmids present in the Agrobacterium vector [usually, Kanamycin (km) for pBin derived vectors and Tetracyclin (tet) for EHA105-pCH32 Agrobacterium strain, or spectinomycin (spec) for pTF102 plasmid and Km for EHA101 Agrobacterium strain. Storage cultures were kept in a -80°C freezer or a fresh streak of the strain was used. A total of 5 plates per strain were grown for 3 days at 19°C (or 2 days at 28°C).

2.9.3.1 Day of inoculation:

To begin an experiment, one full loop (3 mm) of bacteria culture was scraped from the plate cultured for 3 days and suspended in 5 mL infection medium (Inf) supplemented with 100 mM AS (acetosyringone) in a 50 mL tube. Then, the tube was affixed horizontally to a shaker platform and shaken at low speed (~75 rpm) for two to four hours at room temperature. Agrobacterium should be growing vigorously.
at this time (Wang 2006). If the *Agrobacterium* culture was growing slowly and the *Agrobacterium* suspension was translucent, the *Agrobacterium* culture is allowed to grow for additional 2 hours. The optical density (OD$_{550}$) of the culture was measured prior to use and adjusted to the desired level (0.3 - 0.4) with additional amounts of fresh medium of IM. This pre-induction step was carried out for all experiments.

For infection, once the pre-induction step was completed, IZEs from one ear (1.5 to 2.0 mm) were dissected directly into a 2-mL Microcentrifuge tube filled with *Agrobacterium*-free infection medium (with100 mM AS). IZEs were washed twice with this medium. The final wash was removed and 1 to 1.5 ml of *Agrobacterium* suspension (OD$_{550}$= 0.3 to 0.4) was added to the embryos. Embryo infection was accomplished by gently inverting the tube 20 times before resting it upright with embryos submerged in the *Agrobacterium* infection medium for five minutes in the dark. Embryos were not vortexed at any time during this procedure. These and all subsequent tissue culture steps were carried out in a laminar flow bench using aseptic technique.

### 2.9.3.2 Co-Cultivation

After infection, embryos were transferred to the surface of co-cultivation medium and excess *Agrobacterium* suspension was pipetted off the medium surface and removed from the area surrounding each embryo. Embryos were oriented with the embryo-axis side in contact with the medium (scutellum side up$^3$).

Plates were wrapped with air permeable adhesive tape (leucopore tape, Melford chemical, UK) and incubated in the dark at 20°C for three days in a secure incubator.

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$^3$ Leave the lid of the co-cultivation plate ajar for up to 1 h to let the medium and embryo surfaces dry further before orienting each embryo scutellum side up with the aid of a stereo microscope.
2.9.4 Callus induction; resting

After three days of co-cultivation, embryos were transferred to the callus induction medium (the resting medium) at 28 °C in the dark for 7 days.

Sometimes a delay in embryo response was noticed, and in some cases, embryo death after 3 days of co-cultivation was registered. Nevertheless, all embryos were transferred as many of the slow responding embryos eventually formed callus. No embryos were discarded prematurely, and the embryos were transferred throughout the following selection steps continuously. Plates were wrapped with a leukopor tape. All embryos were transferred from co-cultivation medium to resting and selection media.

After 1 week on resting medium, embryogenic callus induction frequency (ECIF) of Agrobacterium-infected embryos which co-cultivated on medium containing 300 mgL⁻¹ cysteine was calculated as a percentage of targeted embryos. ECIF was measured as the number of co-cultivated immature zygotic embryos that had initiated embryogenic callus formation at their scutellum base after 7 d on a resting medium, expressed as a percentage of the total number of embryos plated on the resting medium.

2.9.5 Selection for stable transformation events

After 7 days on resting medium, embryos, responding or not, were transferred to selection I medium (35 IZEs per plate) containing 1.5 mgL⁻¹ filter-sterilized bialaphos in order to select of transformed calluses for 2 weeks. Callus pieces were transferred every 2 weeks to a fresh selection medium. Selection medium (SM) was similar to that used for callus initiation, but selected antibiotic (bialaphos or kanamycin) was added to the SM. This was the first selection. After that, 2-4 subcultures, depending
on the genotypes, were carried out on a fresh medium containing the same amount
of antibiotics for selection. IZEs were then sub-cultured for two more 2-week
passages on Selection II medium containing 3 mgL⁻¹ bialaphos (25 callus per plate),
and this selection was the second selection. Embryogenic calluses that were
antibiotic/bialaphos-resistant were produced on selection II medium.

An individual transformed clone was identified as carrying the plate number that
it derived from and transferred to another plate containing fresh media. The callus
clump from which it originated was transferred to the same plate as well to ensure
that a single transformation event was picked only once. Within 11-13 weeks of
transformation, antibiotic-resistant clones which emerged from the selected callus
pieces were putatively transgenic proliferated type II calli. However, samples from
these antibiotic-resistant clones were collected for molecular analysis using
polymerase chain reaction (PCR) and for histochemical staining of GUS activity
using a GUS reaction mixture with X-Gluc solution.

Plates were wrapped with Parafilm throughout the selection and incubated at
28°C in the dark. Throughout the selection stages, 100 x 20 mm petri dishes (Sigma-
Aldrich, UK, P5606-400EA) were used to culture the materials.

Putatively transformed events were visible as rapidly growing Type II callus
emerging from a subset of embryos. This event can be recognized with the naked
eye several weeks after infection. It has previously been established that the rate of
clone emergence is construct dependent. For example, putative Type II callus Hi II
events can be visible as early as five and as late as 10 weeks after infection (Frame
et al. 2011).
Stable transformation efficiency (%) was calculated as the number of bialaphos resistant callus events recovered per 100 embryos infected after 4 to 6-week of selection.

2.9.6 Regeneration of transgenic plants

2.9.6.1 Shoot formation; Mature somatic embryos production

Regeneration of R₀ transgenic plants from Type II embryogenic callus was attempted next. From the literature, this should be accomplished by a 2 to 3-week maturation step on Regeneration I Medium RM₁, containing no hormones but with 0-3 mgL⁻¹ bialaphos. This is the third selection followed by germination in the light on Regeneration Medium II- as described in Frame et al. (2000). For some events, it was noticed that no somatic embryos were formed. In these cases, calluses were first transferred to RM amended with 0.25 mgL⁻¹ 2,4-D (pre-regeneration medium) and 1 mgL⁻¹ bialaphos at 25°C in the dark for 2-weeks (Armstrong 1994; Frame et al. 2000).

Regeneration of transgenic Type II callus ( friable, stocked somatic embryos present) was accomplished by transferring many small pieces (approximately 4 mm) of somatic embryo-enriched callus from pre-regeneration medium to Regeneration Medium I and incubating for 2-3 weeks. Mature somatic embryos were produced and shoot formation was begun at this stage. About 10 to 15 small pieces of highly embryogenic callus placed per petri dish. Petri-plates were wrapped with vent tape (leucopore tape) and incubated at 25°C in the dark.
2.9.6.2 Plantlet formation; Rooting of shoots and elongation

After 2-3 weeks on Regeneration I medium, the majority of callus pieces producing one or more mature somatic embryos should appear swollen, opaque and white. After that, by using a stereo microscope, mature somatic embryos were transferred from the dark on Regeneration I medium to the light on the surface of Regeneration II medium for germination in an illuminated incubator as described by Frame et al. (2000). Mature somatic embryos were transferred to the light (80–100 \( \mu \text{E m}^{-2} \text{s}^{-1} \) light intensity, 16:8 h photoperiod) on RM II media containing 3% sucrose and no hormones where they germinated for 7 to 21 days to form plantlets. Again the petri plates were wrapped with leucopore tape and incubated at 25°C, in the light. Plantlets sprouted leaves and roots derived from mature somatic embryos through further maturation and germination on this medium for 1 week.

When the plantlets formed fully formed roots and shoots with a leaf length of 2 to 4 cm, they were transferred individually into a culture tube (150 x 25mm glass vials) containing 15 mL of 2/3 strength MS (Murashige and Skoog, 1962) solid media for further elongation. After that, these plantlets were grown in glass vials with MS for up to 14 days until they reached a size of approximately 10cm.

Frame et al. (2011) have mentioned that Hi II plantlets sprouted leaves and roots on Regeneration II medium within 1 week and were ready for transfer directly to soil about 3 days later (10 days after transfer to this medium).

Regeneration percentage (%) was measured as the number of independent transgenic clones successfully regenerated to plants per 100 events for which regeneration was attempted.
2.9.7 Plant hardening in a controlled environment: Acclimation of plantlet

When plantlets were of 6-10 cm leaf length and had roots which were well established, they were transplanted into pre-wetted soil mixture (Multi-purpose compost and John Innes Compost No.2; ratio 2:1) in small pots (5 cm) in a growth chamber (170 μE m\(^{-2}\) s\(^{-1}\) light intensity, 22°C, 16:8 h photoperiod) as described for the growth of donor plants (Chapter 3). Roots were rinsed thoroughly with distilled water prior to transplanting. Small plants were initially covered with a plastic Humi-dome with one ventilation hole opened and placed in a growth chamber. After 6 to 7 days and before plants reached the top of the Humi-dome, the plastic dome was removed, and four to seven days later the plants were transplanted into bigger pots (10 cm) and kept in the growth chamber for 7 days. After that, the plants were taken out of the growth chamber for left for seven to ten days in the lab. When the plants could be easily lifted out of the small pot with roots holding the soil, each plantlet was transplanted to a larger pot (40 cm) in the laboratory, and transferred to the glasshouse. This step is restricted under UK GM regulations so only non-transgenic plants were transferred to the glasshouse and GM plants were grown on in the growth chamber. Young plants were fertilized regularly with Miracle-Gro (NPK fertilizer (24-8-16) with Micro-nutrients), and treated with the calcium/magnesium recipe by watering them once every 2 weeks with this mix as described for the donor plants care (Chapter 3).
2.10 Molecular techniques

The presence of the CBF gene and the bar gene were confirmed in both A. tumefaciens strains used in this study EHA101 and EHA105; the transformation process was confirmed using the GUS assay and by the polymerase chain reaction (PCR).

2.10.1 Genomic DNA extraction from maize callus and leaf tissue

For the events generated using the CBF and bar gene construct, genomic DNA was used for PCR and qRT-PCR. Leaf samples and callus pieces were collected from transformed and non-transformed events simultaneously. For each event, after 3 to 4 sub-cultures on media containing a suitable antibiotic or bialaphos as a selection agent, three samples of surviving callus were selected and ground immediately in liquid nitrogen using a pestle and mortar. Samples of the powder were stored at -20°C after the complete evaporation of the liquid nitrogen. Moreover, leaf samples were detached from each transformed plant and non-transformed plants and placed immediately in liquid nitrogen, then ground and stored. In both the callus and transgenic plants, the total genomic DNA for molecular analysis was extracted from the sample powders using a Genelute plant genomic DNA miniprep kit (Cat. No. G2N70. Sigma Aldrich. UK), and then quantified spectrophotometrically ($A_{260}/A_{280}$) (as outlined in chapter 6).

2.10.2 Polymerase chain reaction PCR on genomic DNA

Integration of T-DNA, carrying the marker gene (bar or NPTII) or the CBF gene, in the genomes of transgenic maize plants was assessed through polymerase chain reaction (PCR) analysis of total isolated DNA (Mamontova et al. 2010) using primers of the selection gene (antibiotic resistance). PCR was performed in 25 μL PCR
master mix containing the appropriate forward and reverse primers and transgenic genomic DNA as template according to the PCR program (presented later in Chapter 6). All samples were stored at -20 °C for later use.

All PCR amplifications were carried out using the Applied Biosystems, Veriti 96 well thermal cycler and a Master Cycler Gradient (Eppendorf, Hamburg, Germany). Gel running was carried out using agarose gel 1% for 1 to 1.5 hours, after which the gel was examined under UV light under a Bio Rad universal Hood II (Gel-Doc XR: 170-8170) to detect the relative bands. Band intensities proportions and locations were compared with the positive sample (Agrobacterium plasmid) and negative sample (control, non-transformed genomic DNA).

2.10.3 Quantitative Real-Time PCR (qRT-PCR) analysis

Quantitative real-time PCR (qRT-PCR) was adopted to estimate the integrated transgene copy number in transgenic callus and genomes of maize plants generated using the two different plasmid vector constructs.

The quantitative PCR (qRT-PCR) amplification was performed in 96-well reaction plates on the StepOnePlus™ Real-Time PCR systems (Applied Biosystems), in a 25 μL final volume, according to manufacturer’s instructions (TaqMan probe, Life Technologies Ltd, UK). Primers and probe used for the endogenous single copy gene “chi” were as reported by Dalla Costa et al. (2009) (see Chapter 6 for details). The StepOne and StepOnePlus systems use fluorescent-based PCR reagents to provide quantitative detection of target nucleic acid sequences (targets) using real-time analysis.

The estimated copy number of the transgenes obtained by quantitative real-time PCR (qRT-PCR ) has been previously shown to yield identical results to the more
established Southern blot analysis (Song et al. 2002). Furthermore, a TaqMan quantitative real-time PCR detection system is suitable for efficient early screening of transgenic clones. Identifying transgene copy number by this system has been found to be more accurate than genomic Southern blot hybridization (Mason et al. 2002) and it is suitable for the determination of transgene homozygotes in segregation populations effectively and reduces the cost and intensive labour requirements (Yi et al. 2008). In addition this method is amenable to identifying transgene copy number for large numbers of transgenic events rapidly while requiring very little tissue (Song et al. 2002). Thus, qRT-PCR represents an efficient means for determining transgene copy numbers in transgenic maize plants using Applied Biosystem, StepOne Plus Real-Time PCR Software v2.0.

### 2.11 Histochemical GUS assays

The histochemical beta-glucuronidase (GUS) assay (Jefferson 1987; Wilson et al. 1995) was used to screen for reporter gene expression in the herbicide-resistant events derived from immature embryos treated with the construct pTF102. This assay was carried out on most of bialaphos-resistant putative transgenic calli recovered from the selection. Different parts of transformed plants and non-transformed plants underwent screening. Stem, leaf, root, and other parts of plant organs were cut from transformed plants and submerged in the GUS reaction mixture as described in (Chapter 6). Furthermore, histochemical staining of GUS activity was performed on leaf pieces of R1 progeny of transformed plants and control plants to confirm expression of the GUS transgene in segregating offspring of studied germplasms.
Shen et al. (1993) has described the expression of GUS reporter gene delivered with high efficiency to maize shoots by *A. tumefaciens*.

### 2.12 Abiotic stress assessment techniques; (physiological assay)

#### 2.12.1 Assessing transformed plants *in-vivo* for salinity resistance

Assessing transformed plants *in-vivo* to salinity resistance was carried out under controlled environment conditions over the entire growth cycle. 

*R*₁ progeny of transgenic clones were grown in the growth chamber at 23°C, 13 h light/11 h dark. Three week-old seedlings of the transgenic plants and of controls were irrigated with distilled water amended with different amounts of sea water under normal conditions for nearly 70 days. Physiological indicators such as: Photosynthetic efficiency as chlorophyll fluorescence \(F_v/F_m\) (energy dissipated via photochemical processes), plant height, plant stem diameter, leaf area index, specific leaf area and dry weight were used for expression analysis. Three biological replicates per treatment were used for analysis of transgenic plants tolerance of salt in comparison with unaltered plants (control). The \(F_v/F_m\) values were calculated before the treatment with the saline water and after every 7-10 days treatment with saline water. The chlorophyll fluorescence was measured using a Pocket PEA photosynthesis system according to the instructions of Hansatech Instruments Ltd, UK. The \(F_v/F_m\) values were analysis using the Pocket PEA Plus Software.
2.13 \( R_1 \) progeny screening for \textit{bar} gene expression

To confirm that the \textit{bar} gene is expressed in the regenerated transgenic plants of studied genotypes; plantlets were sprayed with a herbicide (bialaphos). Two weeks old and 3-4 weeks old plantlets were sprayed with different concentrations of bialaphos solution and 0.1\% (v/v) Tween 20. One leaf from each plant was painted using a paint brush, and for other treatments, the whole plant was sprayed with bialaphos solution. Plants continuing to express the \textit{bar} gene providing resistance to bialaphos or phosphinothricin were bialaphos-resistant plants and remained alive. Death of the non-transformed plants was indicated that these plants were very sensitive to the herbicide and could not detoxify the herbicide. Therefore, the transformed plants expressed the \textit{bar} gene that encodes a phosphinothricin acetyl transferase (PAT) enzymes that modifies phosphinothricin and detoxifies the herbicide, conferring cellular resistance to bialaphos as well as phosphinothricin or glufosinate.

2.14 Statistical Analysis

Analysis of variances (ANOVA) was carried out on experiments using Minitab v.16 Statistical Software. Results are presented as means + standard error (s.e). The means were compared using least significant difference (LSD) test. Correlation among the different parameters was assessed using Excel curve fitting, and values of the correlation coefficient for different levels of significance investigated according to Fisher and Yates (1949).
Chapter 3:

Growth of donor plants and glasshouse care
3.1 Growth of Donor Plants

Harmonization of male and female flowering

Harmonization of male and female flowering for each maize genotype needed to be established in the glasshouse. Pollen from tassel shed is required at the same time of silk emergence within each genotype for cross-fertilization in order to ensure maximum kernel set; cobs production (Westgate et al. 2003). The synchronization of silking and pollen shedding of maize genotype with other important factors such as the distance from the pollen source and wind direction affected the rate of cross-fertilization in maize (Ma et al. 2004). Naturally pollen shed and silk emergence are slightly asynchronous and this may be accentuated by plant stress such as drought or high plant density (NeSmith and Ritchie 1992; Traore et al. 2000; Frankel and Galun 2012). A husbandry care plan of donor maize plants was needed in order to produce sufficient high quality immature embryos for laboratory experiments. Plants of each genotype were initially cultured in a controlled environment (temperature and light) and then transferred to the glasshouse and potted on as outlined below.

3.1.1 Seed germination and plant hardening under controlled environment conditions

Germination of selected Syrian maize genotypes and “Hi II” grains was conducted under laboratory conditions (Plate 3. 1). Germinating grains were then sown in 9-cm round pots (Capacity: 0.36 L), or in 10-cm square pots (approximately 700 mL volume) containing a loam based cultivation substrate (John Innes seed sowing compost) and then placed in a growth chamber (22/20 °C day/night, 13 hours light, 170 μmol s\(^{-1}\)m\(^{-2}\) photon flux density) for three weeks by which time the seedlings had reached 3-4 leaf stage with a good root mass (Plate 3. 2).
Soil moisture was checked daily by observing the darkness of the soil surface and each plant was watered individually according to need in order to avoid over-watering (Frame et al. 2006b; Jones 2008). It was important not to over water plants at any stage (especially at early stages before internode elongation) to ensure the development of a good root system. Excessive watering or a poor drainage promotes rot/fungal growth at the stem/root junction so adequate drainage is important.
Plate 3. 1: Establishing maize plants in the growth chamber, 4 days old.

Plate 3. 2: Maize plants at 3-4 leaf stage in the growth chamber (left), plantlets with soil adhering to well established roots 1 week after transferring to glasshouse (right).
3.1.2 Hardening and transplanting young maize plants to the glasshouse

An important process for preparing the plantlets for transplanting into the glasshouse is a “hardening off” procedure that acclimates the plantlets prior to its placement in a new environment. Hardening normally takes 1 week with the plantlets being placed in the glasshouse and in a shaded environment during this period. In addition, watering was provided properly and no fertilizer was given to the plants, the objectives being to slow additional height growth, although the plantlets themselves became larger and the roots established well.

When the plants could be easily lifted out of the pot with soil adhering to the roots, they were transferred to a glasshouse in full daylight (25/21°C day/night, 16 hours light). Whilst the daytime temperature was set at 25°C and the night-time temperature at 21°C with the current cooling system in the glasshouses, daytime temperatures in summer could range as high as 37°C regardless of thermostat settings (Figure 1). Plants were allowed to equilibrate for a further week, and then each maize plant, with an intact root ball, was transplanted into a 35-cm pot (20 L) containing a suitable loam-based substrate mix (multipurpose John Innes compost and the John Innes No.2 soil in a 2:1 ratio volumetrically) and 60 g Osmocote Pro⁴ (a general long-term slow release fertilizer that contained (19% N, 6% P and 12% K) was added per pot for long-term fertilization. With Osmocote plant food, plants get continuous and constant nutrition for up to four full months.

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⁴ Each Osmocote granule is coated with a unique resin that controls nutritional release. This means that plants get what they need, when they need it. Osmocote longevity is 3 to 4 months at 21°C, 1 to 2 months at 32°C (Product Specification Sheet, Scotts Miracle-Gro).
3.1.2.1 Transplant the young maize plants

Young plants (4 weeks old) were transplanted into 35 cm pots in the glasshouse according to the steps outlined below:

- The multiple purpose compost was mixed with the John Innes No.2 soil (2:1 ratio). Then, 50% of the pots volume was filled with the soil mixture.
- Before finishing the rest of the pot, Osmocote 60 g was mixed gradually with the soil to finish filling the pot by adding compost until 2-3 cm from a top edge of the pot.
- Young plants were moved with soil adhering to the root ball from the small pots into the middle of the big pots. The roots were covered with soil and plants were planted deeply enough to be sure they will not tip over when they grow taller, and can form good roots.
- Each pot was drenched with water alone without fertilizer until the water reached the top edge of the pot, and it drained completely.
- Each pot was labelled and fixed using a stick immersed in the soil beside the plant.

3.1.2.2 Watering the plants

The maize plants require significant quantities of water, but do not tolerate excess, since the roots will not be able function under anaerobic over-watering conditions. Normally, no watering was needed during the first week after transplanting the plants into big pots, but this depended on the season and prevailing temperatures. About 7 days after transplant, or at the 6-7 leaf stage (Plate 3. 3), each plant was thoroughly watered and each pot was individually watered according the need. Small plants needed special attention as they prefer a drier root
environment to stimulate and develop a good and strong root system. Controlled soil water has been shown to stimulate the development of a root system and significantly enhance root to shoot ratio (Kang *et al.* 2000). The only way to develop a good rooting system was by giving the plant the water when it was really needed. A constant check was needed to determine whether the plants needed water or not, by checking the soil moisture through the drainage holes in regard to the dry soil surface. Developing a healthy root system at this early stage of growth was the most critical issue for maize plant breeding in the glasshouse. There are many factors affect the size of the root system; it is not only genetically determined but, is also influenced by the moisture level, the structure and texture of the soil and its temperature and chemical factors (pH, electrical conductivity EC, and levels of essential and toxic elements) (Jones 2008).

During the vegetative growth period the young plants were kept as close to the greenhouse lights as possible by keeping them on tables. To ensure adequate root system development, particular care was taken to avoid over-watering plants at this stage. Maintaining the lowest moisture of soil around the stem and through the plants canopy will keep the plant canopy dry and reduce the possibility of fungal diseases.

Further fertilization was applied to the plants when a good root and leaf system was established using a soluble plant food (Miracle-Gro solution) (one large 15 mL spoonful in 4.5 litres of water).
Plate 3. 3: Young maize plants at 6-7 leaf stage in the glasshouse.

3.1.2.3 Watering after 6 to 7 leaf stage

Plants needed more water at the 8 to 11 leaf stage as internodes elongated and the tassel/ear was formed (Plate 3. 4). The soil moisture was checked daily and whenever the soil was dry, plants were watered again individually. The period between irrigations was reduced to provide more water. A second dose of Miracle-Gro solution fertilizer was added to the plants at the 8-11 leaf stage of growth.
Plate 3. 4: Maize plants at 11 leaf stage, growing in the greenhouse beside young plants.

Throughout the development of the plants, male and female flowering times were monitored and recorded (Table 3. 1). After this, the differences between male and female flowering were considered for all future experiments of growing. Phased plantings of maize were grown to ensure the harmonization of male and female flowering within a genotype (Plate 3. 4).

Table 3. 1: Time of male and female flowering under glasshouse conditions.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number days from planting</th>
<th>Delay of silks (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tassel shed ($\bar{X}$)</td>
<td>Silks emerging ($\bar{T}$)</td>
</tr>
<tr>
<td>Basil.1</td>
<td>80</td>
<td>82</td>
</tr>
<tr>
<td>Basil.2</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>Ghota.1</td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>Ghota.82</td>
<td>99</td>
<td>102</td>
</tr>
<tr>
<td>Inbred line.3: 565-06</td>
<td>108</td>
<td>&gt;115</td>
</tr>
<tr>
<td>Inbred line.4: 792-06</td>
<td>87</td>
<td>94-96</td>
</tr>
</tbody>
</table>
Every pot was assigned a serial number and genotype name and dated clearly. All plant numbers were scheduled in a logbook and data recorded for every individual pot including the flowering time, watering, nutrient deficiency, fertilization, pest control, pollination and sampling/harvesting.

3.1.3 Production of immature embryos for *Agrobacterium*-mediated transformation

Plants are required as embryo donors for genetic transformation studies which utilised immature zygotic embryo explants. Initial work aimed at establishing a suitable system for the growing of Syrian maize genotypes plants in the glasshouse for this purpose. Maize seeds were sown with intervals, according to the delay of silks as mentioned in the (Table 3. 1), to ensure the synchronization of silking and tasselling within a genotype plants. Thus, a constant supply of available pollen could be ensured for cross-fertilization.

For all transformation experiments, each time three pots of each genotype were grown. On average about 30 maize plants delivered 20 useable ears to the lab at staggered times.
3.1.3.1 Pollination

Flowering was monitored daily for glasshouse-grown plants. When the silks started emerging (Plate 3. 5), each ear was covered with a white paper bag (shoot bag) to prevent contamination of the silks with unwanted pollen before hand-pollinating⁵. The shoot bags were tightened firmly over each ear every morning. The shoot bags were affixed carefully to avoid destroying silks. At the same time, once tassels started to shed pollen, they were covered with plain brown paper bags to collect pollen from the donor plants and to prevent pollen release (Plates 6, 7 and 8). The shoot bags were affixed carefully to avoid destroying of silks or remove the ear leaf when we try to get at the emerging silks.

Plate 3. 5: Silks emerging, 1 day old in the glasshouse.

⁵ If the emerging silks will be “contaminated” with unwanted pollen, the ears will have to be discarded.
Plate 3. 6: Covering of 2-3 days-old Silks emerging in the glasshouse.
Plate 3. 7: Beginning of maize tassels shedding pollen and pollen collection in the glasshouse.
The most effective means to ensure complete tassel pollination in the glasshouses is by hand pollinating (Stanley and Linskens 2012) by releasing the pollen over the silks several times over a 3 to 7 day period to ensure pollination of as many ovules as possible (Plate 3. 9). Every morning, the flower pollination was carried out when the glasshouse temperature was at its lowest due to the pollen longevity and pollen vigour affected by the temperature (Hoekstra et al. 1992). However, temperature could be used to favour pollen longevity with early maturity to increase the vigour of pollen (Bajaj et al. 1992; Ottaviano et al. 2012). Ma et al. (2004) confirmed that the cool temperature and high humidity favour the pollen
Pollen that was collected in the brown bags was spread over the emerging silks of plants of the same genotype. The most successful fertilisations irrespective of genotype, was obtained when the silks were 2-3 days old and short. However, if the silks were long and up to a week old, successful crosses could still be obtained if the silks were cut back (Plate 3. 10), re-covered again with the shoot bag and the fresh growth of silks was pollinated the next day.

Pollen shed could be begin before silking and can continue over a week or longer depending on the environmental conditions (Ritchie et al. 1993). If the silk emergence was delayed and the tassels were flowering and ready to release pollen, crystalized yellow pollen was often collected in the brown bags and was not viable. Generally, maize pollen remains viable to pollinate silks only for 1–2 hours after pollen shedding (Luna et al. 2001). Westgate et al. (2003) estimated that individual tassels of three hybrids of (Pioneer Hi-Bred Intl., Johnston, IA) produced 4.5 × 10^6 pollen grains on average, and pollen shedding lasted for 5-6 days with a peak intensity 2 to 3 days of shedding after anthesis. White and light yellow pollen was successfully used for pollination of maize genotypes.
Plate 3.9: Hand pollinating of female flower (silks, 2-3 d old) (a), the fresh stubs catching pollens (b) and covering the ear with the pollination bag (c).
Plate 3. 10: Revitalization of old silks by cutting back using a sterilized scalpel.

Sometimes, if the ears were large enough, and to make the silks more uniform for pollination the top of the emerging ear was cut off with a sterilized scalpel\(^6\) before the silks emerged.

After pollen was collected from the tassel of a donor plant, the bag was marked with a male sign ($\varpi$) followed with the genotype name to indicate that pollen had already been used for this plant. For recording all crosses, the female plant ID (the plants whose ear was being pollinated) was listed first, and the male or pollen donor second (female $\varphi$ x male $\varpi$), followed by the date of pollination. Black permanent marker pen was used for recording in a glasshouse work because all other colours will eventually fade due to sunlight. The ears that had been pollinated were kept bagged with the brown bags (Plate 3. 9,c).

\(^6\) Make sure to clean the scalpel with 70% ethanol before cutting each ear to avoid contamination with or spread of mold or bacteria between ears.
3.1.3.2 Cob harvest with non-transgenic immature embryos

After pollination, plants still needed special care of to ensure high quality embryo production. Both callus induction and subsequent transformation efficiency are reported to be influenced by the quality of the immature embryos (Frary and Earle 1996; Frame et al. 2000; Loganathan et al. 2010). Vega et al. (2008) reported that the transformation frequencies were affected by the used immature embryos grown in various seasons with the highest average of transformation frequencies (18%) achieved using Agrobacterium tumefaciens-mediated transformation of immature zygotic embryos of Hi II maize.

- During the 2 weeks after pollination, plants were watered about 3 times over a 10 day period (depending on the temperature and the season). At 16-25 days after pollination, plants were provided with water twice every 10 days.
- At 15 days post-pollination, pollination bags were removed and the ears exposed to the air to let them dry and to prevent the development of fungal growth and rot.

Embryo size was checked 13 days after pollination by removing individual kernels from the middle of the ear and dissecting out the embryo. Surface sterilization of the ear/cob was carried out using 70% ethanol in the dissection position of the cob in the glasshouse. Kernels were removed using a sterilized scalpel blade and dissected to obtain the intact immature embryo which was measured using a ruler (Plate 3. 11). Afterwards, the husks in the position of dissection were cleaned using ethanol and taken back to cover the cob’s seeds. When the immature embryo size was about 2.00 mm, ears were harvested and stored at 4°C in the fridge for up to three days to further dissection.
Plate 3. 11: Checking embryo size on the plant prior to cob harvesting.

The time taken to develop usable immature embryos which sized 1.5-3.0 mm in length was different (Table 3. 2). This variation was correlated with genotype and also with glasshouse temperature (average of 19.7°C) (Figure 3. 1), and light.

Table 3. 2: Number of days post pollination to harvest the cobs containing immature embryos.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number of the days from planting until</th>
<th>Pollination (♀ x ♂)</th>
<th>Cob Harvesting</th>
<th>Number of days post pollination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil.1</td>
<td></td>
<td>85</td>
<td>105</td>
<td>20</td>
</tr>
<tr>
<td>Basil.2</td>
<td></td>
<td>88</td>
<td>109</td>
<td>21</td>
</tr>
<tr>
<td>Ghota.1</td>
<td></td>
<td>95</td>
<td>116</td>
<td>21</td>
</tr>
<tr>
<td>Ghota.82</td>
<td></td>
<td>104</td>
<td>126</td>
<td>22</td>
</tr>
</tbody>
</table>
Figure 3.1: Glasshouse temperature, May to August 2011, during the growth of donor plants to produce immature embryos, the first experiment.

Five to 10 ears were required for the lab work to carry out the transformation experiments each week and this required attention to detail in the plant growing schedule. However, when there was a delay in transformation experiments in the lab and the number of maize ears that were almost ready to transfer and exceeded the lab work capacity, the glasshouse temperature was lowered to slow development down by opening the glasshouse doors and some maize plants were transferred outside the glasshouse where the temperature was lower than the glasshouse temperature. By judicious manipulation of sowing dates and glasshouse temperature the laboratory was constantly provided with suitable ears for transformation according the lab capacity to manage the Agrobacterium transformation experiments properly.
3.2 Harvesting of maize seeds in Glasshouse grown plants

The supply of Syrian genotypes was limited at the beginning of the study and there was no opportunity to obtain further seed due to the civil unrest in Syria and so a protocol had to be established to produce seed for experiments later in the programme of study. It is well known that the production of seeds in the glasshouses can vary greatly according the genotypes and from season to season. Furthermore maize seed production is acknowledged to be very difficult in the glasshouse and needs a lot of care because it is very susceptible to insect and disease injury. To reduce the risks of transformation failure that can result from poor seed giving poor explant material, special care of and daily monitoring was carried out inside the glasshouse.

It is important that the seed source be reliable and that the seeds harvested are not contaminated by disease organisms. For production of mature maize seeds in the glasshouse, a few routine steps regarding the irrigation and temperature were followed:

1. First of all, after pollination, the ears that were covered with bags for 2 weeks were exposed to air by raising the pollination bag. When mold appears on the husk within 2 wk covering, pollination bag should be lifted earlier.

2. Four to 5 weeks post-pollination watering was stopped to let plants dry down.

   At the same time, at 25-30 days, the cobs were exposed by pulling down the husks to allow the kernels to further dry.
3. Around the sixth week after the pollination, signs of plant maturity began to appear. Thereafter, seeds were checked on the ears by observing their softness/solidity. Finally, cobs were harvested once seeds showed a black layer at the seed base (Plate 3. 12). This sign is an indication that carbohydrate nutrition has stopped being sent to the seed from the rest of the plant. Mature ears on the plant should be bagged to protect them from birds when the glasshouse door is opened (Plate 3. 13).

4. The harvested maize cobs were left on the shelves in the glasshouses for a few days to dry further, then bagged and stored in a cool, dry place in a capped jar (Jones 2008) in preparation to grow them for the next experiment. However, standard grain storage procedures can prevent the development of fungal disease in stored grains.

5. Generally, 13 to 14% of the grain moisture is the recommended level of moisture for long-term maize grain storage (Munkvold and Desjardins 1997).

6. Sometimes, ears were contaminated by fungus that continues to grow with seeds and this affected seed vitality. Such seeds were cleaned externally using a 70% ethanol dip before storage.
Plate 3. 12: Harvesting of immature cobs and mature maize seeds in the glasshouse.

Plate 3. 13: Yield losing caused by birds consuming on uncovered ears.
3.3 Troubleshooting in the Glasshouse

Crop protection is essential to safeguard maize seed production from pests and pathogens. Pests and diseases impact on seed quality, and also reduce plant resource-use efficiency.

Pests and diseases can thrive and multiply in warm glasshouse conditions and can be difficult to eradicate. The main pests under glass are aphid and red spider mites. Aphids are members of the superfamily Hemiptera: Aphidoidea, are also known as greenflies or blackflies in Britain.

There are diverse ways that through which aphids weaken their host plants. Firstly, during the feeding phase on the plant, they divert nutrients that are necessary for plant growth for their own profit through sucking the plant sap. Moreover, once aphids have pierced the phloem with their stylets, they inject their saliva into the plant which could be phytotoxic. Secondly, aphids can transmit plant viruses in their saliva. Numerous viruses are transmitted by aphids and nearly 50% of insect-borne viruses (275 out of 600) are transmitted by aphids (Nault 1997). Finally, infection of numerous aphids hinders the photosynthetic activity by forming black, sooty molds that frequently grow on the honeydew excreted by the aphids and which can cover the leaves. Aphids are serious pests because their high asexual reproductive potential and their adaptability to environmental conditions in order to survive. Due to their unusual lifecycle, they can reproduce very quickly. In summer, aphid populations can double every 3-4 days under ideal conditions. In the worst cases of aphid infestation, flowering may fail (Plate 3. 14).
The mechanisms of plant resistance to aphids can be described as: (i) antixenosis, implying that pests move away from the plant as it is rapidly recognised as a poor host by the pest; (ii) antibiosis, when the survival and fertility of pests feeding on the plant are affected significantly; (iii) tolerance, when the susceptible plant is more affected by pest feeding than the tolerant plant that infested by pest but can still multiply and survive (Webster 1991). These types of resistance have been reported for interactions between the aphids and crops for most species.

Plate 3. 14: Failure of male flowering resulting from serious aphid infestation in the glasshouse.
3.4 Biological control and cultural management practices

In the hot and humid environment of the glasshouses, aphid pests can quickly become a serious problem. In spite of the availability of insecticide treatments to control aphids and other plant pests, biological control and cultural management practices can be used for pest control in the glasshouse where semi-containment of insect populations is possible. Nowadays the hazards of pesticides both to health and environment are well recognised (Geiger et al. 2010). Furthermore, the use of pesticides reduces the opportunities for biological pest control as they are generally indiscriminate in their target species. Crop protection against aphids must be planned according to Integrated Pest Management (IPM) rules. The IPM concept has been elaborated by many researchers (Jacobsen 1997; Way and van Emden 2000; Jacobsen et al. 2004). Nowadays insecticides are used as the last resort in commercial glasshouses. In IPM systems which aim to minimize dependence on pesticides, it is essential that the conservation of natural enemies to pest species is undertaken.

Aphids are attacked by a large range of natural enemies. These include parasitoids (Frank 2010; Boivin et al. 2012), predatory species (flying predators e.g. ladybird beetles, (Bianchi and Van der Werf 2003), and others (Schmidt et al. 2003; Rutledge et al. 2004) and pathogens like fungi, bacteria, nematodes and viruses (Helyer et al. 2014).

An integrated pest management system was employed to control the aphids using biological control based on ladybirds which are a member of the Coccinellidae and are a family of small beetles, ranging from 0.8 to 18 mm (Seago et al. 2011).
Once a sign of aphid infestation was registered on the maize plants (Plate 3. 15), biological control and practical cultural practices were conducted.

Plate 3. 15: The first sign of aphid infestation under the maize leaf in glasshouses.

Ladybirds were collected from local gardens between March and May as the adults were available in this period according to their life cycle (Figure 3. 2). They were released in the glasshouse in the early morning (at a temperature of about 20 °C) by gently scattering over affected plants near the aphids infestation. Once the ladybirds were released, they immediately started searching for their food (Plate 3. 16).
Figure 3.2: Illustration of the life cycle of a 7-spot ladybird. Modified from the UK Ladybird Survey (http://www.ladybird-survey.org/lifecycle.aspx 2014).
Ladybirds and ladybird larvae consume large numbers of aphids daily. The adults of ladybirds also lay 20 plus eggs per day under the leaves of plants. When these eggs hatch, the larvae will commence feeding immediately. Adults tended to move on when their food source became scarce, while the larvae remained and searched for more prey. As a result, introducing a range of ladybirds through the growth stage of donor plants in the glasshouses was helpful to control aphid pests.

It became apparent through observation for pest infestations that the maize genotypes showed different susceptibility to aphids. Some genotypes (Syrian varieties) were observed to have high resistance to aphids while others were susceptible (the inbred lines). Maize resistance to aphids infestation has been identified to be a genetic trait (So et al. 2010), and the inheritance of the corn leaf aphid (CLA) resistance in many plants like barley (Verma et al. 2011) is related to the inheritance of recessive genes.
3.5 Cultural practices management

The key for controlling the problems caused from pests inside glasshouses is to keep the glasshouses clean and tidy and to keep a watchful eye on the plants and dealing with outbreaks of pests and diseases early on.

Starting off with a clean glasshouse before transferring the plants is one of the most important strategies for the production of healthy plants and controlling pests. Glasshouses were cleaned by removing any weeds, dead plants or rotting leaves, and then the glass was washed with warm soapy water. Sometime, pest and disease problems can be carried over from the previous experiments. As a precaution, glasshouse equipment and the old pots were washed with garden disinfectant (Jeyes fluid)\(^8\). A new experiment was established using fresh compost and clean pots. All these procedures should help to prevent pest infection and fungal diseases.

In summer when the temperature increases, the glasshouse becomes hot and dry (temperature and humidity are generally inversely related in temperate climates). Consequently, glasshouse red spider mite populations can build up and thrive rapidly. Glasshouse red spider mite is one of the most troublesome pests of glasshouses plants. It is a sap-sucking mite that attacks the foliage of plants causing mottled leaves and early leaf loss. Smooth webbing on the plants and yellow or bronze speckling on the upper leaves are signs of a red spider infestation (Plate 3. 17). When inspection, the underside of the leaves have many tiny yellowish green mites. Eventually the leaves lose most of their green colour and dry up. Glasshouse red spider mite was only a problem in the periods between May and August when the glasshouses conditions became hot and dry.

\(^8\) For cleaning the glasshouse a long handled tools were used for safe working from ground level. Eye protection and gloves were worn for safety.
To control the red spider mite, cultural management practices (non-chemical control) were carried out as an alternative to using insecticides. This avoids insecticide resistance problems and the risk of spray damage to the plants and to other biological control species. In some instances it was necessary to move most of the plants outside the glasshouse during the summer months (Plate 3. 18). For those that remained inside the glasshouse, they were sprayed with water using a hand sprayer to keep the humidity level high which helps to reduce or prevent red spider mite. Also, the floor of the glasshouse was sprayed with water to increase the humidity.
When signs of insect infestation were registered on the leaves at an early stage, suitable procedures were conducted for pest control:

1- The infected section of the maize plant was snipped off using garden shears and dropped into a plastic bag and discarded to prevent further spreading of aphids.

2- Aphids and spider were crushed using fingers or were killed using a tissue wipe wetted with 50% alcohol. It is difficult to kill large infestations simply by crushing them, but killing some of them sends off an alarm pheremone chemical signal that warns other aphids to stay away. Routine patrolling and crushing proved to be very effective at reducing the aphid population. This procedure helped reduce the pest populations and when combined with the other effective practices mentioned, a very effective pest control strategy was achieved.

3- Keeping a small and weak plant in the glasshouse encouraged aphids to move away from the adult plant to more appetizing plants (small plants). Then, this sacrificial plant was destroyed in a plastic bag.
Plate 3. 18: Plants located outside the glasshouse to avoid a red spider infection.

Improved plant protection strategies to prevent pest infection and pest damage can increase production and make the immature embryos more susceptible to _Agrobacterium_-mediated transformation and to callus induction.

It is vital that this issue is addressed, not only to enhance the productivity of healthy embryos, but also to preempt problems of emerging invasive pests and diseases.

3.6 Nutrient deficiency-symptoms and treatment: Calcium deficiency

Mineral elements are very important factors to plant growth. Macronutrients (N, P and K), also Mg and Ca are essential factors that plants require in relatively large quantities for growth and development. Nitrogen and Phosphorus are involved in the
composition of amino and nucleic acids, phosphoproteins, phospholipids, dinucleotides, and adenosine triphosphate. Potassium plays a vital role in photosynthesis, protein synthesis and glycolytic enzymes (Hu and Schmidhalter 2005). Micronutrients are also very important elements required in smaller quantities but are also essential for healthy plant growth. Nutrients are required for many biological processes including photosynthesis, regulation of biological processes in the plant and storage and transfer of energy and carbohydrates. Often, signs of mineral element deficiency for a particular plant species appear as a result of the interaction of several minerals and strategically important developmental pathways. Deficiency signs differ both according to the nutrient element and the severity of element deficiency and plant genotype. The uptake of nutrient by plants is genetically determined and the plant ability of transport and accumulation of nutrients can differ both between and within plant species (Marschner and Marschner 2012).

Calcium plays an essential role in plant growth. Plants can both sense and resist abiotic and biotic stress through the effects of calcium on membrane structure. Also stomatal function, cell division and cell-wall synthesis and many physiological processes that influence both growth and plant response to stress have a calcium component (McLaughlin and Wimmer 1999). Calcium deficiency has been recognized in maize plant leaves as a characteristic symptom, in which maize plants developed rippled edges of leaves known as he "bull-whip" symptom (Kawasaki and Moritsugu 1979).

The genotypes studied showed differential response to calcium deficiency. The Lines were more susceptible to calcium deficiency than the varieties. When calcium deficiency occurred, plants of Syrian Lines and Hi II hybrid leaves became curled with rippled edges. Affected plants developed un-pigmented patches or lesions on
the leaves. In some cases, when the calcium deficiency was very severe, the leaves
developed in a whorl wrapped tightly around each other and eventually rotted (Plate
3. 19). Severe calcium deficiency caused of susceptible plants to be severely stunted.
The difference in the plant capacity to absorb calcium appears to have differed with
the different cultivars of maize plants since plants were grown in homogenized
compost. The emergence of this characteristic symptom of a calcium deficiency in Hi
II and Lines plants was more frequent than the varieties and the hybrids were more
tolerant of calcium deficiency. This observation of varied plant tolerance of nutrient
deficiency indicated a genetic dependence and this was consistent with that
postulated by Marschner Horst and Petra Marschner (2012).

Plants suffering from calcium deficiency were treated with a calcium/magnesium
recipe by implementing the following steps.

- Calcium and magnesium solution. Firstly, 2 stocks of nutrient solution were
  prepared separately as follows:

  Stock.1: Calcium nitrate Ca(NO₃)₂. 4H₂O: Molar mass is: 236.15 g mol⁻¹
  720 gL⁻¹ (Fisher Scientific, Product Code: 10000080)
  Stock.2: Magnesium sulfate MgSO₄.7H₂O: Molar mass is: 246.48 g mol⁻¹
  370 gL⁻¹ (Fisher Scientific, Product Code: 10264630)

- 10 mL was added from stock 1 and stock 2 to the final irrigation solution water
  of 10 litres. This recipe was recommended by the Department of Agronomy,
  Iowa State University, USA.

- Plants were watered with this final solution (mixed Ca⁺⁺/Mg⁺⁺), beginning
  when plants were transplanted into big pots in the glasshouse at which time
  plants were normally at the 6-7 leaf stage. All plants were watered once every
  2 weeks with calcium and magnesium as a precautionary measure.
Plate 3. 19: Signs of calcium deficiency in maize plants.
Chapter 4:

*Agrobacterium* growth and maintenance
4.1 *Agrobacterium* growth and maintenance

To date, reproducible and efficient generation of transgenic maize plants has been dependent on the employment of *Agrobacterium tumefaciens* (*A. tumefaciens*) strains harbouring binary vectors and carrying virulence (Vir) genes such as VirG, VirS and VirE in the Vir region of their Ti plasmid, which render the bacteria hypervirulent (Komari *et al.* 1996; Dym *et al.* 2008; Denkovskienė *et al.* 2015). It has been confirmed that these virulence genes enhance the efficiency of *Agrobacterium*-mediated gene transfer (Park *et al.* 2000). Such *A. tumefaciens* strains have been successfully used for gene transfer to both immature embryos (Shrawat and Lörz 2006; Valdez-Ortiz *et al.* 2007; Zhang *et al.* 2010) and androgenetic pollen cultures (Kumlehn *et al.* 2006). The *A. tumefaciens* strains EHA101 and EHA105, which are widely used for gene transformation, and harbour these additional alleles on their Ti-plasmid-derived helper plasmid (the agropine/L, L-succinamopine-type Ti-plasmid pTiBo542 or pCH32 respectively) need to be maintained by culturing them on appropriate media containing antibiotics to select for all plasmids present in the *Agrobacterium* vector.

The *A. tumefaciens* strains with DNA plasmid constructs used for maize transformation in this study harbor a helper plasmid and an expression vector plasmid. Therefore, not only the maintenance of the *Agrobacterium* with the expression plasmid, but also the maintenance of the helper plasmid is very important to achieve the effective positive transformation.
4.2 Objectives

The purpose of these experiments was to maintain the Agrobacterium strains containing the plasmids vectors by developing of Agrobacterium maintenance procedures in-vitro.

4.3 Materials

Two strains of A. tumefaciens EHA101 and EHA105 harbouring different vectors pTF102 and pBINPLUS/ARS respectively were used in this study.

4.4 Establishment of an Agrobacterium growth system using different components of culture media

Experiments were focused on identifying culture media regimes to optimize A. tumefaciens-mediated stable transformation of Syrian maize genotypes.

The yeast extract peptone (YEP) medium, Luria-Bertani (LB) medium and two different media modified from standard LB medium, LB without glucose LB\textsuperscript{G} and another modified LB medium (LBm) were used to determine the best medium for the A. tumefaciens strains (EHA101 and EHA105) used in this protocol (Table 4.1). All media that were used to culture the Agrobacterium contained appropriate antibiotics to select the Agrobacterium strain with the plasmid contained in the Agrobacterium vector (Table 4.2).
Table 4.1: The ingredients of media used for *A. tumefaciens* growth.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>LB Standard (LB&lt;sup&gt;S&lt;/sup&gt;)</th>
<th>LB without glucose (LB&lt;sup&gt;G&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBm</td>
<td>- Pure water 1L</td>
<td>- Pure water 1L</td>
</tr>
<tr>
<td></td>
<td>- 5g Yeast extract</td>
<td>- 5g Yeast extract</td>
</tr>
<tr>
<td></td>
<td>- 10g Tryptone</td>
<td>- 10g Tryptone</td>
</tr>
<tr>
<td></td>
<td>- 5g Sodium chloride</td>
<td>- 5g Sodium chloride</td>
</tr>
<tr>
<td></td>
<td>- 2g Glucose</td>
<td>- 18g Bacto-agar</td>
</tr>
<tr>
<td></td>
<td>- 15g Bacto-agar</td>
<td>- 15g Bacto-agar</td>
</tr>
</tbody>
</table>

4.4.1 Standard growth curves of *A. tumefaciens* strain EHA101 (harboring pTF102 or APX/ pCGN1578 plasmid) and the *Agrobacterium* strain EHA105 (harboring CBF/pBINPLUSARS plasmid)

Bacterial growth is the division of one bacterium cell into two daughter cells in a process called binary fission (Rogers 2011) but both daughter cells from the division do not necessarily survive. However, if the number of bacteria cells surviving exceeds the average, then the bacterial population undergoes exponential growth. There are several methods to measure an exponential bacterial growth curve through bacterial enumeration (cell counting), direct and individual methods (microscopic, flow cytometry) (Skarstad *et al.* 1983), direct and bulk (biomass). As
well as, indirect and individual (colony counting), or indirect and bulk (most probable number, turbidity, nutrient uptake) methods (Zwietering et al. 1990).

An indirect and individual method was followed in the following protocol for the determination of a calibration curve which is a common method for estimating the concentration of bacterial cells in an unknown sample by comparing the unknown to a set of standard samples of known concentration:

1. The *Agrobacterium* was streaked out from the glycerol stock culture on three LB plates containing appropriate antibiotics for both *Agrobacterium* strains EHA101 and EHA105 harbouring the different plasmids (Table 4.2). Two different types of streaking methods were used to streak a plate using a sterile inoculation loop (Black 2008), the three-phase streaking pattern, known as the triplet streak (T-Streak), and a different style in streaking direction as a criss-cross streaking (Zigzag Inoculation) which is that used in the Appalachian Fruit Research Station, USDA-ARS (from where the EHA105 CBF strain was obtained). After that, the plates were incubated for 2 days at 28°C (Plate 4.1).

![Plate 4.1: Examples of plates of A. tumefaciens strain EHA101 on LB standard medium after 2 days incubation from streaking.](image-url)
2. After two days when the Agrobacterium had grown and produced well-formed colonies, the cells were harvested by placing 2 mL of LB + antibiotics on each plate. Then, bacterial cells were scraped together with a sterile spreader and transferred to a sterile labelled tube. The optical density (OD\textsubscript{600 nm}) was measured spectrophotometrically (Helios Epsilon spectrophotometer) at 600 nm.

3. A serial dilution (Figure 4.1) was conducted in thirteen tubes containing 9.0 mL of LB liquid medium amended with appropriate antibiotics for each strain. One mL of Agrobacterium suspension was then taken from each tube of the serial dilution to measure the OD\textsubscript{600 nm}. Then, 0.1 mL of each tube was cultured in three replications on LB + antibiotics agar plates and cultured at two different incubation temperatures (28°C and 19°C) in the dark for 3 and 5 days respectively. Colony forming units (CFUs) were counted and the relationship between the number of bacteria colonies and their OD was observed to draw the standard curve of Agrobacterium growth. All steps were carried out in a sterile environment near a flame.

Data showed that the number of bacterial colonies CFUs grown on the agar media was positively correlated with the OD of the bacterial suspension for each dilute in the serial dilution (Figures 4.2 and 4.3).
Figure 4. 1: A serial dilution of the broth culture of the *Agrobacterium* strains used in this study for maize transformation.

It is possible to estimate the number of viable bacterial cells in the *Agrobacterium* broth by reading the optical density and plate the result on the standard growth curves that obtained for both *Agrobacterium* strains EHA101 and EHA105 (Augustin *et al.* 1999). Thus, the efficiency of *Agrobacterium* involved in the infection of immature embryos can be estimated by knowing the OD of *Agrobacterium* inoculum used in maize transformation experiments.
Table 4.2: Appropriate antibiotics used for maintenance the *Agrobacterium* with the vector plasmids and the helper plasmid containing in the studied constructs.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Antibiotics</th>
<th>Kanamycin 50 mgL⁻¹</th>
<th>Gentamycin 50 mgL⁻¹</th>
<th>Tetracycline 10 mgL⁻¹</th>
<th>Spectinomycin 100 mgL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHA101 (pTF102)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHA105 (CBF/pBINPLUSARS)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHA101 (APX/pCGN1578)*</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHA105 (SDO/BINPLUS/ARS)</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EHA105 (SDO/pCGN1578)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*APX and SOD constructs were not used in transformation experiments in this study. But, the pTF102 and CBF constructs were used to transfer Syrian maize. APX and CBF constructs were used to identify a suitable culture media regime in order to maintain the *A. tumefaciens* EHA101 and EHA105. These constructs were kindly provided by Professor Michael Wisniewski and Dr Timothy S. Artlip, Appalachian Fruit Research Station- United States Department of Agriculture /Agricultural Research Service (USDA-ARS).

Figure 4.2: The relationship between bacteria colonies forming and the optical density of *Agrobacterium* EHA101 strain.

\[
y = 0.6241x + 0.0004 \\
R^2 = 0.9293
\]
Figure 4. 3: The relationship between colony-forming units and the optical density of *Agrobacterium* EHA105 strain.

### 4.4.2 Differentiation of *A. tumefaciens* strains growth on different media

A 50 μL glycerol stock of EHA101 c and EHA105 strain was grown in a 10 mL tube of LB medium overnight on a shaker (ca. 100 rpm) at 28°C. On the second day, 100 μL of *Agrobacterium* suspension was resuspended with 9.9 mL of the studied liquid media mentioned above (Table 4. 1) which was amended with 50mgL⁻¹ kanamycin (to maintain the *Agrobacterium* strains EHA101 and EHA105 and for pBINPLUS/ARS vector), and 50mgL⁻¹ gentamycin (to maintain the pCGN1578 plasmid) or 10mgL⁻¹ Tetracycline (for pCH32) (Table 4. 2).

Three tubes of each studied medium (LB⁻ᴳ, LBm, YEP and LBS as a control) were placed on the shaker (ca. 100 rpm) at two different temperatures (28 and 19)°C. The optical density of *Agrobacterium* cultures was measured at three concentrations of the media pH (6.8, 7.0 and 7.2) for each medium after 24h, 48h and 72h of the bacteria incubation.
4.5 Results

4.5.1 Media effects

Results showed highly significant differences between the culture media used for both Agrobacterium strains (Table 4.3). The YEP medium has the highest value of optical density of the Agrobacterium strain EHA101 with significant differences in comparison with the LBm (Figure 4.4). The response of Agrobacterium EHA101 to growing in the YEP medium was higher than the “control” treatment LB$_S$. The Agrobacterium strain EHA105 responded significantly to the modified LB medium (LBm). The number of bacteria growing in the LBm medium was higher than the bacterial colonies number growing in the control or in the YEP and LB$_G$ with significant differences (Figure 4.5).

Table 4.3: Fishers significance test (p value) and Least Significant Difference (L.S.D) values of the optical density of Agrobacterium strains at 5 and 1% level of significance (two-tailed).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>P value</th>
<th>L.S.D (0.05)</th>
<th>L.S.D (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EHA101/EHA105</td>
<td>EHA101/EHA105</td>
<td>EHA101/EHA105</td>
</tr>
<tr>
<td>Media</td>
<td>0.000***</td>
<td>0.016/0.009</td>
<td>0.021/0.011</td>
</tr>
<tr>
<td>pH</td>
<td>0.634/0.75</td>
<td>N.S</td>
<td>N.S</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.000***</td>
<td>0.011/0.006</td>
<td>0.015/0.008</td>
</tr>
<tr>
<td>Time</td>
<td>0.000***</td>
<td>0.014/0.007</td>
<td>0.018/0.010</td>
</tr>
</tbody>
</table>

Significant - * Very significant - ** Highly significant - *** N.S. Non-significant
Figure 4. 4: The effects of culture medium on the *Agrobacterium* EHA101 growth.

Figure 4. 5: The effects of culture medium on the growth of the *Agrobacterium* strain EHA105.
4.5.2 Temperature effects

Results showed that the *Agrobacterium* growth was significantly influenced by the temperature of incubation (ANOVA $p< 0.001$). After 24 hours of incubation, the bacterial density at 28°C was higher than the bacterial density at 19°C for both *Agrobacterium* stains. But within 48 h of incubation the optical density of bacteria incubated at 19°C increased rapidly overtaking that at 28°C for the strain EHA101. After 48h the bacteria were still growing but the rate of growth started to slow down at both temperatures as the cultures moved into the stationary phase and growth became stable. So, the optimal time for *Agrobacterium* growth was between 24 and 48 h of the incubation. It was suggested that bacterial cells should be harvested after 24h at 28°C incubation in order to maintain competency of the cells for transformation. The bacterial cells density was still low after 24h at 19°C and for that reason; it is suggested to harvest the bacteria cells after 48h at 19°C to obtain the optimal density of bacteria EHA101 (Figure 4. 6). But, the optimal bacterial cells density of *Agrobacterium* strain EHA105 could be obtained after 48h at 28°C (Figure 4. 7).
Figure 4. 6: Combination of temperature and time of bacteria incubation, and their effects on the optical density of *Agrobacterium* EHA101.

Figure 4. 7: Combination of temperature and time of bacteria incubation, and their effects on the optical density of *Agrobacterium* EHA105.
4.5.3 pH effects

The total means of the optical density, expressing bacterial cell growth, did not differ significantly with the pH treatments (ANOVA p > 0.05) (Figure 4. 8). However, there were high significant differences in the time of incubation according to the optical density of bacteria cultures (Table 4. 3).

![Figure 4. 8: The effects of media pH values on the growth of Agrobacterium strains.](image)

4.6 Discussion

It was observed that the *A. tumefaciens* growth curve was affected by temperature, time of incubation and the kind of media incubation.

After 48h of bacteria incubation, the *Agrobacterium* growth curve showed a slowing down in growth probably as result of limited nutrient medium. This means that the optimal time for the growth under both temperature tested was 48h incubation which was the maximum growth rate. The slope of the straight line of
Exponential growth is the specific growth rate of the organism. The actual rate of this growth (i.e. the slope of the line in the figure 8) depends upon the growth conditions, which affected the frequency of cell division events and the probability of both daughter cells surviving.

Temperature had a statistically significant effect on *Agrobacterium* growth, conforming with Peleg and Corradini (2011), and Rossetti *et al.* (2011). It was demonstrated that differences in *Agrobacterium* growth attributed to differences in temperature were not the sole explanation for the evolution of life cycles dominated by time and media. Moreover, at the beginning, the number of bacterial cells growing at 19°C was lower than cells growing at 28°C, but the growth curve was superior at 19°C after 48h of incubation (Peleg and Corradini 2011). These findings indicated that the strain EHA101 was more adapted for 19°C after 48h, while the *Agrobacterium* strain EHA105 adapted to 28°C after 48h (Figure 4.7).

There were no significant interactions among different media to all pH treatments (P > 0.05). This result refers that *Agrobacterium* strains adapts a wide range of pH (6.8, 7.0 and 7.2), but it tends to grow best on a slightly acidic medium (pH, 6.8).
4.7 Conclusion

The results showed that the *Agrobacterium* strains EHA101 and EHA105 were able to grow on the studied media but, there were small but significant differences between treatments regarding to the *Agrobacterium* ability of growth. YEP medium was more suitable for the strain EHA101 with the highest value of the optical density whereas growing the strain EHA105 on the modified LB medium resulted in the highest value of bacterial density. The optical density of bacteria cells of EHA101 and EHA105 was obtained for 48h of incubation at 19°C and 28°C respectively. That means, bacteria could be incubated and grown at 19°C or 28°C for 48h to harvest the maximum of bacteria cells for initiation of infection experiments. Bacteria were able to grow after 48h, but their activity declined. So, the optimum time of growth was 48h of incubation. In spite of this, the *Agrobacterium* strains had ability to grow in all studied media with varying pH, but they trended to grow slightly better in the acidic medium (pH= 6.8).

It can be concluded that both temperature and the time of incubation affected *A. tumefaciens* growth within the studied media. Finally, bacteria are maintained in exponential growth phase by culturing it on a suitable medium with pH= 6.8 at 19°C for 48h on YEP for EHA101, and at 28°C for 48h on LBm medium for the *Agrobacterium* strain EHA105.
4.8 Care and maintenance of recombinant *A. tumefaciens*

It is well known that recombinant bacterial strains containing introduced plasmids can be environmentally “less fit” than “wild types” and there is a tendency for recombinant stains to lose their plasmids and convert to wild type. Recombinant plasmids therefore have antibiotic resistance genes inserted in them and recombinant bacterial strains are then kept under constant antibiotic challenge in order to kill wild type revertant cell lines and in reverse maintain a pure transgenic culture. In order to maintain the recombinant strains for a long time without losing the plasmids or losing the strain entirely from a laboratory accident such as incubator malfunction, glycerol stocks are prepared and subsequently maintained in the freezer for future use.

Whilst subsequent regrowth on an antibiotic containing medium reaffirms the presence of the plasmid, it is pertinent to either check the culture from time to time to ensure that the transgene is still present and intact by PCR or by DNA preparation.

4.9 Preparation of a -80°C freezer stock of *A. tumefaciens*

The protocol for making a -80°C freezer stock was as follows:

- The transgenic *Agrobacterium* was streaked out on three LB + appropriate antibiotic plates; and then incubated for 2-3 days at 28°C.

- When the colonies had grown well, 1 mL of LB + 60% glycerol was placed on each plate and cells were scraped together from the surface of the agar with a sterile spreader and then transferred to a sterile microfuge tube with a screw-cap or cryotube specifically made for freezer stocks that had been labelled.
Chapter 4

➢ The microfuge tube/cryotube was then plunged into liquid nitrogen to effect an immediate rapid freeze and to promote a “glassy” state with no/small ice crystals which could rupture the bacterial cells, and then transferred directly to a -80°C freezer.

For reuse, a freezer stock tube was allowed to thaw slightly, and a small amount of the stock solution was gathered up on the tip of a sterile pipette and transferred to an LB + antibiotic plate; to initiate a new experiment, a triple streak was done as above; and incubated for 2-3 days. All steps were carried out in sterile transfer conditions (benchtop flame aseptic technique).

4.10 Plasmid Extraction

Plasmid extraction was carried out in order to confirm the presence of the expression vector introduced into A. tumefaciens strains used in this project.

Plasmid DNA from recombinant Agrobacterium strains was isolated following the method of Voo and Jacobsen (1998). An overnight recombinant Agrobacterium culture (5 mL) was harvested by centrifugation at 12,000 x g for 1 minute, and the pellet subjected to a modified alkaline-SDS Lysis procedure followed by adsorption of the DNA onto silica in the presence of high salts. Contaminations (biochemical and cell debris) were then removed by a spin-wash step. Finally the bound DNA was eluted in molecular biology grade water following the instructions from the kit supplier (Sigma cat PLN70) in the following steps:

1. Harvest and Lyse bacteria cells: Agrobacterium cells were pelleted from 5 mL “LB broth” of an overnight recombinant culture by centrifugation at 12,000 x g for 1 minute. After discarding the supernatant completely, the pelleted bacterial cells were re-suspended with 200 μL of the resuspension solution by
pipetting up and down to thoroughly homogenise the cell suspension. Then, 200 μL of Lysis solution was added and the re-suspended cells mixed by gently inverting the tube 6-8 times until the mixture became clear and viscous (around 5 minutes).

2. **Prepare cleared Lysate**: 350 μL of the Neutralization binding solution was added to the cell debris, and the tube was inverted gently 4-6 times. Then, the cell debris was pelleted by centrifuging at 12000 x g for 10 minutes.

3. **Prepare binding column**: 500 μL of the Column Preparation Solution was added to each miniprep binding column and centrifuged at 12,000 x g for 1 minute. The liquid flowing through the column was discarded.

4. **Bind plasmid DNA to the column**: The cleared lysate was transferred to the column prepared in the previous step, and centrifuged at 12,000 x g for 30 seconds to 1 minute.

5. **Wash to remove contaminants**: 500 μL of wash solution was added to the column. The flow-through liquid was discarded after 1 minute of centrifuging. 700 μL of diluted wash solution (with 100% ethanol) was added to the column to remove the residual salts and other contaminants introduced during the column load by centrifuging at ≥ 12,000 x g for 30 sec to 1 minute. After that, the column was spun again at maximum speed for 1 to 2 minutes to dry the column and thoroughly remove excess ethanol.

6. **Elute purified plasmid DNA**: the column was transferred to a new collection tube. 100 μL of molecular biology reagent grade water was added to the sample to elute the DNA by centrifuging at 12,000 x g for 1 minute. Finally the pure plasmid DNA was either run out on agarose gels or stored at -20°C for future use.
Agarose-gel results examined under UV light were showed a highly resolved molecular weight band indicated presence of relevant plasmids in the studied *Agrobacterium* strains (Figure 4. 9). A photograph was made using the gel documentation system (Section: DNA agarose gel electrophoresis, Chapter 6).

Figure 4. 9: Agarose gel to confirm the presence of the relevant plasmids in the studied *Agrobacterium* strains EHA101 (pTF102) and EHA105 (CBF). Lane L, 100 bp ladder; Lane MW, the control (molecular water).
Chapter 5:

Production of genetically altered maize plants using

*Agrobacterium*-mediated transformation
5.1 Production of genetically altered plants using *Agrobacterium*-mediated transformation

The genetic transformation methods often used by genetic engineers are many and varied. Genetic transformation using direct or indirect methods is an effective means to integrate beneficial genes from wild relatives or unrelated species into crop plants for the production of genetically altered plants with improved specific traits. Despite an initial recalcitrance to *Agrobacterium* in recent years, genes and techniques have become available using *Agrobacterium tumefaciens* mediated transformation of cereals (Ke *et al.* 2001; Repellin *et al.* 2001; Sahrawat *et al.* 2003). Frame *et al.* (2011) have reported an approach for an *Agrobacterium*-mediated transformation protocol used efficiently to transform of two distinct maize genotypes, Hi II hybrid and inbred B104 line through direct targeting the immature zygotic embryos (IZEs). However, *Agrobacterium* mediated genetic transformation of cereals has been largely confined to particular genotypes that combine the amenability to gene transfer by *Agrobacterium* with adequate in-vitro regeneration potential. Such restricted genotype limitation severely limits the wide use of this technique.

Transformation frequency of *Agrobacterium*-mediated plant transformation is influenced by several factors such as: bacterial, plant and environmental factors (Tzfira *et al.* 2002). At the plant level, genotype and concentration of AgNO₃, 2,4-D in the culture medium are the important factors that need to be optimized in *Agrobacterium*-mediated transformation systems (Wei 2009). On the bacterial side, strains ability to attach and transfer its T-DNA to the host cells (Cheng *et al.* 2004), and the density of the bacterial culture (Cheng *et al.* 2004; Opabode 2006), immersion time (Xing *et al.* 2007), co-cultivation conditions such as period of co-
cultivation (Vasudevan et al. 2007), and temperature conditions (Salas et al. 2001) were described to influence the transformation frequency.

An efficient transformation protocol is one of the basic needs in genetic improvement of crops via *Agrobacterium*-mediated transformation. But, amenability of genotypes to gene transfer by *Agrobacterium* with adequate regeneration potential is also an essential requirement to achieve successful transformation (Lawrence and Koundal 2001). It is important to mention that protocols efficiently used for cereal transformation generally rely on the use of hypervirulent *Agrobacterium* strains such as EHA101 (Frame et al. 2002; Reyes et al. 2010) and EHA105 for maize (Zhang et al. 2010).

To date it is not known whether or not indigenously bred Syrian maize genotypes have suitable *in-vitro* regeneration capability or transformation potential. The main intention of this chapter is to investigate the amenability of Syrian genotypes to gene transfer using *Agrobacterium tumefaciens* strains, EHA101 and EHA105, and to know whether or not Syrian maize genotypes can be transformed via *Agrobacterium tumefaciens*. Comparison between Syrian varieties and hybrids responses to transformation with anti-stress genes was investigated. Also, the transformation ability and the regeneration potential between the Syrian genotypes and the “cv line” (Armstrong et al. 1992; Shrawat and Lörz 2006), Hi II hybrid maize were carried to demonstrate which has a good efficiency of transformation by *Agrobacterium*. Thereby, the key question, can Syrian maize genotypes be transformed via *Agrobacterium tumefaciens*- mediated transformation? can be answered.
5.2 Objectives

The purpose of this study was to firstly determine if Syrian maize genotypes would respond to callus induction, or not, using maize immature zygotic embryos as this is a pre-cursor to maize transformation by *Agrobacterium tumefaciens*. Secondly, can these genotypes be transformed via *A. tumefaciens*-mediated transformation?

These experiments were also conducted to understand the potential of the Syrian maize genotypes for *in-vitro* regeneration and to produce abiotic stress-tolerant plants that could serve as an effective tool for improving the maize tolerance to drought and salinity in dry and semi-arid areas. Future development of maize breeding in Syria could thus involve the production of transgenic plants from genotypes which are already adapted to local conditions, recognizing the importance of development of local varieties in plant breeding programmes.

Maize transformation was conducted at the School of Biological Sciences Facility, Plymouth University using maize immature embryos harvested from the glasshouses at the university. The protocols were approved by the School Biological Safety Committee and full GM safety procedures were followed throughout. During the conduct of this project, the procedures and facilities were inspected twice by the HSE Inspector and approved.

5.3 Materials

5.3.1 *Agrobacterium tumefaciens* strains: Transformation of maize was conducted using two strains of *Agrobacterium*, EHA101 and EHA105. The *A. tumefaciens* strain EHA101 (Hood et al. 1986) harbouring a standard “11.6 kb” binary vector pTF102 (Figure 5. 1) was used to establish a general protocol of
Syrian maize transformation in this study. The pTF102 contains a spectinomycin-resistant marker gene (aadA), the “bar” selectable marker (phosphinothricin acetyltransferase gene driven by the cauliflower mosaic virus CaMV35S promoter) and a P35S-Gus-int reporter gene cassette (β-glucuronidase [GUS] gene with an intron driven by the CaMV35S promoter).

The second Agrobacterium strain was the A. tumefaciens strain EHA105 harbouring the pBINPLUS/ARS:pRTL2:CBF vector (14.58 Kb) containing the enhanced 35S-PpCBF1 fragment (a CBF transcription factor).

Figure 5.1: T-DNA region of standard binary vector pTF102. LB, Left border; RB, right border; bar, phosphinothricin acetyltransferase gene; gus-int, β-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator; H, HindIII. Adapted from Frame et al. (2002).

5.3.2 Plant materials: F2 immature zygotic embryos (IEs) (embryo lengths <1.5mm, 1.5 to 2mm and >2mm) of the maize Hi II hybrid genotype and IEs of Syrian maize hybrids (Basil.1 and Basil.2) and varieties (Ghota.1 and Ghota.82) were used in this study.

5.3.3 Plant tissue culture media: The nutrient media used are summarised in the general materials and methods (section 2.5). According to the protocol obtained from (Frame et al. 2005), infection medium (IM), solid co-culture media (CCM) as well as solid media for callus induction (CIM) or (resting
medium “RM”), Selection Medium I “SM₁”, Selection medium II “SM₂” and regeneration media (RM) are required. IM, CCM and CIM were based on Chu N6 mineral salts and vitamins, (Olhoft and Somers 2001) with cefotaxime and vancomycin antibiotics used instead of carbenicillin. Regeneration medium RM was based on MS mineral salts (Frame et al. 2006a) supplemented with additional components as previously shown in the general materials and methods. The pH was adjusted prior to filter sterilisation of the solutions.

5.4 Procedures

5.4.1 Isolation and preparation of immature embryos

The ears of the selected maize genotypes were harvested 20–22 days after pollination when immature embryos IEs were about 1.5–3.0 mm in length. For surface sterilization ears were immersed in a sterilizing solution [see “embryos dissection” in the general materials and methods] for 20 minutes and finally washed 3 times in sterile distilled water for 5 minutes each. The ears were ready for embryo dissection after thorough draining from the final rinse (Plate 5. 1). This procedure was carried out under sterile conditions within a laminar flow cabinet.
After removing the top of the kernel crowns with a sterile scalpel in a laminar flow cabinet using aseptic technique, IEs were dissected with a lancet and up to 100 IEs collected in a 2 mL screw cap microtube containing 2 mL of IM and washed twice with this medium (Plate 5. 2).
5.4.2 Growth of *Agrobacterium* and co-cultivation of immature embryos (Inoculation)

The *A. tumefaciens* strain EHA101, harbouring the vector pTF102, was pre-cultivated for 2-3 d on solid YEP medium (An et al. 1989) containing 100 mgL$^{-1}$ spectinomycin (for maintenance of pTF102) and 50 mgL$^{-1}$ kanamycin (for EHA101) at 28°C in the dark. The *A. tumefaciens* strain EHA105, harbouring the pBINPLUS/ARS vector (Belknap et al. 2008, Wisniewski et al. 2011) was cultivated on LB medium containing 50 mgL$^{-1}$ kanamycin (for EHA105) and 10 mgL$^{-1}$ tetracycline (to maintain the vector). On the day of transformation, for inoculation, 1 to 1.5 ml of *Agrobacterium* suspension with an OD$_{550}$= 0.3-0.4 (prepared in IM supplemented with AS, see the general materials and methods; “*Agrobacterium* infection”) was added to the embryos IEs after discarding the second wash of
Agrobacterium-free infection medium. Then, embryos were mixed by gently inverting the tube 20 times. After an incubation period of 5 minutes at room temperature, the IEs were transferred to four dry 4.5 cm filter paper disks to remove excess solution. Subsequently, 40 IEs each were placed with the scutellum side up (embryo-axis side in contact with the medium) onto petri dishes containing CCM (Plate 5. 3).

Plate 5. 3: Co-cultivation of immature embryos infected with A. tumefaciens.

Embryogenic callus emerged after co-cultivation from the scutellum cells in the basal scutellum region, and these were targeted for transformation because of their known ability to produce embryogenic callus (Frame et al. 2002). Petri dishes were then sealed with air permeable tape and incubated in the dark at 20ºC for three days.

In parallel, culturing of non-transformed embryos (controls) was conducted on CCM with the same co-cultivation conditions for comparison between the behaviour of transferred embryos infected with A. tumefaciens and non-infected embryos.
behaviour for callus induction and selection events in the presence or absence of bialaphos (bar resistance).

All subsequent procedures (Selection for stable transformation events, Regeneration of transgenic plants and Plant acclimatization) were followed as previously described in the general materials and methods.

5.5 Results

5.5.1 Callus Induction, Development, Regeneration and Rooting

The response of Syrian maize genotypes for callus formation was investigated using three different sizes of immature embryos IEs (<1.5mm, 1.5 to 2.0 and >2.0mm). After three days of co-cultivation stage of Agrobacterium-infected immature embryos on the CCM (fresh medium)\(^9\), infected IEs were transferred and incubated first on the callus induction medium (resting media RM), as described in the methodology, for 7 days to initiate callus formation (Plate 5. 4). Then, after 1 week on the resting medium, embryogenic callus induction frequency (ECIF) for infected studied genotypes embryos co-cultivated on co-cultivation medium containing 300 mgL\(^{-1}\) cysteine was calculated as a percentage of targeted infected embryos.

\(^9\) In infection experiments, fresh co-cultivation medium 1-4 day old was generally used.
Results from these initial experiments showed significant differences ($p < 0.001$) in callus induction within treatments according to IEs size (Table 5. 1). The response of IEs size (1.5 - 2.00 mm) for callus formation was the best (callus induction 76%) (Figure 5. 2). The results of the experiments reported were in agreement with other studies which indicated that embryo size is one of the most significant factors that influences successful Agrobacterium-mediated genetic transformation of cereals (Wu et al. 2003; Shrawat et al. 2007). In addition, the type of plant tissue used for transformation is one of the crucial factors of successful Agrobacterium-mediated transformation of cereals (Nadolska-Orczyk et al. 2000).
Figure 5.2: Effect of immature embryos sizes on callus formation. Values are the means of five replications. Different letters above the standard error bars indicate significant differences between treatments (P< 0.001).

Table 5.1: Fisher’s significance test (p value) and Least Significant Difference (L.S.D) values of callus induction and callus weight at (5 and 1)% level of significance (two-tailed).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Traits</th>
<th>P value</th>
<th>L.S.D (0.05)</th>
<th>L.S.D (0.01)</th>
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<tr>
<td></td>
<td>Callus induction</td>
<td>0.000***</td>
<td>0.001**</td>
<td>8.14</td>
</tr>
<tr>
<td>Treatments (IEs size)</td>
<td>Callus weight</td>
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<td></td>
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</tr>
<tr>
<td>Genotypes</td>
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<td></td>
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</tr>
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<td>Callus weight</td>
<td>0.172</td>
<td></td>
<td>N.S</td>
<td>N.S</td>
</tr>
<tr>
<td>Treatments x Genotypes</td>
<td>Callus induction</td>
<td>0.309</td>
<td></td>
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<tr>
<td></td>
<td>Callus weight</td>
<td>0.172</td>
<td></td>
<td>N.S</td>
</tr>
</tbody>
</table>

Significant -* Very significant - ** Highly significant - *** N.S. Non-significant

Although there were no significant differences between the studied genotypes (p= 0.441) according to the immature embryos response to callus induction based on IEs size, the response of the hybrids (Basil.1 and Basil.2) for callus formation (77 and 81)% derived from the IEs size (1.5-2.00mm) was slightly higher than for the varieties (Ghota.1 and Ghota.82) (74 and 73)% respectively (Figure 5.3).
Previous studies indicated that there are a highly significant impacts of genotype on both induction of embryogenic callus and callus differentiation (Wei 2009). The use of hybrids therefore is recommended for callus induction to initiate *Agrobacterium*-mediated transformation experiments slightly more than varieties.

**Figure 5. 3: The response of selecteed maize genotypes to embryogenic callus induction frequency (ECIF) according to immature embryos sizes. Values are the means of five replications. N= 100-150 IEs.**

### 5.5.1.1 Callus weight (g)

The response of maize genotypes for callus formation and callus development was investigated using callus weight (g) derived from two different sizes of immature embryos IEs (a: 1.5-2.00 mm and b >2.00 (2.00-4.00) mm embryo size. Calluses were weighted after 1 week of embryo culture on the resting medium. Embryogenic callus weight was calculated as a percentage of calluses weight of callus formation derived from targeted infected embryos.

Results showed very significant differences between two different sizes of embryos (p= 0.001) (Table 5. 1) and the weight of callus that formed from different sizes of immature embryos was significantly affected by the embryo size (Figure 5. 4). Embryos of a size bigger than 2.00 mm produced callus that weighed more than...
callus initiated from the 1.5 to 2.0 mm embryos. However, the percentage of callus formation of the big embryos was less than the callus formation % of embryos sized 1.5-2.0 mm which also had a higher percentage of shoot formation (Table 5.2 and Plate 5.5).

![Figure 5.4: Effect of immature embryos sizes on callus weight. Values are the means of five replications each embryo size for five genotypes. Different letters above the standard error bars indicate significant differences within treatments (P= 0.001).](image)

**Table 5.2: Shoot formation % of different sizes of immature embryos.**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Immature embryos (IEs) size</th>
<th></th>
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<th></th>
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<tr>
<td></td>
<td>a: 1.5 – 2.00 mm</td>
<td>b: &gt; 2.00 mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No. of IEs</td>
<td>No. of shoots*</td>
<td>Shoots %</td>
<td>No. of IEs</td>
<td>No. of shoots</td>
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<td>3.6</td>
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<td>Gh.1</td>
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<td>32</td>
<td>9.0</td>
<td>399</td>
<td>189</td>
</tr>
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<td>Gh.82</td>
<td>166</td>
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<td>6.6</td>
<td>242</td>
<td>125</td>
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<tr>
<td>Hi II</td>
<td>188</td>
<td>15</td>
<td>8.0</td>
<td>99</td>
<td>44</td>
</tr>
</tbody>
</table>

* In some cases, shoots were formed combined with callus initiation, so the event counted as callus induction and shoot formation.
Plate 5. 5: The response of different sized immature embryos of the hybrid Basil.2 to callus formation and shoot formation.

There were significant differences within genotypes regarding the mean weight of callus formed from both sizes of immature embryos (Table 5. 1). Syrian genotypes (Ghota.1 and Ghota.82), according to the ANOVA analysis, were significantly superior the Basil.1 plants in their ability to callus formation regarding the callus weight (Figure 5. 5). The results showed, also, that the line Hi II plants were superior the hybrid Basil.1 in callus weight (Figure 5. 5).

![Callus formation graph](image-url)

Figure 5. 5: The weight of callus formation and the response of targeted embryos of maize genotypes to callus formation (g).
5.5.2 Callus development and selection for stable transformation events

Transformation experiments with pTF102 construct of

*Agrobacterium tumefaciens*

5.5.2.1 Stage 1 of selection (1.5 mgL$^{-1}$ bialaphos):

For the first selection of two weeks, immature zygotic embryos IZEs (35 IZEs per plate) were transferred from 7 days on the resting medium to callus induction media (Selection I medium- SM$_1$) containing 1.5mgL$^{-1}$ bialaphos (Sigma Aldrich, UK). Plates were wrapped with Parafilm throughout selection and incubated at 28°C in the dark.

Depending on the genotypes (Ishida *et al.* 2007), 2-3 sub-cultures (for Syrian hybrids) to 4-5 sub-cultures (varieties) were conducted on SM$_1$ until non-growing callus Type I, which is slightly yellow, started to grow rapidly forming callus type II which is white and friable (Plate 5. 6). Callus pieces (on SM$_1$) were transferred every 2 weeks to fresh selection media until putative transformation events (bialaphos-resistant clones) were distinguishable as clusters of white, rapidly growing callus. The appearance of type II embryogenic callus in this stage was a visible indication of transformation events since it indicated that the type I callus was indeed resistant to the bialaphos and was able to develop to type II callus.
5.5.2.2 Stage 2 of selection (3 mgL\(^{-1}\) bialaphos):

In the second selection stage, 20 embryos were cultivated per dish on SM\(\text{II}\) supplemented with 3 mgL\(^{-1}\) bialaphos at 28°C in the dark. Individual clones from the first selection were transferred and sub-cultured two more times (2 x 14d) on SM\(\text{II}\) media (Plate 5. 7).

The medium was replaced every 14 d for up to one month until immature somatic embryos emerged (the rate of clone emergence can be “construct” dependent). The studied genotypes needed at least 11 weeks and as much as 15 weeks to produce type II callus. On the other hand, putative Type II callus of Hi II events (Plate 5. 8) have been reported to be visible as early as 5 weeks and as late as 10 weeks after infection (Frame et al. 2011). The results reported here demonstrated that the different genotypes produced different callus types over
different number of sub-culturings and these results were consistent with (Ishida et al. 2007).

Plate 5. 7: Surviving embryogenic callus Type II of Ghota.82 variety on selection II medium.
Production of genetically altered maize plants

5.5.3 The control response

A good response of callus induction was detected for non-transformed embryos on callus induction medium without bialaphos (SM control) (Plate 5. 9.a) and after 3-4 subcultures on callus induction medium without bialaphos, callus developed and shoots formed successfully (Plate 5. 9.b). However, callus derived from the control (non-transformed embryos) initially stopped when cultured under bialaphos stress on SM\textsubscript{I} supplemented with 1.5 mgL\textsuperscript{-1} bialaphos. After 2 sub-cultures of the control event, the colour of non-growing callus changed to a slightly brown colour indicating the beginning of cell necrosis. Callus of control died outright on SM\textsubscript{II} with 3.0 mgL\textsuperscript{-1} bialaphos and not all putatively resistant (transformed) calluses survived after sub-culturing on this medium (Plate 5. 10). Surviving embryogenic calluses which were

Plate 5. 8: Friable embryogenic callus Type II of Hi II transformed events surviving on selection II medium.
bialaphos–resistant were selected and transferred to the next stage to attempt regeneration of plantlets using regeneration media (Plate 5. 11).

Plate 5. 9.a: Callus induction derived from non-transformed embryos of the hybrid B.2 (the control), and the hybrid Hi II (right) on a selection medium without bialaphos (SMc).

Plate 5. 9.b. Callus development and shoot formation of non-transformed callus on selection medium without bialaphos.
Plate 5. 10: Death of non-transformed callus on SM/bialaphos (lift), surviving of transferred calluses with a selectable marker gene; bar under bialaphos stress (right).

Plate 5. 11: Selection of bialaphos-resistant callus on selection II medium.
5.5.4 Stable transformation frequency

Stable transformation frequency % was calculated as the number of independent bialaphos-resistant callus events recovered after sub-culturing on SM\textsubscript{II} with 3 mgL\textsuperscript{-1} bialaphos per 100 immature zygotic embryos infected and selected.

Results from 23 transformation events (11 for hybrids, 7 for varieties and 5 for Hi II) showed that the stable transformation frequency of Syrian varieties was higher than the transformation frequency of Syrian hybrids. Transformation frequency, in these experiments, averaged 5.2% and ranged between 2.2% and 10.9% for hybrids (B.1 and B.2 respectively) and averaged 6.5% and ranged between 2.5% and 10.1% for varieties (Gh.1 and Gh.82 respectively) compared with an average transformation frequency of 14.5% for hybrid Hi II (Tables 5. 3 and 5. 4).

Stable transformation frequency based on GUS gene expression was calculated as the number of GUS positive callus or explants which expressed positive GUS per 100 embryos infected. Whereas 81% of bialaphos-resistant events recovered expressed the GUS gene for varieties, 92% of the bialaphos-resistant events of hybrids showed a positive GUS gene expression (Tables 5. 3 and 5. 4). However the percentage of recovered events expressing the GUS gene of total Hi II events resistant to bialaphos was 76%. Consequently it was concluded that the \textit{A. tumefaciens}-mediated transformation was efficient to produce transgenic events in maize.
Table 5.3: Efficiency of *Agrobacterium tumefaciens* EHA101-mediated transformation of Syrian maize hybrids, Basil.1 and Basil.2. NA, not applicable; NT, not tested.

<table>
<thead>
<tr>
<th>Genotypes /construct</th>
<th>Expt. date</th>
<th>No. of inoculated immature embryos</th>
<th>No. of callus formation</th>
<th>No. of Bialaphos-resistant events recovered</th>
<th>No. of callus events expressing GUS gene</th>
<th>No. of events regenerated to plants</th>
<th>No. of events attempted</th>
<th>Frequency of regeneration b</th>
<th>D, %</th>
<th>Transformation Frequency c,d</th>
<th>B/A, %</th>
<th>C/A, %</th>
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<td>5</td>
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</table>

a: Number of callus initiated from different sizes of IEs on callus induction medium which based to calculate the callus formation %.

b: Frequency of regeneration= (No. of events regenerated to plants/No. of events attempted) x 100.

c: Transformation frequency = independent bialaphos (3 mgL$^{-1}$) resistant events recovered/ total of embryos infected (X100).

d: Transformation frequency was calculated as the number of GUS positive callus or explants among the total number of embryos infected,%. Results were scored 3–5 subcultures after treatment with *A. tumefaciens* or when the explants were produced.

Table 5.4: Efficiency of *Agrobacterium tumefaciens* EHA101-mediated maize transformation of Hi II and Syrian varieties, Ghota.1 & Ghota.82.

<table>
<thead>
<tr>
<th>Genotype/construct</th>
<th>Expmt. date</th>
<th>No. of inoculated IEs</th>
<th>No. of callus formation</th>
<th>No. of Bialaphos resistant events recovered</th>
<th>No. of callus events expressing GUS gene</th>
<th>No. of events regenerated to plants/No. of events attempted</th>
<th>Frequency of regeneration D, %</th>
<th>Transformation Frequency c.d</th>
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5.5.5 Regeneration and rooting of transgenic plants

5.5.5.1 Shoot formation; Mature somatic embryo production

Regeneration of transgenic bialaphos-resistant Type II callus that were friable and had somatic embryos present (Plate 5. 12, a) was accomplished by culturing callus on RM₁ supplemented with 1.5 mgL⁻¹ bialaphos for 2-3 weeks. Firstly, immature somatic embryos were produced from small callus pieces (approximately 4 mm) of somatic embryo-enriched callus (Plate 5. 12, b). Then, the production of mature somatic embryos was accomplished by further maturation for a further week after sub-culturing on fresh RM₁ medium.

Results were promising, with maize genotypes producing mature somatic embryos using RM₁ which appeared swollen and white or purple (Plate 5. 13) and shoot formation was also apparent at this stage sprouting from different shapes of mature somatic embryos (Plate 5. 14).
Plate 5. 12: Formation of immature somatic embryos on regeneration I medium. Somatic embryo-enriched callus type II (a); immature somatic embryos production (b). About 10 to 15 small pieces of highly embryogenic callus placed per petri dish. Petri-plates were wrapped with vent tape (leucopore tape) and incubated at 25°C in the dark.
Plate 5.14: Different shapes of mature somatic embryos coloured white, white yellowish or purple (a, b, and c), beginning of shoot formation of mature embryos (d)
5.5.5.2 Shoot formation and plantlet formation

Mature somatic embryos showed a high ability to regenerate transgenic plants of maize by forming shoot and roots. Over 37 transgenic events were regenerated to plants and grown on. This was accomplished by production of shoots and roots of mature somatic embryos through further maturation and germination in the light on RM II followed by further elongation on MS solid media. Shoots were formed from clusters of maize calluses with mature somatic embryos (Plate 5. 15) and from individual mature somatic embryo (Plate 5. 16) that germinated on the surface of RM II in an illuminated incubator for 2-4 weeks.

Plate 5. 15: Plantlet formation. Shoots and roots formation from segments of embryogenic callus. Plantlet formation of Ghota.1 variety (a), and shoot formation of Ghota.82 variety (b) and of Hi II hybrid (c).
Up to 15 mature embryogenic callus, or individual mature somatic embryos were derived from one clone placed per petri dish wrapped with vent tape and incubated at (24°C, 16:8 photoperiod).

Plate 5. 16: Shoots formation derived from individual mature somatic embryos. Shoot formation of Hi II hybrid (a), and plantlet formation of Syrian varieties (b) and Syrian hybrids (c).

When the plantlets developed fully formed roots and shoots (Plate 5. 17) they were transferred individually to glass vials (150 x 25mm) containing 15 mL of 2/3 strength MS solid media for further elongation for 10-14 days (Plate 5. 18) until they reached a total shoot length of approximately 10 cm. Regeneration (%) was calculated as the number of independent transgenic clones successfully regenerated to plants per 100 events for which regeneration was attempted.
Results showed that there were differences between genotypes regarding the ability to regenerate and produce transgenic plants from mature somatic embryos. Frequency of regeneration for Syrian varieties was higher (57%) than that for hybrids (12%) (Tables 5.3 and 5.4). But, of the 26 survived callus events with mature somatic embryos for which regeneration was attempted, 21 transgenic events of Hi II regenerated to transgenic plants successfully (Table 5.4). The average percentage of regeneration of transformed events attempted with Hi II was the best (81%) compared with that from non-transformed events (96%). However, Syrian varieties also showed a good efficiency of regeneration with 54-60% for transgenic events from Ghota.1 and Ghota.82 respectively (Table 5.4).

**Plate 5.17:** Production of plantlet with fully formed roots and shoots on RMII, 24 °C, 16 hours light (170 μmol s⁻¹m⁻²). Explant produced from 1 transgenic clone on RM II were transplanted individually per boiling tube.
Plate 5. 18: Plantlets with fully formed roots and shoots in the boiling tubes, 24 °C, 16 hours light (170 μmol s⁻¹ m⁻²).
5.5.6 Plant Acclimatization in the growth chamber

Transformed maize plants were acclimatized successfully in a growth chamber. When roots were well established in the glass vials, plantlets with 6 to 10 cm leaf length were transferred to soil in small pots (7.5 cm) for plant hardening in the growth chamber in the light. Plantlets were covered with a plastic Humi-dome (Plate 5. 19, a) with one ventilation hole opened, or planted within a plastic container for 14 days (Plate 5. 19, b).

After 1 - 2 week of hardening within a humi-dome in the growth chamber (Plate 5. 20), plants with roots which had “bonded” with the soil were transplanted into bigger pots (10 cm) of soil in the growth chamber (Plate 5. 21) for a further 7 to 10 days of hardening before removing from the growth chamber to the growth room.

The results of hardening showed an acceptable percentage of hardened transformed plants that succeeded to continue growing in the growth room. Of 8 transformed plants subjected to hardening, 5 plants of Syrian hybrids were grown successfully and survived and 18 transformed plants of varieties survived after the acclimatization of 25 transformed maize plants in the growth chamber (Table 5. 5). However, the hybrid Hi II showed a good ability of acclimatization. The average percent of successfully weaned plants reached 75% under the hardening condition in the lab. 33 plants succeeded to continue growing to the maturity stage of 44 transformed Hi II plants that were acclimatized in the growth chamber (Table 5. 5).
Plate 5.19: Plant hardening in the growth chamber in small pots of soil. Hardening of transformed plants using the Humi-dome (a), and using plastic containers (b). (22/20 °C day/night, 13 hours light, 170 μmol s⁻¹m⁻² photon flux density).

Table 5.5: Hardening and production of transgenic maize plants in growth chamber.

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<th>Genotypes</th>
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<td>Gh.82</td>
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<tr>
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a: Different clones were derived from different embryos infected at the same experiment or different experiments of transformation.

b: Independent bialaphos resistant events regenerated to plants from the transgenic clones recovered.

c: explants were produced from the regenerated plants by subculture the explants individually on a fresh MS medium.
Production of genetically altered maize plants

Plate 5. 20: Plantlets in small pots of soil after 2 weeks of hardening within a humid dome in the growth chamber.

Plate 5. 21: Plantlets in 10 cm pots of soil for plant hardening in the growth chamber. Plants are 3 weeks old ready to transplant in the growth room.
5.5.7 Production of R1 progeny of transgenic plants

R1 progeny of transformed maize plants was produced by culturing the R0 progeny plants that hardened in the growth chamber for 2 to 3 weeks. Transformed R0 plants that acclimatized, at 4-5 leaf stage with a good root mass, were transplanted into a 35-cm pot (20 L) in a walk-in growth room at (25/17°C day/night, 16 hours light 170 μmol s⁻¹ m⁻² photon flux density) until full maturity. In addition, at the same time, R1 maize grains of non-transformed plants "controls" were grown in 9 cm pots (0.25 L) in a growth chamber at 22/20 °C day/night, 13 hours light, 170 μmol s⁻¹ m⁻² photon flux density, for three weeks. At this time, transformed and non-transformed plants of similar growth stage were planted alongside each other in the growth room in a cultivation substrate mix (John Innes compost and sand, 2:3 ratio) and 60 g of a general long-term slow release fertilizer, Osmocote Pro, per pot. The control non-transformed plants were used to pollinate the transformed plants whose tassels were removed before flowering (for bio-security reasons). The tassels of control plants were covered with plain brown bags to collect the pollen from the donor plants. Three non-transformed plants were used to pollinate each transformed plant to make sure pollen was available when the silks of transformed plants emerged because usually there are some differences in the time of male and female flowering in maize. The pollination was carried out when the silks were 2-3 days old using fresh pollen of control plants. Care of plants in the growth room was carried out according to the general glasshouse care of plants (as mentioned in the chapter 3: Growth of donor plants and glasshouse Care). Cobs of R1 progeny (Plate 5. 22) were harvested from the mature transformed plants and the results showed a high germination ability of R1 seeds. Transformed R0 and R1 plants successfully produced transgenic cobs in the growth room (Table 5. 6). These transgenic seeds of R1
progeny were used in the next experiments of assessment of abiotic stress resistance of transformed maize plants by physiological assays by culturing this progeny in the growth room and exposing the plants to abiotic stress (salt stress).

Plate 5. 22: Production of R₁ progeny of transformed plants in the growth room (a). R₁ progeny of B.2 (b), and the progeny of Gh.1 (c).
Table 5. 6: Fertility of R\textsubscript{0} & R\textsubscript{1} progenies of transgenic maize plants derived from EHA101/pTF102 and EHA105/ PpCBF1. NT, not tested.

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The female plant was pollinated from the same plant or crossed with another non-transformed plant for each genotype.

a: Control data were collected from non-transgenic plants derived from embryogenic callus.
b: In some cases, the pollen was not available to pollinate all the female silks efficiently.
c: Harvested seeds were dried at room temperature before the weight.
d: Percentage of R\textsubscript{1} plants harvested seed obtained from data based on 2- 5 plants.
5.5.8 Transformation experiments by the *Agrobacterium* strain EHA105 containing pBINPLUSARS plasmid with CBF gene

Initially, maize transformation with *A. tumefaciens* harbouring the pTF102 plasmid carrying the *bar* gene that confers resistance to herbicide phosphinothricin (bialaphos) was investigated. After a routine and efficient protocol using an *A. tumefaciens* standard binary vector system was established and developed to transfer Syrian maize genotypes, this protocol was translated to transfer of Syrian genotypes with anti-stress genes, the stress regulon transcription factor CBF gene, and compared with the hybrid line Hi II which is considered the germplasm used as a model (Armstrong *et al.* 1991) in a routine transformation of maize (Armstrong *et al.* 1992; Shrawat and Lörz 2006). These experiments aimed to produce abiotic stress-resistant plants of Syrian genotypes using *Agrobacterium*-mediated transformation of immature embryos to be promising core in the Syrian maize breeding against the abiotic-stress.

The main aims of these experiments were to optimise a tissue culture system using immature zygotic embryos to initiate callus and to induce somatic embryogenesis in these cultures and then attempt to regenerate the somatic embryos from these cultures that transformed with anti-stress gene using the *A. tumefaciens* to produce genetically altered plants of maize that resistant to abiotic stresses.

In these transformation experiments, immature somatic embryos of Syrian varieties and hybrids and the hybrid Hi II were transformed with *A. tumefaciens* strain EHA105 harbouring a pBINPLUSARS plasmid carrying CBF.

Results showed that 31 kanamycin-resistant events of Syrian hybrids were recovered on selection media supplemented with 50mgL⁻¹ Kanamycin. While 29 events of Syrian varieties were recovered on the selection medium against 21 transformed
events of Hi II were registered that kanamycin-resistant events after (2-5 x 14d) subcultures on selection media (Tables 5. 6 and 5. 7). After that, mature somatic embryos were initiated from the surviving clusters of kanamycin-resistant callus (Plate 5. 23) and regenerated successfully (Plate 5. 24). This regeneration of transgenic callus was accomplished by culturing the surviving friable calluses on regeneration medium supplemented with 25mgL⁻¹ Kanamycin for 2-4 weeks. Firstly, immature somatic embryo-enriched calluses were produced. Then, by one to two sub-cultures on a fresh regeneration medium, mature somatic embryos appeared white and swollen were identified. Every somatic embryogenic cluster of callus produced many mature somatic embryos that were then used to produce transformed plants of maize.
Plate 5. 23: Mature somatic embryos formation.

Plate 5. 24: Regeneration of mature somatic embryos derived from Ghota.1 variety transformed with CBF gene by Agrobacterium EHA105.

5.5.8.1 Transformation frequency

Transformation frequency percentage was calculated as the number of independent kanamycin-resistant callus events recovered after sub-culturing on a SM_II supplemented with 50 mgL^{-1} kanamycin per 100 immature zygotic embryos infected with A. tumefaciens EHA105 harbouring the pBINPLUSARS plasmid carrying the CBF gene.
Results of 14 transformation experiments of Syrian hybrids (6 transformation events for the hybrid B.1 and 8 transformation events for the hybrid B.2) showed that of 458 inoculated immature embryos of the hybrid B.1, 8 transformed events were kanamycin-resistant events while 23 transformed events of the hybrid B.2 were recovered on Kanamycin resulting from transformation of 577 immature embryos (Table 5. 7). Therefore, the stable transformation frequency of Syrian hybrid B.2 was 3.99% and was higher than the transformation frequency of the hybrid B.1 at 1.75%. Transformation frequency in these experiments averaged 2.87% and ranged between 1.39% and 10.29% for hybrids (B.1 and B.2 respectively) (Table 5. 7). Transformation frequency calculated from data of 11 transformation experiments of varieties (6 for Ghota.1 and 5 for Ghota.82) demonstrated that of the 563 transformed immature embryos of the variety Ghota.1, 18 transformed events showed resistance to kanamycin, while 11 transformed events of the variety Ghota.82 were recovered from 305 inoculated immature embryos on a SM with 50mgL\(^{-1}\) kanamycin (Table 5. 8). There were slight differences between the transformation frequencies of the two Syrian varieties with the transformation frequency of Ghota.1 at 3.2% and 3.61% for the variety Ghota.82. Overall, the transformation frequency of Syrian varieties averaged 3.41% and ranged between 1.67 and 7.14% for the varieties Ghota.1 and Ghota.82 respectively (Table 5. 8). The results showed that the highest frequency of transformation by the \textit{A. tumefaciens} EHA 105 in these experiments was 6.31% for the hybrid Hi II (Table 5. 8). These differences of transformation frequency confirmed the significant impact of genotype on successful \textit{Agrobacterium}-mediated transformation of maize as stated by Wei, (2009).
Table 5. 7: Efficiency of Agrobacterium tumefaciens EHA105-mediated transformation of Syrian maize hybrids, Basil.1 and Basil.2.

NA, not applicable; NT, not tested.

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<td>1/4 /50</td>
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<td>1/4 /25</td>
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<td>2/18</td>
<td></td>
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<td>50</td>
<td>2/12</td>
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<td></td>
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</tr>
<tr>
<td>Total</td>
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<td>231</td>
<td>164</td>
<td>7/54</td>
<td></td>
<td></td>
<td>12.96</td>
</tr>
</tbody>
</table>

a: Number of callus initiated from different sizes of IEs on callus induction medium which based to calculate the callus formation %.

b: Frequency of regeneration= (No. of events regenerated to plants/ No. of events attempted) x100.

c: Transformation frequency= independent Kanamycin (50 mgL⁻¹) resistant events recovered/ total of embryos infected (X100).
Table 5.8: Efficiency of *Agrobacterium tumefaciens* EHA105-mediated transformation of Syrian maize varieties, Ghota.1 and Ghota.82, and the hybrid Hi II.

NA, not applicable; NT, not tested.

<table>
<thead>
<tr>
<th>Genotypes/construct</th>
<th>Experiment date</th>
<th>No. of inoculated immature embryos</th>
<th>No. of callus formation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of Kanamycin-resistant events recovered</th>
<th>No. of events regenerated to plants/ No. of events attempted&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Frequency of regeneration&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Transformation Frequency&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gh.1/CBF</td>
<td>21/08/12</td>
<td>120</td>
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<td>2/3</td>
<td>66.67</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23/08/12</td>
<td>59</td>
<td>31</td>
<td>3/3</td>
<td>33.33</td>
<td>1.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>42</td>
<td>26</td>
<td>2/2</td>
<td>100</td>
<td>4.76</td>
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</tr>
<tr>
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<td></td>
<td>40</td>
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<td>1/1</td>
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<td>3.33</td>
<td></td>
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<tr>
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<td>1/1</td>
<td>100</td>
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</tr>
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<tr>
<td></td>
<td>02/10/12</td>
<td>62</td>
<td>39</td>
<td>5/5</td>
<td>83.33</td>
<td>5.06</td>
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<tr>
<td>Total</td>
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<td>2/3</td>
<td>66.67</td>
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<td>5/5</td>
<td>100</td>
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<td></td>
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<td>22</td>
<td>1/1</td>
<td>100</td>
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<tr>
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<td>108</td>
<td>69</td>
<td>12/11</td>
<td>72.73</td>
<td>11.11</td>
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<td>0/2</td>
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<td>39</td>
<td>10/10</td>
<td>100</td>
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<td></td>
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<td>39</td>
<td>31</td>
<td>9/9</td>
<td>100</td>
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<td></td>
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<tr>
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<td>37</td>
<td>12/13</td>
<td>92.31</td>
<td></td>
<td></td>
</tr>
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<td>33</td>
<td>11/11</td>
<td>100</td>
<td></td>
<td></td>
</tr>
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<td>140</td>
<td>42/43</td>
<td>97.67</td>
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</table>
5.5.8.2 Regeneration and rooting of transgenic plants

It has been reported that the regeneration ability of genetically transformed cereals of different germplasm is varied and an adequate regeneration potential of genotypes is required for successful Agrobacterium-mediated genetic transformation (Tzfira et al. 2002). Results presented here confirmed that Syrian maize genotypes varied in their regeneration ability in in-vitro. The frequency of regeneration of varieties was higher than the regeneration frequency of hybrids (Tables 5. 7 and 5. 8). Of the 5 kanamycin-resistant events recovered of Basil. 1 for which the regeneration was attempted only 2 transformed events with CBF gene regenerated to plants. Also, only 3 transformation events of the hybrid Basil.2 were regenerated successfully to plants from 13 transformed events which failed to regenerate (Table 5. 7). But, of 17 survived transformation events of the variety Ghota.1 for which the regeneration was attempted, 11 were regenerated to plants on the regeneration media. Moreover, 5 events of 9 transformed events of the variety Ghota.82 were also regenerated to plants (Table 5. 8). The average regeneration frequency was 65-56% for the varieties Ghota.1 and Ghota.82 respectively while; the percentage of frequency of regeneration was 19% for the hybrid Basil.2. The average percent of regeneration frequency of the hybrid Basil.1, based on 5 transformed events that subjected to regeneration, was 40%. On the other hand, the percentage of recovered events regenerated to plants of total Hi II events expressing resistance to kanamycin was 75% (Table 5. 8). Results of regeneration of 20 kanamycin-resistant events of the hybrid Hi II showed that 15 transformed events were regenerated successfully.
5.5.8.3 Plantlet formation and plant acclimatization in the growth chamber

Results showed that transformed plantlets of R₀ progeny of genotypes were formed successfully. Most of regenerated plants were transformed explants. Explants of the R₀ progeny were subjected to hardening in a growth chamber for 14 to 21 days before transplanting them to the growth room. Over 44 transformed plants were derived successfully from 34 transgenic events regenerated (Table 5.9).

Table 5.9: Hardening and production of transformed maize plants in the growth chamber.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Hardening of regenerated maize plants transformed with CBF construct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of transgenic clones</td>
</tr>
<tr>
<td>B.1</td>
<td>2</td>
</tr>
<tr>
<td>B.2</td>
<td>3</td>
</tr>
<tr>
<td>Gh.1</td>
<td>11</td>
</tr>
<tr>
<td>Gh.82</td>
<td>8</td>
</tr>
<tr>
<td>Hi II</td>
<td>10</td>
</tr>
</tbody>
</table>

a: Different clones are derived from different embryos inoculated with EHA105/CBF construct during different experiments of transformation.

b: Independent Kanamycin-resistant events regenerated to plants from the transgenic clones recovered.

c: Explants were produced from regenerated events by sub-culturing them individually on a fresh MS medium. Those explants were acclimatized in a growth chamber.

Generally the maize genotypes showed a good response to acclimation in the growth chamber and produced mature transformed plants in the growth room. Of the 9 transformed plantlets of the Syrian hybrids, 6 transformed plants survived and produced mature plants and 23 transformed plants of Syrian varieties were acclimatized successfully in the growth chamber, while only 6 transformed plants failed to continue growing. Moreover, of the 18 transformed explants of the hybrid Hi
II for which the acclimatization was attempted, 15 events (83%) produced mature transformed plants (Table 5.9).

5.5.9 Transformation efficiency using _Agrobacterium tumefaciens_ mediated transformation

Transformation efficiency % was calculated as the number of independent bialaphos/ kanamycin-resistant events recovered after sub-culturing on SMII supplemented with 3 mg L\(^{-1}\) bialaphos (for pTF102), or with 50 mg L\(^{-1}\) Kanamycin (for CBF) per 100 immature embryos infected.

Results of ANOVA analysis of 23 transformation experiments using the _Agrobacterium_ strain EHA101 (pTF102), and 30 experiments transformed by the _Agrobacterium_ EHA105 (CBF) showed that differences in the transformation efficiency of _Agrobacterium_ strains was highly significant (Table 5.10). Transformation efficiency using the _Agrobacterium_ strain EHA101 was higher than the transformation efficiency of EHA105 (Figure 5.6 and Table 5.11). Whereas this was 3.23% of transformed kanamycin-resistant events recovered using the CBF construct, 7.65% of the pTF102 events were bialaphos-resistant events derived from Hi II and Syrian genotypes (Figure 5.6). There were no significant differences between genotypes regarding their transformation ability (Figure 5.7 and Table 5.5). However the percentage of recovered events of Hi II using EHA101 was the highest at 12.1%, while the transformation efficiency of Basil.2 was 5.7%. For EHA105, the highest transformation efficiency was 4.7% for Hi II compared to 1.8% of Basil.1 (Figure 5.7).
Figure 5. 6: Transformation efficiency of *Agrobacterium tumefaciens* strains-mediated transformation of Hi II and Syrian maize genotypes. Values are the means of four replications for each genotype. Different letters above the standard error bars indicate significant differences within treatments (P= 0.001).

Figure 5. 7: Transformation efficiency of Hi II and Syrian maize genotypes using *Agrobacterium tumefaciens* strains. Values are the means TE% of four replications each genotype.
Table 5. 10: Fisher's significance test (p value) and Least Significant Difference (L.S.D) values of transformation efficiency %, TE at (5 and 1)% level of significance (two-tailed).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>P value</th>
<th>L.S.D (0.05)</th>
<th>L.S.D (0.01)</th>
</tr>
</thead>
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<tr>
<td>Treatments (Agrobacterium strains)</td>
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<td>2.36</td>
<td>3.18</td>
</tr>
<tr>
<td>Genotypes</td>
<td>0.184</td>
<td>N.S</td>
<td>N.S</td>
</tr>
<tr>
<td>Treatments x Genotypes</td>
<td>0.569</td>
<td>N.S</td>
<td>N.S</td>
</tr>
</tbody>
</table>

Significant -* Very significant - ** Highly significant -*** N.S. Non-significant
Table 5.11: Frequencies of transformation and regeneration of Syrian genotypes transformed by *A. tumefaciens* EHA101/pTF102 and *Agrobacterium* EHA105/PpCBF1.

Bial: bialaphos, Km: kanamycin.

<table>
<thead>
<tr>
<th>Agrostrains/construct</th>
<th>Varieties Gh.1 &amp; Gh.82</th>
<th>Hybrids B.1 &amp; B.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of inoculated immature embryos</td>
<td>No. of Bial/Km-resistant events recovered</td>
</tr>
<tr>
<td>EHA101/pTF102</td>
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<td>23</td>
</tr>
<tr>
<td>EHA105/PpCBF1</td>
<td>868</td>
<td>29</td>
</tr>
<tr>
<td>Total (^c)</td>
<td>1229</td>
<td>52</td>
</tr>
</tbody>
</table>

\(^a\) Frequency of regeneration= (No. of events regenerated to plants/No. of events attempted) x100.

\(^b\) Transformation frequency= independent Kanamycin (50 mgL\(^{-1}\)) or bialaphos (3 mgL\(^{-1}\)) resistant events recovered /total of embryos infected (X100).

\(^c\) The sum of immature embryos of Syrian varieties and hybrids infected with EHA101 (pTF102) and EHA105 (PpCBF1).
5.6 Discussion

5.6.1 Callus induction, genotypes response and the effect of immature embryo size on callus formation

Genotypes were varied somewhat in their response to callus formation but there were no significant differences between genotypes regarding callus induction. Significant differences were found between genotypes regarding callus weight and subsequent callus development according the callus weight initiated after 7 to 10 days on callus induction media. The Syrian varieties succeeded to develop the callus more than the hybrids indicating that there are significant impacts of genotype on both embryogenic callus induction and callus differentiation and these results correspond with the results of Wei (2009). Syrian varieties were significantly superior the hybrid B.1 regarding callus weight initiated on callus induction media after 10 days of inoculation. However, the performance of the variety Ghota.82 to develop callus was similar to Hi II hybrid response to callus weight and was better than for the variety Ghota.1 (Figure 5. 5). Results showed that callus induction is genotype dependent as stated in (Frame et al. 2011).

Callus induction and callus weight was significantly affected by the initial embryo (explant) size. It has been previously demonstrated that embryo size is one of the most significant factors that influences the callus induction and successful Agrobacterium-mediated transformation of cereals (Wu et al. 2003; Shrawat et al. 2007) and this was supported here. The embryo size of 1.5-2.00mm showed the best response of callus induction (Figure 5. 2) but the callus derived from embryos >2.00mm weighed significantly more than those initiated from the size 1.5-2.00mm (Figure 5. 4). However, the percentage of callus induction from 1.5-2.00mm was
significantly superior to the callus induction percentage of > 2.00mm embryos. These results showed that callus induction is embryo size dependent.

5.6.2 Stable transformation frequency

The efficiency of transformation varied between genotypes. Whereas, transformation frequency of Syrian hybrids averaged 5.2% using Agrobacterium strain EHA101, it was 6.5% for the varieties. These results emphasize that the transformation frequency can be genotype dependent. The best response of Agrobacterium-mediated transformation was with Hi II. These results confirm that genotype is one of the most important factors affecting the efficiency of transformation by Agrobacterium (Wei 2009; Cao et al. 2014). Results showed that the response of genotypes to transformation by the Agrobacterium strain EHA105, also varied but the transformation frequency of Syrian varieties was still higher than the transformation frequency of hybrids (Tables 5.7 and 5.8). This confirms that the plant germplasm and the type of plant tissue used in the Agrobacterium-mediated transformation systems are two of the most crucial factors for successful transformation of maize as reported by Nadolska-Orczyk and Przetakiewicz (2000). Comparison between the Agrobacterium efficiency of transformation revealed that the efficiency of Agrobacterium strains to transform maize genotypes also varied significantly (Table 5.10). The transformation frequency using the Agrobacterium strain EHA101 (5.21, 6.47)% of Syrian genotypes (hybrids and varieties) respectively was higher than the transformation frequency of Agrobacterium strain EHA105 (2.87, 3.41)%. Therefore, even though A. tumefaciens has been successfully used to transform immature embryos (Shrawat and Lorz 2006; Valdez-Ortiz et al. 2007; Zhang et al. 2010), the choice of appropriate strains of Agrobacterium harbouring binary vectors and carrying virulence genes is very important to achieve an efficient
transformation of a wide range of germplasm (Hiei et al. 1994; Komari et al. 1996). The frequency of transformants was significantly influenced by the *Agrobacterium* strains used in this study, as Cao et al. (2014) reported. An efficient gene transfer may be achieved using different *Agrobacterium* strains by empirical experimentation of different germplasm. Frame et al. (2002 and 2011) previously used the *A. tumefaciens* EHA101 harbouring the standard binary vector pPTF102 to transform immature zygotic embryos of the hybrid line Hi II efficiently and this successfully served as the “proof of system” adopted in this study. The *Agrobacterium* strain EHA105 harbouring the pBINPLUS/ARS vector containing the enhanced 35S-PpCBF1 fragment however was developed to successfully transform apple by (Wisniewski et al. 2011) and had not been used for maize previously. In other studies, it has been found that the *Agrobacterium* strain EHA105 was the most efficient to transform the microalga *Chlamydomonas reinhardtii* with a higher rate of transformation frequency than EHA101 and LBA4404 (Pratheesh et al. 2012) and in the transformation of pea (*Pisum sativum L.*), the efficiency of transformation with hypervirulent EHA105 strain was higher than for strains LBA 4404 and C58C1 (Nadolska-Orczyk and Orczyk 2000). The work presented here showed that EHA105 was able to successfully transform maize and deliver the CBF gene construct. It is confirmed that the hypervirulent *Agrobacterium* strains EHA101 and EHA105 are effective in developing efficient maize transformation protocols agreeing with previous studies (Hood et al. 1986; Frame et al. 2002; Ombori et al. 2013).
5.6.3 Regeneration, rooting and production of transformed maize plants

Transformed Syrian genotypes were successfully regenerated in this study. The frequency of regeneration of Syrian varieties Gh.1 and Gh.82 (56.5-61.5)% was higher than the regeneration frequency of hybrids B.1 and B.2 (11.5- 23.8)% using both strains of Agrobacterium EHA101/pTF102 and EHA105/PpCBF1 respectively (Table 5.11). The positive control hybrid line Hi II showed the highest frequency of regeneration 77.9%. These results confirm that a genotype dependency for potential regeneration following transformation by Agrobacterium agreeing with the literature (Hensel et al. 2009; Frame et al. 2011). It appears that cereal crops are hardly able to regenerate plants from leaf tissue but in contrast using immature embryo explants as the gene transfer target has proven both the transformation and regeneration efficiency in cereals (Shrawat and Lörz 2006).
5.7 Conclusion

In an effort to develop the transformation protocol of maize (*Zea mays*) for a wider choice of germplasm, a routine and efficient protocol was established and developed for the production of transgenic maize plants using an *Agrobacterium tumefaciens* standard binary vector system. The present study was focused on the transformation technique of immature zygotic embryos of the hybrid line Hi II which is widely reported in the literature and then translated to diverse genotypes of Syrian maize compared.

The effects of *A. tumefaciens* strains, genotypes and embryo size on transformation efficiency were investigated and it was shown that embryo size and source of germplasm are the most significant factors that influence successful callus induction following *Agrobacterium*-mediated genetic transformation of maize.

Results showed that the response for callus formation of the Syrian hybrids (Basil.1 and Basil.2) was greater than for the varieties (Ghota.1 and Ghota.82). There were significant differences within treatments according to immature embryo size with the 1.5 to 2.0 mm embryo size giving the best response with up to 70% callus induction. Most of immature embryos size <1.5 mm did not develop callus very well and embryo death after 3 days of co-cultivation on fresh media containing high concentration of cysteine (300 mg L\(^{-1}\)) was common. The response of larger IEs, >2.00 mm, was lower than the response of the small and mid-size IEs <1.5 and 1.5 - 2.00 mm, and most of these IEs produced shoots.

Different genotypes required different numbers of sub-cultures of callus to produce the friable embryogenic Type II callus, a discovery only found out by empirical persistent sub-culturing. However, depending on the genotype, different callus types were identified for selection after 2-3 subcultures for hybrids and 3-4
subcultures for varieties. This tissue culture system was the first step to facilitate *Agrobacterium*-mediated maize transformation. The rate of recovery of bialaphos/kanamycin-resistant clones from Type II callus was affected by both maize genotype and *Agrobacterium* strain used in the transformation. Both strains of *Agrobacterium* EHA101 and EHA105 used in this study were capable of inducing transformation in Hi II and in immature embryos of Syrian maize genotypes. *Agrobacterium* strain EHA101 showed a higher transformation frequency compared to the *Agrobacterium* strain EHA105. The *Agrobacterium* strain EHA101 should be preferred over other *Agrobacterium* strains in future genetic transformation experiments in Syrian maize. The transformation frequency of Syrian varieties was higher than the transformation frequency of hybrids with a higher capability of regeneration. Syrian varieties have combined the amenability to gene transfer by *Agrobacterium* with adequate regeneration potential. Reproducible and efficient generation of transgenic maize plants has been achieved by the *Agrobacterium*-mediated maize transformation.

To the best of knowledge, this research marks the first report of a reproducible method for Syrian maize transformation using an *A. tumefaciens* standard binary vector system and establishes a protocol that can be routinely applied in Syrian plant breeding programmes.
Chapter 6:

Molecular analysis and histochemical GUS assays of abiotic stress-resistant maize plants transformed by *Agrobacterium*
6.1 Introduction

The molecular basis of genetic transformation via *Agrobacterium*-mediated transformation of plant cells is the transfer of a copy of a region of a large tumour-inducing (Ti) plasmid containing the gene of interest from the bacterium into the plant nuclear genome. Inserted genes can be expressed transiently or constitutively in transgenic plants where the functional role of the gene in the plant genome is expressed. Most gene transfer is accompanied by the insertion of a marker gene that is either constitutively expressed or co-expressed with the gene of interest and a commonly used marker gene is GUS. The integration of the gene of interest can be evaluated by the histochemical GUS assay often followed by molecular analysis after polymerase chain reaction (PCR) amplification of selected portions of genomic DNA.

For purposes of maize plant genetic transformation, gene of interest with selectable marker gene and reporter gene containing in the T-DNAs of *Agrobacterium* plasmid were introduced into plant genome with the capacity to encode multiple gene products in a biosynthetic pathway. Different strains of *A. tumefaciens* could be used to deliver different T-DNAs to the same plant cells (Neve *et al.* 1997; De Buck *et al.* 2000; Zhi *et al.* 2015). However, by using a binary vector, very large DNA segments (150-200kbp) can be introduced into the maize genome via *Agrobacterium*-mediated transformation (Miranda *et al.* 1992; Hamilton *et al.* 1996).

In this study, transformation efficiency of two different *Agrobacterium* strains carrying different T-DNAs (selectable marker gene *bar* and a reporter gene *gus*) was studied. Delivery of those genes including the anti-stress gene CBF to the plant cells was confirmed by molecular analysis and the histochemical assay of GUS expression in transgenic calli, leaves, roots, silks and tassels of transgenic plants. Many studies confirm the efficiency of transformation mediated by the *Agrobacterium* using the
GUS assays and polymerase chain reaction (PCR) using specific primers for the selection gene (Crow et al. 2006; Nott et al. 2011; Gallagher 2012).

6.2 Aims and objectives

The main aim of this study was to confirm the gene transformation process to $R_0$ transgenic maize plants using the histochemical GUS assay and polymerase chain reaction (PCR). Furthermore, to investigate the insertion and integration of specific DNA fragments in the transgenic genome of $R_1$ progeny of transformed plants by the GUS assay and a glufosinate herbicide leaf-spray test. More specifically the objectives were:

- To confirm the presence of the \textit{bar} gene and the \textit{CBF} gene in the both \textit{Agrobacterium tumefaciens} strains EHA101 and EHA105 containing DNA plasmid constructs (pTF102 and PpCBF1) respectively used in this study.
- To confirm the stable expression of \textit{bar} and \textit{CBF} transgenes in transformed maize plants by PCR amplification, and by presence/absence experiments using TagMan reagents.
- To estimate the copy number of transgenes in the transgenic genome(s).

6.3 Materials and methods

6.3.1 Plant materials

$R_0$ transgenic callus leaves and roots of transformed (surviving selective screen) and non-transformed maize plants were initially used for molecular analysis and histochemical GUS assay. In addition, anthers of $R_1$ progeny of transformed and non-transformed plants were used in this analysis. Immature zygotic embryos of F2 non-transformed maize plants that inoculated with the \textit{Agrobacterium} inoculum were also stained using the histochemical GUS assay.
6.4 Molecular analysis techniques and methodology

6.4.1 Genomic DNA extraction from maize callus and leaf tissue

For the transformation events generated using the CBF and bar gene construct, genomic DNA was extracted from callus and young leaves of plants based on the methods of Sambrook et al. (1989) and Bruce and Eric ((1993) cited by Birren and Lai (2012)) using PCR and qRT-PCR. Leaf samples and callus pieces were collected from transformed and non-transformed plant materials simultaneously. For each event, after 3 to 4 sub-cultures on media containing a suitable antibiotic or bialaphos as a selection agent, samples of surviving callus were selected and ground immediately in liquid nitrogen using a pestle and mortar. The powder of samples was stored at -20°C after the complete evaporation of the liquid nitrogen for immediate use. Also leaf samples were detached from transformed and non-transformed plants and placed immediately in liquid nitrogen, then ground into a fine powder using a pestle and mortar in liquid nitrogen. After that, aliquots of 100 mg of fine tissue powder for each sample were weighed when the liquid nitrogen evaporated completely. Samples were transferred to 1.5 mL RNase and DNase free microcentrifuge tubes (Cat. No. T9661. Sigma Aldrich. UK), and stored at -80°C to prevent DNA degradation before the DNA isolation to be used in PCR amplification.

For both the callus and leaf tissue of transgenic and non-transgenic events, the total genomic DNA for molecular analysis was extracted from the sample powder using a Genelute Plant Genomic DNA Miniprep kit (Cat. No. G2N70. Sigma Aldrich. UK) based on the methods of Sambrook and Russell David (1989), and Birren and Lia, (1993) cited by the manufacturers of the extraction kit (Sigma). In accordance with the protocol provided by the kit manufacturer, 100 mg of the powder was transferred to a 1.5 mL microcentrifuge tube on ice and 350 μL lysis solution (part A),
and 50 μL of lysis solution (part B) were added to the tube to lyse the cells. To dissolve any precipitate, the mixture was incubated at 65°C for 10 minutes. Then, 130 μL of precipitation solution was added to the mixture to precipitate the cellular debris, proteins and polysaccharides followed by centrifugation of the mixture at maximum speed (12 000-16 000 xg) for 5 to 7 minutes. A further purification was carried by filtration of the supernatant using a GenElute filtration column with a 2 mL collection tube provided by the kit. Then, the supernatant was centrifuged at maximum speed for 1 minute to remove any cellular debris that had not been removed before. Binding of the genomic DNA was done by adding 700 μL of binding solution directly to the filtered flow-through liquid using the GenElute Miniprep Binding column. After that, the binding column was washed twice by adding 500 μL of the diluted wash solution to the column for each wash. Pure genomic DNA was collected in a 2 mL collection tube by adding 100 μL of pre-warmed (65°C) Elution solution to the binding column and centrifuged at maximum speed for 1 minute as outlined in the preparation instructions of the kit. The elution process was repeated with 50 μL MW, and the column was prevented from contacting the flow-through. Genomic DNA was stored at -20°C for long-term storage or for short-term storage of DNA, 2-8°C is recommended. To avoid freezing and thawing which causes breaks in the DNA strand, small aliquots of DNA were stored at -20°C.

6.4.2 Determination the quality and quantity of genomic DNA

The purity and concentration of the genomic DNA extracted from the plant tissues was measured using a computerized Nano-Drop spectrophotometer (Lab Tech, UK). The Nano-drop spectrophotometer was calibrated with aliquots of a 2 μL buffer using the Elution solution or molecular water depending on the buffer that was used in the extraction the DNA. Then, concentrations of a 2 μL of nucleic acid DNA
samples were measured and the ratio of DNA/protein (A$_{260\text{nm}}$/A$_{280\text{nm}}$) and the ratio of DNA/other contaminants were measured at A$_{260\text{nm}}$/A$_{230\text{nm}}$. Duplicate measurements were taken for each sample, and the concentration of extracted genomic DNA was recorded in ng $\mu$L$^{-1}$. The ratio of pure DNA should be in the range between 1.8 and 2.05 (Brown 2010) (Figures 1, 2 and 3, Appendix 2). In addition, the DNA quality was checked further by running samples of the extracted DNA into agarose gel electrophoresis, before the amplification of the DNA by PCR. The purified DNA samples were electrophoresed and images were captured after electrophoresis using UV gel documentation system Bio Rad universal Hood II (Gel-Doc XR: 170- 8170), or EC3 Imaging System from UVP as described in section (DNA agarose gel electrophoresis).

6.4.3 Measuring the DNA concentration using the Qubit® Fluorometer

To determine the purity and quantity of genomic DNA samples, the Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific, Life Technologies. Cat. No. Q32850) was used with the Qubit® 2.0 Fluorometer according to the manufacturer instructions (Xiong et al. 2013). The assay was performed at room temperature using the Qubit® Fluorometer to establish the concentration measurements of 2 μL of the genomic DNA. These measurements are a much better indication of sample purity than that produced by measuring the A260/A280 ratio spectrophotometrically. The Qubit reagent was diluted 1:200 using the buffer provided to prepare the Qubit working solution. The standard solutions were prepared by adding 10 μL of each Qubit standard (Qubit® dsDNA BR Standard #1 and Standard #2) to the appropriate tube of 190 μL of Qubit® working solution, and mixed by vortexing for 2–3 seconds. Then, 2 μL of each sample was added to the 198 μL of the Qubit working solution and mixed in Qubit assay tubes (Life Technologies Cat. No. Q32856) and incubated at
room temperature for 2 minutes. The final volume in each tube must be 200 μL. After that, values of the genomic DNA concentration were read using the Qubit® 2.0 Fluorometer. The Qubit assay is highly selective for double stranded DNA (dsDNA), and based on target-specific fluorescent dyes, this assay offer accurate DNA quantification values, even when samples contain common contaminants such as salts, free nucleotides, detergents, solvents or protein which are well tolerated in this assay (Couto et al. 2010; Bundo et al. 2012).

6.4.4 Conventional polymerase chain reaction PCR amplification

6.4.4.1 Primers and probes design

The full length sequences of complementary DNA (cDNA) of the genes of interest (CBF and bar genes), and the GUS gene were identified using GenBank submission tools by the GenBank accession number for the interest genes. An appropriate primer in terms of sequence, number of nucleotides and melting temperature for each gene was designed using the primer-BLAST software of the National Centre of Biotechnology Institute (NCBI). The annealing temperature of the primers that were used was between 60 and 65°C, and the primers designed to produce amplicons of sizes 150-500 bp to determine the correct amplicon size of genes inserted in the plant genome.

Probes and primers for RT-PCR amplification were designed using the Primer Express Software v3.0. The GC content % and the length of sequence were considered during the design. The annealing temperature of primers designed was between 56 and 61°C, and the annealing temperature of probes ranged between 48°C and 58°C. The primers and probes were commercially synthesised by oligonucleotide synthesis (Eurofins MWG Operon). Lyophilized primers were
dissolved in appropriate volumes of molecular biology grade water (MBW) to prepare 10 pmol μL⁻¹. Then, aliquots of probes and 200 μL of primers concentrated 10 pmol μL⁻¹ were stored at -20°C.

6.4.4.2 Polymerase Chain Reaction PCR amplification of genomic DNA

Integration of T-DNA, carrying the marker gene (bar or NPTII) or the CBF gene, in the genomes of transgenic maize plants was assessed through PCR analysis of total isolated DNA (Mamontova et al. 2010) using primers of the selection gene (antibiotic resistance). Amplicons of the genes of interest were amplified using genomic DNA extracted and prepared in the manner described in the section “Genomic DNA extraction”. Forward and reverse gene-specific primers designed using the Primer-Blast software were used to verify CBF, gus and bar gene as follow:

CBF-85f, 5’ ATGGTCATGGACATGATCTTCG 3’, and CBF-557r, 5’ AGCTAAGCATTGGGGTGAGAAAG 3’.

bar-f 5’ TCTACACCCACCTGCTGAAGTC 3’, and
bar-r 5’ AAACCCACGTGTCATTCC 3’.

GUS-f 5’ CAACGTCTGCTATCAGCGGAAT 3’, and
GUS-r 5’ TATCCGGGTTGGCAATC 3’.

Genomic DNA was used as template DNA for the PCR detection system. The reaction components were prepared using DNase free PCR tubes (200 μL) as follows:
The master mix consisted of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>2 μL</td>
</tr>
<tr>
<td>Red-Taq PCR reaction buffer (1 x), (Sigma: Cat. No. D 4309)</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>dNTPs (Deoxynucleotide Mix) (200 μM ), (Sigma: Cat.No.D7295)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Red-Taq DNA polymerase (0.05 U μL⁻¹)</td>
<td>1.25 μL</td>
</tr>
<tr>
<td>M.H20</td>
<td>16.25 μL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25 μL</strong></td>
</tr>
</tbody>
</table>

The contents of the master mix were mixed thoroughly by pipetting, and centrifuged briefly to collect the mixture at the bottom of the tubes. Then the reaction components of master mix 25 μL were added respectively for individual reaction. PCR controls were set using molecular water and non-transgenic DNA as a negative control instead of transformed genomic DNA. Standard PCR reactions were run using the Master Cycler Gradient (Eppendorf, Hamburg, Germany) under the following conditions of thermal cycle; initial denaturation at 94°C for 2 min followed by 40 cycles of (denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s), and a final extension at 72°C for 7 minutes followed by a hold of samples at 4°C until collected.

Samples of PCR products were mixed with loading buffer and run into 0.8% agarose gels in TAE buffer along with a 100 bp ladder, after which the gel was examined in a gel documentation system using SYBR safe dye under UV transillumination light under a Bio Rad universal Hood II (Gel-Doc XR: 170-8170) to detect the relative bands. DNA bands were visualized and band images were
captured to determine the correct amplicon. Band intensities proportions and locations were compared with the positive sample (*Agrobacterium* plasmid) and negative sample (control, non-transformed genomic DNA).

### 6.4.5 DNA agarose gel electrophoresis

The agarose gel electrophoresis was used to investigate the quality and quantity of genomic DNA of transformed and non-transformed events. Gel electrophoresis was conducted using 0.8% (w/v) agarose gels prepared by dissolving 0.4 g of agarose (Molecular biology grade, DNase and RNase free, Melford-Chemical and Biochemical manufacturer, cat# MB 1200) in 50 ml of TAE buffer (1×) (Tris-Acetate + EDTA, Invitrogen cat# 15558-026) (Appendix 2) in a 250 ml conical flask using a microwave on mid power for 2-3 minutes until the agarose completely dissolved. Then, 3 μL of SYBR safe dye (Fisher Scientific, UK. cat # VXS33102) was added to the molten agarose solution once the solution became cooler (50°C) and mixed well to make a homogeneous mixture. The molten agarose was poured into an end taped tray with an inserted comb, and allowed to solidify at the room temperature. After 20-30 minutes the set gel was placed into an electrophoresis tank (Pharmacia GNA100, UK) and submerged with 350 mL of (1×) TAE buffer. DNA samples were prepared by mixing 10 μL of each sample with 3 μL of DNA loading buffer. Samples were loaded into the gel's wells. Five μL of the molecular weight markers of 100 bp (Fisher Scientific, Cat. BP2571-100) was loaded in the first well as a reference ladder to estimate the size of the DNA product. The gel was run at 70 V until the dye front reached the end of gel and then the DNA was visualized using a UV transilluminator (390 nm) and photographed with the gel documentation system Bio- Rad universal Hood II (Gel-Doc XR: 170- 8170) (Bio-Rad, UK) (Appendix 2).
6.4.6 Purification of the genomic DNA (PCR products)

PCR products of the genomic DNA extracted from transgenic clones were purified directly from an amplification reaction using the Wizard SV Gel and PCR Clean-Up System kit. PCR products were purified using the membrane-based system which removed excess nucleotides and primers, and allowed recovery of PCR products up to 95% as described by the manufacturer and by following the following steps, (given in brief):

**Preparation of PCR products**

1. An equal volume of Membrane Binding Solution was added to the sample of PCR amplification.

**Binding of DNA**

2. SV Minicolumn was inserted into the Collection Tube.

3. Prepared PCR product was transferred to the Minicolumn assembly. Then, the Minicolumn containing the sample was incubated at the room temperature for 1 minute.

4. Samples were centrifuged at 16,000 × g for 1 minute. Flow-through liquid was discarded and the Minicolumn was reinserted into a new collection tube.

**Washing**

5. 700 µL of Membrane Wash Solution (80% ethanol) was added to the samples. Flowthrough discarded after centrifugation at 16,000 × g for 1 minute. Then, Minicolumns were reinserted into the collection tube.

6. Washing was repeated with 500 µL Membrane Wash Solution by centrifugation at 16,000 × g for 5 minutes.
7. Flowthrough liquid was discarded from the collection tube and the column assembly recentrifuged for 1 minute using the microcentrifuge with the tube lid open (or off) to allow evaporation of any residual ethanol.

**Elution of genomic DNA**

8. Carefully Minicolumns transferred to a clean 1.5 mL microcentrifuge tube. 50 µL of Nuclease-free water (Molecular biology water) was added to the Minicolumn. Samples were incubated at room temperature for 1 minute, and centrifuged at 16,000 × g for 1 minute.

9. Purified DNA eluted by centrifugation was stored at -20°C. Both the purity and quantity of DNA products were measured using a Nano-drop spectrophotometer (Lab Tech, UK).

This procedure describes the purification of specific DNA fragments employing the Wizard SV Gel and PCR CleanUp System from Promega (Zimmermann et al. 1998) cited by Promega. Com, and (Grundemann and Schomig 1996). Purified DNA can be used for automated fluorescent DNA sequencing without further manipulation in order to classify of genomic DNA transferred into the plant genome (Tan et al. 2013).

**6.4.7 Sequencing of PCR products**

The DNA sequencing of PCR products was performed by the genome analysis of plant genomic DNA to confirm the transient gene in transgenic plants. Purified PCR products (20 µL) at a concentration of 10-20 ngµL⁻¹ and 2 µL primers (forward and reverse 10 pmol µL⁻¹ each) were placed separately in 1.5 mL Eppendorf tubes and sent to the BioScience Innovation Centre (LBIC), (GATC Biotech, UK) for sequencing. The partial sequencing results obtained via the GATC Biotech website (www.gatc-biotech.com/en/index.html) were matched to those standard Nucleotide
BLAST sequences in available databases using the Basic Local Alignment Search Tool (BLAST, GeneBank) services (www.blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the phylogenetic relationships.

6.4.8 Quantitative Real-Time PCR (qRT-PCR) analysis

The StepOne and StepOnePlus systems use fluorescent-based PCR reagents to provide quantitative detection of target nucleic acid sequences (targets) using real-time analysis. Quantitative real-time PCR (qRT-PCR) was adopted for further confirmation of gene integration into transgenic genomes and to estimate the integrated transgene copy number in transgenic genomes of maize plants generated using the two different plasmid vector constructs.

The quantitative PCR (qRT-PCR) amplification was performed in 96-well reaction plates on the StepOnePlus™ Real-Time PCR systems (Applied Biosystems), in a 25 μL final volume, according to manufacturer’s instructions (TaqMan probe, Life Technologies Ltd, UK). Probes and gene-specific primers were designed using the Primer Express Software v3.0 (Table 6.1). Primers and probes used for the endogenous single copy gene “\textit{chi}” were as reported by Dalla Costa et al. (2009).
Table 6.1: Probes and primers sequences, length and annealing temperature, which designed and used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequences (5'-3'), length</th>
<th>Tm °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBF- f</td>
<td>CGTCGAGCAGTCCGAAGAA (19)</td>
<td>58.8</td>
</tr>
<tr>
<td>CBF- r</td>
<td>ACCGGGTGCCTCGTCTCT (18)</td>
<td>60.5</td>
</tr>
<tr>
<td>Chi- f</td>
<td>CATC GGCAAGCATGGTGTGTT (19)</td>
<td>56.7</td>
</tr>
<tr>
<td>Chi- r</td>
<td>TCCGATAATCTTGCTGCAAA (21)</td>
<td>55.9</td>
</tr>
<tr>
<td>bar- f</td>
<td>CGTCAACCACATCGAGACAA (23)</td>
<td>60.6</td>
</tr>
<tr>
<td>bar- r</td>
<td>GTCCACTCCTGCGGTTCCT (19)</td>
<td>61.0</td>
</tr>
<tr>
<td>CBF Probe</td>
<td>CGGGAAGGAAAGTTT (15)</td>
<td>47.8</td>
</tr>
<tr>
<td>Chi Probe</td>
<td>CCTGAAGCGAAGAAGA (16)</td>
<td>49.2</td>
</tr>
<tr>
<td>bar Probe</td>
<td>ACTTCCGTACCGAGCGG (17)</td>
<td>57.6</td>
</tr>
</tbody>
</table>

6.4.8.1 Prepare reaction mixes (Master Mix)

Each plate contained samples of CBF, chi and bar gene along with the negative controls of each gene. 21 x 25 μL of chi reaction mixture was prepared. 18 x 25 μL reaction mixes were prepared as well for the CBF gene and another for the bar gene (including samples and controls). For 25 μL of master mix, the following reagents were combined in a clean 1.5 mL Eppendorf tube, and reaction components added respectively, to the each reaction well as below:

Master Mix components:

<table>
<thead>
<tr>
<th></th>
<th>X1</th>
<th>X21</th>
<th>X18</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Universal MasterMix</td>
<td>12.5 μL</td>
<td>262.5 μL</td>
<td>225 μL</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>1 μL</td>
<td>21 μL</td>
<td>18 μL</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>1 μL</td>
<td>21 μL</td>
<td>18 μL</td>
</tr>
<tr>
<td>Probe</td>
<td>1 μL</td>
<td>21 μL</td>
<td>18 μL</td>
</tr>
<tr>
<td>M.H20</td>
<td>7.5 μL</td>
<td>157.5 μL</td>
<td>135 μL</td>
</tr>
</tbody>
</table>
The master mix was multiplied up for the required volume. All reaction mixes were mixed by vortex and stored on the ice before proceeding further.

6.4.8.2 Prepare DNA standards

A standard curve is needed for each primer/probe set for each gene. The concentrations of genomic DNA were measured using the Qubit® Fluorometer. One aliquot of genomic DNA was thawed in an Eppendorf tube as a known-concentration stock S0. After that, a standard DNA dilution series was made by adding 45 μL of molecular water to 5 Eppendorf tubes labelled S1, S2, S3, S4 and S5. Then, 5 μL of stock DNA (transgenic DNA) was added to the tube S1, mixed well to produce 50 μL of the first sample (10⁻¹ concentration). After that, 5 μL of S1 was added to the tube S2 and mixed well to produce dilution of 10⁻² of the stock. By the same way, all the dilutions were performed. Furthermore, a serial dilution of non-transgenic DNA was made using a stock of non-transgenic DNA to use as a control.

6.4.8.3 Run the reaction

Tenfold dilutions of the target sample were prepared in triplicate of 25 μL using the MicroAmp® optical 96 well reaction plate that covered with adhesive film (Applied Biosystems, USA). Quantitative PCR was performed in the StepOne™ Plus real-time PCR system (ABI Applied Biosystems, USA). The thermal PCR conditions were included denaturation for 2 minutes at 94°C, followed by 40 cycles of (denaturation at 94°C for 30 sec, primer-specific annealing temperature at 60 or 62°C for 1 min, and extension stage at 72°C for 30 sec), and a final extension at 72°C for 7 minutes.
To detect the quantity of target gene among the experiments, the slopes of the standard curves were determined by performing a linear regression test with StepOne™ software version 2.3 (Applied Bio-systems).

6.5 Histochemical GUS assays

β-Glucuronidases (GUS) are histochemically and fluorometrically detectable enzymes that hydrolyze the glycosidic bond between the glucuronic acid and other molecules of endogenous and exogenous compounds in the living organisms.

In plant molecular biology, the GUS gene is used as a reporter gene to study gene regulation in transformed plants, and its activity can be detected histochemically allowing the assessment of gene activity in transgenic plants (Jefferson 1989; Gallagher 2012).

The histochemical GUS assay (Jefferson 1987; Wilson et al. 1995) was used to screen for reporter gene expression in the herbicide screen survivors (herbicide resistant) derived from immature embryos treated with the construct pTF102. This assay was carried out on most of the bialaphos-resistant putative transgenic calli recovered from the selection of genotypes. Various parts of transformed plants and control plants of R₀ progeny underwent screening. Stem, leaf and root were cut from both transformed and non-transformed plants and submerged in the GUS reaction mixture as described by Jefferson (1987) protocol with some modification according the following procedures:

1. Firstly, GUS buffer solutions (staining solutions) were prepared containing X-Gluc and another solution without X-Gluc (Appendix 2).
2. Samples were placed on the ice for 30 minutes followed by samples washing with 1x- Phosphate buffer for 20 - 30 minutes on cold-ice with a change of buffer 2 times.

3. Samples were washed with distilled water. Then, plant tissues were washed in the GUS buffer without X-Gluc.

4. Samples were next immersed in staining solution buffer with X-Gluc, and subjected to vacuum infiltration for two to three minutes using Microfuge tubes which work well to immerse of small tissue samples, but for larger tissue samples, 24-well plates were used.

5. Samples were incubated in the GUS solution with X-Gluc overnight at 37°C and at room temperature in darkness to develop the blue colour of product.

6. Staining solution (GUS solution) was discarded and tissues were rinsed with distilled water, and then washed with several changes of 50% ethanol (EtOH). Samples were incubated approximately 12 hours between each 50% EtOH change until the tissues became clear from chlorophyll. After that, samples were checked for GUS staining activity.

6.6 Stable GUS expression in the R₁ progeny

Furthermore, histochemical staining of GUS activity was performed on leaf pieces and other parts of plant organs such as tassels and silks of R₁ progeny of transformed plants and control plants to confirm expression of the GUS transgene in offspring of studied germplasms.

Leaves from PCR positive transgenic plants were cut radially and washed with several changes of ice-cold distilled water. The tissue was washed in staining
solution buffer without X-Gluc. After that, tissues were incubated with X-Gluc according the general procedures.

6.7 Stable bar gene expression in transgenic plants of R₁ progeny

To confirm that the bar gene is expressed in the R₁ progeny of transgenic plants, 14-30 days after being transplanted into the soil, plants were sprayed with different concentrations of bialaphos (glufosinate) solution at concentrations of 150, 250 and 350 mgL⁻¹. A glufosinate leaf-spray test (Brettschneider et al. 1997) was carried out three to five times at 1 to 2 day intervals using the bialaphos solutions. Bialaphos solutions were prepared from the glufosinate-ammonium/Phosphinothricin herbicide (Sigma Aldrich- UK, CN. 45520) containing drops of 0.1% Tween. Plants were scored for herbicide resistance 2, 3, 4 and 5 days after spraying the plants, bialaphos-resistant plants were obvious as they retained normal leaf colour whilst the susceptible plants showed leaf bleaching.

6.8 Statistical analysis

All the numerical data were analysed using Minitab v.16 statistical software. Balanced analysis of variances (ANOVA) and one way analysis of variance were used for data analysis.
6.9 Results

6.9.1 Analysis of stable events

Histochemical GUS assays of $R_0$ progeny

The histochemical GUS assays were carried out on bialaphos-resistant callus to confirm the transgene into the transformed plants. Three different protocols of the GUS histochemical staining with X-Gluc (Table 6.2) were carried out using transformed and non-transformed calli induced from immature embryos mediated by $A.\ tumefaciens$ with the GUS reporter gene. Samples were incubated in the GUS solution with X-Gluc overnight at room temperature, and at 37°C in the dark. Then, plants tissues were visualized and the blue colour was histochemically detected.
Table 6.2: Details of the different protocols used in this study of GUS assay using histochemical staining with X-Gluc.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Ingredients</th>
<th>Concentrations</th>
<th>Stock</th>
<th>Want</th>
<th>Per 10 ml final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol, 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. H$_2$O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7100.0 μL</td>
</tr>
<tr>
<td>b. Sodium Phosphate buffer* pH 7.0</td>
<td>1.0M 0.1M</td>
<td></td>
<td></td>
<td></td>
<td>2000.0μL</td>
</tr>
<tr>
<td>- NaH$_2$PO$_4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[380.0 μL+</td>
</tr>
<tr>
<td>- Na$_2$HPO$_4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>620.0 μL]</td>
</tr>
<tr>
<td>c. NaEDTA</td>
<td>0.5M 10.0mM</td>
<td></td>
<td></td>
<td></td>
<td>200.0 μL</td>
</tr>
<tr>
<td>d. Triton X-100</td>
<td>10% 0.1%</td>
<td></td>
<td></td>
<td></td>
<td>100.0μL</td>
</tr>
<tr>
<td>e. K$_3$Fe(CN)$_6$ Potassium ferricyanide</td>
<td>50mM 1.0 mM</td>
<td></td>
<td></td>
<td></td>
<td>200.0μL</td>
</tr>
<tr>
<td>f. K$_4$Fe(CN)$_6$ Potassium ferrocyanide</td>
<td>50mM 1.0 mM</td>
<td></td>
<td></td>
<td></td>
<td>200.0μL</td>
</tr>
<tr>
<td>g. X-Gluc</td>
<td>0.1M 2.0mM</td>
<td></td>
<td></td>
<td></td>
<td>200.0μL</td>
</tr>
</tbody>
</table>

| Protocol, 2 |                            |                         |           |       |                       |
| a. H$_2$O |                           |                         |           |       | 8300.0 μL             |
| b. Sodium Phosphate buffer pH 7.0 | 1.0M 0.1M              |                         |           |       | 1000.0μL              |
| - NaH$_2$PO$_4$ |                           |                         |           |       |                       |
| c. NaEDTA | 0.5M 10.0mM |                         |           |       | 200.0 μL             |
| d. Triton X-100 | 10% 0.1% |                         |           |       | 100.0μL              |
| e. K$_3$Fe(CN)$_6$ Potassium ferricyanide | 50mM 1.0 mM |                         |           |       | 200.0μL              |
| f. X-Gluc | 0.1M 2.0mM |                         |           |       | 200.0μL              |

| Protocol, 3 |                            |                         |           |       |                       |
| a. H$_2$O |                           |                         |           |       | 7500.0 μL             |
| b. Sodium Phosphate buffer pH 7.0 | 1.0M 0.1M              |                         |           |       | 2000.0μL              |
| - NaH$_2$PO$_4$ |                           |                         |           |       | [1000.0 μL+         |
| - Na$_2$HPO$_4$ |                           |                         |           |       | 1000.0 μL]             |
| c. NaEDTA | 0.5M 10.0mM |                         |           |       | 200.0 μL             |
| d. Triton X-100 | 10% 0.1% |                         |           |       | 100.0μL              |
| e. X-Gluc | 0.1M 2.0mM |                         |           |       | 200.0μL              |

\*sodium phosphate buffer was prepared and considered as 2x- buffer stock.
Results were scored as the number of GUS-positive staining events of calli among the total number of callus immersed in the staining solution, %. Results showed highly significant differences between protocols used in this study (p< 0.001). Protocol 2 scored the highest percentage of GUS-positive staining events in both treatments (at room temperature and 37°C) (Plate 6.1). While transformed calli scored GUS-positive staining of the GUS gene expression in bialaphos-resistant callus (Plate 6. 2. a) the control (non-transformed callus) expressed no GUS staining activity (Plate 6. 2. b).

The transfer of gus gene to immature somatic embryos infected with A. tumefaciens carrying pTF102 plasmid containing a gus reporter gene was also investigated by the histochemical GUS assay after 3-4 days of co-cultivation on medium containing 300 mgL⁻¹ Cys (Plate 6. 3. a). Transient gus gene expression and distribution of blue foci in putative transformed callus type I induced from Syrian maize immature zygotic embryos infected with Agrobacterium EHA101, was also investigated after 7-10 days of callus induction on callus induction medium (Plate 6. 3. b). Results were obtained using protocol 2 by incubation of plant tissues in the GUS solution with X-Gluc for up to 12 hours at 37°C. There were no significant differences between the incubation at 37°C or at the room temperature in the dark as results showed in this study (p= 0.761).
Plate 6.1: Analysis of transgenic materials by different protocols of GUS histochemical staining in transgenic maize callus events infected with the *Agrobacterium tumefaciens* EHA101 transformed with pTF102 construct. 1a, shows histochemical analysis of GUS reporter gene expression in maize calli and leaves of R₀ progeny at 37°C in the dark. 1b, shows the GUS histochemical staining in transgenic maize callus events of Syrian genotypes using three different protocols of the GUS assay at room temperature. The non-transgenic control was labelled as NTC.
Plate 6. 2: Histochemical GUS staining of 2 independent callus events. 2a, transgenic callus expressing GUS reporter gene (GUS-positive staining). 2b, shows a non-transgenic control calli stained for GUS expression (GUS-negative staining for the gus gene activity).
Plate 6. 3: Transient expression of the gus gene after co-infection with A. tumefaciens strain EHA101 containing pTF102. 3a, shows GUS histochemical staining in maize embryos after 3-4 days of infection with Agrobacterium. 3b, transient expression of the gus gene in maize calli induced after 7 to 10 days of Agrobacterium infection.

On the other hand, results indicated that vacuum infiltration positively affected leaf staining. There were highly significant differences between treatments regarding the vacuum filtrations of leaf samples (p< 0.001). Leaves samples that exposed to infiltration of GUS staining solution for three minutes using a vacuum showed positive results of tissue staining with X-Gluc (Plate 6. 4. a) but negative results were registered for plant leaf tissues not subjected to vacuum infiltration (Plate 6. 4. b). However, the infiltration of GUS solution during staining was not necessary for callus
staining and no significant differences were found for callus treatment with the vacuum.

Plate 6. 4: Expression of transient *gus* gene in leaves of $R_0$ plants. 4a, showing the positive expression of the reporter gene in leaves subjected to a vacuum infiltration. 4b, shows the GUS-negative staining of leaves without vacuum infiltration. 4c, represents the non-transgenic control. Scale bar: 3.0mm.

A further investigation of transient *gus* reporter gene was carried out using the transformed roots of $R_0$ progeny to confirm the transformation efficiency. Results confirmed that transgenic plants were GUS-expressing plants, while non-transformed plants were non-expressing (Plate 6. 5).
Plate 6.5: Distribution of blue foci (transient *gus* gene expression) in shoots of $R_0$ transgenic maize plants infected with *A. tumefaciens* standard binary vector system, pTF102. 5a, shows the blue staining in transgenic roots expressing GUS reporter gene. 5b, shows non-transgenic control roots stained for the GUS expression. Scale bar= 3.0mm.

6.9.2 Confirmation of transgene integration in $R_1$ generation of transformed maize plants

To confirm the stable GUS expression in $R_1$ progenies of putative transgenic plants, further investigations of transient and stable GUS expression in the $R_1$ progeny of transgenic plants were conducted by histochemical GUS analysis. Leaf tissues and male and female flowers of transformed and non-transformed plants of $R_1$ progeny were tested to confirm expression of the *gus* transgene in progenies of the studied germplasms. Samples were submerged in the GUS substrate according to the general protocol of GUS staining assay using X-Gluc, vacuum infiltrated for 2-3 min, and incubated at 37°C overnight. Blue staining cells were visualized after removing chlorophyll from leaves tissues by soaking tissues in several changes of 50% ethanol. Plant tissue pieces were scored as positive or negative for GUS expression.
Results confirmed the stable GUS transgene expression in the leaf cells of R\textsubscript{1} progeny of transformed plants (Plate 6. 6). Moreover, the transient GUS gene expression and distribution of blue foci in transformed tassels and silks confirmed the stable transformation in R\textsubscript{1} generation of transformed maize plants. Non-transformed silks and tassels of the control plants were GUS negative (Plates 6. 7 and 6. 8). However, all the blue spots of tissues derived from bialaphos-resistant callus, or from R\textsubscript{0} leaves and roots of transgenic plants (Plate 6. 9), and the tissues of silks and tassels of R\textsubscript{1} progeny of transgenic plants that were GUS-positive areas confirmed the stable of transgene expression referring to an efficient transformation protocol with pTF102-plasmid using A. tumefaciens EHA101. In contrast, non-transformed callus and control explants tissues were GUS negative (Plate 6. 9).

Stable bar gene expression in transgenic plants of R\textsubscript{1} progeny was confirmed by the bialaphos (glufosinate) leaf-spray test through screening R\textsubscript{1} progenies (section: R\textsubscript{1} progeny screening for the bar gene expression).
Plate 6. 6: Stable expression of the transgene GUS in R₁ progeny of transgenic maize plants. Transient GUS gene expression and distribution of blue foci in transformed leaves, a GUS-positive staining (a), and the GUS-negative staining in leaves of non-transformed plants (b). Scale bar= 5.0mm.

Plate 6. 7: Stable GUS transgene expression in transgenic male flowers of R₁ progeny of maize plants infected with the pTF102. Transient GUS gene expression and distribution of blue foci in transformed tassels, GUS-positive staining (left), and GUS-negative staining of non-transformed tassels (right). Scale bar= 3.0mm.
Plate 6. 8: Transient and stable GUS transgene expression in transgenic female flowers of R₁ progeny of maize plants. 8a, shows transient GUS gene expression and distribution of blue foci in transformed silks, GUS-positive staining. 8b, shows non-transformed silks expressed GUS-negative staining of the GUS gene expression. Scale bar= 5.0 mm.

Plate 6. 9: GUS histochemical staining in transgenic maize callus, leaves and roots transformed with pTF102 expressing GUS gene of R₀ and R₁ progenies. Scale bar= 5.0 mm.
6.9.3 \( R_1 \) progeny screening for the \textit{bar} gene expression

To confirm the stability of the transgene integrated in \( R_1 \), 3 putative transgenic plants of the \( R_1 \) progeny that derived from GUS-positive \( R_0 \) progeny were bred in the laboratory alongside non-transformed plants as a control. The plants were screened for \textit{bar} gene expression by a bialaphos (glufosinate) leaf-spray test (Brettschneider \textit{et al.} 1997) to identify putative transgenic plants by the stable \textit{bar} gene expression in transgenic plants.

Results recorded after 5 days of plants spraying with the bialaphos solution showed that those which expressed the transgene, stayed green and alive after the spraying, whereas non-transgenic plants turned yellow and showed herbicide sensitivity and eventually died (Plate 6. 10). The bialaphos-resistant plants that expressed positive \textit{bar} gene expression in \( R_1 \) progeny also expressed GUS-positive activity for both generation \( R_0 \) and \( R_1 \).

The resistance of transgenic plants to bialaphos was different according the plant growth stage. Transgenic plants were resistant of 250 mgL\(^{-1}\) bialaphos after 2 weeks of transplanting in the soil. But, 3-4 weeks after being taken to the soil, transgenic plants showed a tolerance of 350 mgL\(^{-1}\) bialaphos. All transgenic plants expressing the \textit{bar} gene and were bialaphos-resistant compared to the non-transformed plants regardless of the concentrations of bialaphos used at each plant growth. The success of transgenic plant growth emphasized the reproducibility and efficiency of the protocol used in this study for maize transformation. Moreover, Syrian maize genotypes produced transgenic plants emphasizing their reproducibility and ability to be efficiently transformed using this protocol.
Plate 6. 10: Transgene expression in R₁ generation of transgenic maize plants; the surviving transgenic plant on the right was a bar-expressing plant (resistant) to glufosinate herbicide spray, whereas the non-expressing plant of bar gene on the left (sensitive) was dead.

6.9.4 Molecular analysis of transgenic plants using PCR

To confirm positive results of stable transformation, putative transformation events that were GUS positive were further analysed by standard PCR reactions with the appropriate primers using 100-300 ng of genomic DNA. Genomic DNA was extracted from bialaphos-resistant callus and from ten transformed plants of R₀ progeny, and purity and quantity of the genomic DNA was measured using a Nano-drop spectrophotometer. Furthermore, the quality of the genomic DNA was checked by the agarose gel electrophoresis before the DNA amplification by PCR. The extracted genomic DNA was visualized and images were captured after electrophoresis using UV gel documentation system (Figure 6. 1). Then, samples of the genomic DNA were subjected to the PCR amplification of a 170 bp fragment.
within the *bar* coding gene. Results obtained from the gel electrophoresis products confirmed the integration of the *bar* gene in the plant genome of transformed R₀ progeny. PCR products clearly appeared in bands which associated with transgenic calli and transgenic DNA derived from transgenic plants (Figure 6. 2).

Further investigation of stable transformation was carried out on the next generation of transgenic plants R₁ progenies. Four putative transformed plants of varieties and hybrids were identified and the genomic DNA extracted from leaves of R₁ progeny plants and amplified by conventional PCR using gene-specific primers. The PCR products were visualized following gel electrophoresis (Figure 6. 3). Results indicated the presence of the *bar* gene in R₁ progenies of Hi II transformed plants and in both the Syrian varieties and hybrids.

![Figure 6. 1: Quality checking of the extracted genomic DNA visualized by the UV gel documentation system after gel electrophoresis without PCR.](image)

To assess the efficiency of transformation and stable expression of the *gus* gene in transgenic plants, the *gus* gene was amplified by PCR using 2 μL, (100) ng of genomic DNA extracted from putative transformed calli and leaves of transformed plants of the R₀ and R₁ progenies of transformed plants. But the results of DNA amplification performed on the R₁ progeny of 2 transformed events showed negative results even though positive results of *bar* and cbf gene amplification were obtained compared with the control. This result means that the specific-gene primers for the
gus gene did not match the gus gene sequence suggesting designing other primers that requires different melting temperature (TM) and containing different sequence of bases that should be a good mix of all 4 nucleotides with a shorter length of sequence (Figure 6. 6). Compared to the negative control of non-transformed plants, transformed plants from both transgenic events showed positive results of bar and cbf gene amplification suggesting that bands related to stable gene expression in the plant genome. Results indicated the integration and presence of the bar gene and gus gene in both R₀ and R₁ progenies of transformed maize plants.

**Figure 6. 2:** PCR amplification of genomic DNA extracted from R₀ progeny of transformed events. Detection of DNA fragments containing the bar gene sequences of transformed calli (lanes: 2, 5 and 6); lane c, negative control (non-transformed DNA of callus). Detection of the bar gene extracted from leaves of the R₀ progeny (lanes: 9 and 10). Lane L, 100 bp ladder; lane 1, positive control pTF102; lanes 7 and 8 non-transformed DNA.
Figure 6. 3: PCR amplification of the genomic DNA of transformed R₁ progenies. Fig. 3A, detection of DNA fragments containing the bar gene sequences of transformed DNA of hybrids R₁ progeny. Lane L, 100 bp ladder; lanes 1 and 5 transformed DNA of regenerated plants; lane 6, negative control (non-transformed DNA) from non-transformed plants. Fig. 3B, containing the detection of transformed DNA of R₁ progenies of varieties. Lane 8 and 9 transformed DNA from varieties; lane 10, non-transformed DNA; lane MW, the control.

6.9.5 The expression of CBF gene in transgenic plants

The putative transgenic plants of R₀ and R₁ progeny transformed with the anti-stress gene cbf were confirmed by the standard PCR. Genomic DNA containing the cbf gene was amplified using the cbf gene-specific primers. PCR results showed bands of 478bp resulted from cbf amplification using the gene-specific primers confirming the integration of the cbf gene in the plant genome in the R₀ progeny (Figure 6. 4). Moreover, the presence of bands in the gel visualized by gel electrophoresis that derived from the DNA amplification of the R₁ transgenic plants confirmed stable cbf gene expression in the progenies of Syrian varieties and hybrids plants transformed with pBINPLUSARS/PpCBF1 construct (Figure 6. 5). PCR results confirmed the integration, presence and stable expression of the bar
and cbf transgenes in R₁ progenies of transformed maize plants compared with the control plants (Figure 6.6).

Figure 6.4: PCR amplification of the genomic DNA of R₀ progenies transformed with CBF gene. Detection of DNA fragments containing the CBF gene sequences of transformed R₀ progeny. Lane L, 100 bp ladder; lane c, negative control; lanes 1 and 2 transformed DNA of transgenic events; lane 3, the control (MW).
Figure 6.5: PCR amplification of genomic DNA with CBF gene sequences extracted from R₁ progeny of transformed events. Fig. 5A, containing the detection of transformed DNA of R₁ progenies of varieties. Lane L, 100 bp ladder, lane 1, non-transformed DNA; lanes 2 and 4 transformed DNA of varieties; lane MW, the control. Fig. 5B, Detection of DNA fragments containing the CBF gene sequences of transformed R₁ hybrids plants. Lanes: 1 and 2 transformed DNA; lane c, negative control (non-transformed DNA); lane MW, the control; lane L, a 100 bp ladder.
Figure 6.6: PCR amplification of the genomic DNA extracted from transformed maize plants of R₁ progenies. Detection of DNA fragments containing the transgenes bar gene and CBF gene of transformed DNA. Lane L, a 100 bp ladder; lanes 1 and 2 transformed DNA with bar gene of regenerated plants; lanes 3 and 4 related to gus gene of transformed DNA; lane 5 and 6 transformed DNA with CBF gene of Gh.1 variety and Gh. 82 variety; lane c, negative control (non-transformed DNA).

6.9.6 Identification of transgene by DNA sequencing

Four samples of PCR products of genomic DNA and DNA plasmid were purified using the Wizard SV Gel and PCR Clean-Up System kit as described in section ‘Purification of PCR products’ and then DNA concentration was measured using a Nano-drop spectrophotometer (Table 6.3). These samples were compared with the DNA plasmid extracted from the Agrobacterium strains containing plasmid constructs pTF102 and PpCBF1.

A dilution of the purified PCR amplicons of genomic DNA samples was carried out using molecular grade water to achieve a working stock of a 20 ng μL⁻¹ of genomic DNA and plasmids. Then, the purified PCR amplicons of genomic DNA of transgenes generated using the gene-specific primers were sequenced by the genome analysis and technology core centre (London Bioscience Innovation Centre,
LBIC-GATC Biotech, UK). The identification of transgenes was performed by Blast the partial sequence (provided in Appendix 2) obtained by the genome analysis using the basic local alignment search tool software from the national centre for biotechnology information (NCBI, http://www.ncbi.nlm.nih.gov/) as described in section ‘Sequencing of PCR products’. Results of the NCBI-BLAST database confirmed that the DNA sequencing of each transgene matched to the accession number of the transgene (Appendix 2) in the GenBank with similarity of 97-100% (http://www.ncbi.nlm.nih.gov/genbank/). These matching sequence regions of each gene could be utilized to design gene-specific primers.

It was found that the bar gene sequence was matched to the *Streptomyces hygroscopicus* strain A10 phosphinothricin acetyl transferase (bar) gene, complete cds with a similarity of 97% and with a similarity of 99% for the sequence of the DNA plasmid. The CBF gene sequence matched to the Loring C-repeat binding factor mRNA, complete cds, extracted from the *Prunus persica* cultivar with a similarity of 99% (CBF gene) and 100% for the PpCBF1 plasmid sequence (Appendix 2).
Table 6. 3: The concentration and purity of genomic DNA and plasmids sequenced using gene-specific primers.

<table>
<thead>
<tr>
<th>Samples origin</th>
<th>DNA concentration ng μL⁻¹</th>
<th>Purity A260/A280, A260/A230</th>
<th>Primers sequence (5'----3'), 10 pmol μL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bar (genomic DNA extracted from Hi II)</td>
<td>136.7</td>
<td>1.89/2.04</td>
<td>bar-f 5'TCTACACCCACCTGCTGAAGTC3'</td>
</tr>
<tr>
<td>Sample No.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bar (Agro plasmid)</td>
<td>149.5</td>
<td>1.91/2.05</td>
<td>bar-f 5'TCTACACCCACCTGCTGAAGTC3'</td>
</tr>
<tr>
<td>Sample No.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBF (genomic DNA, Gh.82)</td>
<td>99.8</td>
<td>1.84/2.04</td>
<td>CBF-557r, 5' AGCTAAGCATTGGGGTGGAGAAAG3'</td>
</tr>
<tr>
<td>Sample No.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBF (Agro plasmid)</td>
<td>140.1</td>
<td>1.96/2.10</td>
<td>CBF-85f, 5' ATGGTCATGGACATGATCTTCG 3'</td>
</tr>
</tbody>
</table>

* DNA concentration and the quality were determined using a Nano-drop spectrophotometer ND-1000, Labtech.

6.9.7 Presence/Absence experiments of transgenes in transgenic plants and the coby number of transgenes

The stable expression of transgenes bar and CBF in the transformed genomes of maize plants was confirmed by the presence/absence experiment using TagMan reagents. The specific sequence of interest genes (targets) was detected using the quantitative real-time PCR (qRT-PCR) designed to amplify and detect only this specific sequence by probes and gene-specific primers. Amplifications curves of transgenes showed the presence of the gene-specific sequence in the amplification plot referring to the stable expression of transgenes in the transformed plant genome (Figure 5, Appendix 2). To estimate the quantitative PCR products of target
genes, the concentration was plotted versus the cycle number at which the fluorescence signal exceeds the threshold cycle (Ct) value by relative PCR. Results showed that the copy number of the target genes \textit{cbf} and \textit{bar} gene could be estimated by the comparison of the standard curves of targets genes amplification with the standard curve of the \textit{chi} gene amplification (Figure 6. 7). The slope of the standard curve for the \textit{cbf} gene was -2.9 referring to a good efficiency of amplification as stated by Okabe \textit{et al.} (2007) that a reaction of amplification with 100\% efficiency will produce a slope -3.32. The efficiency of amplification (Eff\%) of the \textit{cbf} gene was determined using the slope of the standard curve. Results of amplification of the \textit{bar} gene showed that the copy number could be a single copy (Figure 6. 8) but the slope was not referring to the high efficiency of amplification so it is worth to investigate detection of the copy number using another probe.

It was found that the fluorescence signal of \textit{bar} gene amplification appeared after 19 cycles compared with the 21 cycles at which the fluorescence signal of \textit{chi} gene amplification appeared and that means the copy number of the \textit{bar} gene in this clone was matched to the copy number of \textit{chi} gene which is present as a single copy in the plant genome (Appendix 2). However, for the amplification of the \textit{cbf} gene, in some amplicons, the fluorescence signal exceeded the threshold cycle (Ct) value after 16 cycles compared with the \textit{chi} amplicons which means the copy number of \textit{cbf} gene exceeded the copy number of \textit{chi} gene in this clone (Appendix 2).
Molecular and histochemical analysis of transgenic maize plants

Figure 6.7: Standard curve of cbf gene amplification by RT-PCR

![Figure 6.7: Standard curve of cbf gene amplification by RT-PCR](image)

**Target: CBF Slope: -2.909 Y-Inter: 15.511 R²: 0.904 Eff%: 120.679**

Figure 6.8: Standard curve of the bar gene amplification using RT-PCR.

![Figure 6.8: Standard curve of the bar gene amplification using RT-PCR](image)

**Target: BAR Slope: 0.258 Y-Inter: 39.686 R²: 0.021 Eff%: -99.987**
6.10 Discussion

6.10.1 Stable expression of transgenes

Transient expression of GUS gene was confirmed in immature zygotic embryos infected with *A. tumefaciens*. Monitoring of the level of the transgene expression in the immature embryos after 3 to 10 days of inoculation gave a very useful indicator for optimization of the transformation protocol. This optimization included the density of *Agrobacterium* inoculum and the duration of inoculation (Amoah *et al.* 2001; Martinez-Trujillo *et al.* 2004; Hosein *et al.* 2012).

The effects of various factors on T-DNA delivery and the transient expression of the *gus* gene were investigated including the concentrations of Potassium ferricyanide, Potassium ferrocyanide and Sodium Phosphate buffer, and vacuum infiltration. The investigations showed the positive effects of 1mM Potassium ferricyanide on the staining of tissues and appearance of the blue colour in tissue cells. Using the GUS staining solution of the Sodium Phosphate buffer component of NaH$_2$PO$_4$, 0.1M (pH 7.0) containing 2mM X-Gluc resulted in a positive staining of *gus* transient expression.

The histochemical GUS staining using vacuum infiltration for leaf tissues caused positive results that facilitated the penetration of GUS solution containing the X-Gluc to the leaf cells. Vacuum infiltration increased *gus* expression in both callus and leaf samples. It was demonstrated that the influence of vacuum infiltration was significantly positive in staining the leaf tissues (p< 0.001). But, the effects of vacuum infiltration on the staining the callus was less than its effects on the leaf tissues with no significant differences (p> 0.05).
A histochemical GUS staining assay carried out on maize bialaphos-resistant callus and transformed organs of regenerated plants including silks, tassels, roots and leaves of transgenic plants to determine whether these express the transgene (the *gus* reporter gene) in their cells demonstrated the efficiency of the transformation protocol in T-DNA delivery. Shen *et al.* (1993) has previously described the expression of GUS reporter gene delivered with high efficiency to maize shoots by *A. tumefaciens*.

The level of GUS expression can be used to evaluate the efficiency of transformation mediated by various strains of *Agrobacterium tumefaciens*. Zheng *et al.* (2009) evaluated transformation efficiencies of rice plants using a vector containing the *Arabidopsis thaliana* Histone Gene *AtHTA1* and another vector with the target gene by the evaluation of GUS expression in transformed calli. Their results showed that the *AtHTA1* gene enhanced rice transformation efficiency. Moreover, the histochemical assay of GUS activity has proved to be a simple and valuable reporter used to check the transient gene expression in ripe fleshy fruit mediated by *Agrobacterium* (Spolaore *et al.* 2001).

The positive results of the histochemical GUS assay in transgenic plants even appeared clearly in roots of R₀, and in flowers of R₁ progeny indicating heritable constitutive expression. Furthermore there was stable expression of the *bar* gene in the R₁ progenies of transgenic plants. The glufosinate herbicide leaf-spray test (Brettschneider *et al.* 1997) was carried out on some of R₁ progenies plants to determine whether those expressing the *gus* reporter gene also expressed the *bar* gene as bialaphos-resistance and stable *bar* gene expression was observed using the glufosinate leaf-spray test. The glufosinate (bialaphos)-resistant plants of R₁ progeny confirmed the stability of the transgene integrated in transgenic plants. The
bar-expressing transgenic plants showed a resistance and survived the glufosinate herbicide spray, whereas the non-expressing plants (the controls) were sensitive and died (Frame et al. 2002). Furthermore, the bar-expressing transgenic plants correlated with the positive-GUS expression for the events evaluated in this study. The GUS expression in leaf tissue of R₁ progeny also correlated with positive or negative expression of the reporter gene in callus that regenerated to transgenic plants in all events.

6.10.2 Confirmation of transformation and copy number of transgenes

Stable integration of the bar, gus and CBF transgenes in the R₀ and R₁ progenies of independent transgenic events transformed with pTF102 and PpCBF1 constructs was assessed by PCR amplifications of genomic DNA. Progeny analyses confirmed the transfer of the transgenes into the maize cells (integration) and expression of the transgenes in R₀ and R₁ generations of transgenic events. Most of these events showed the expected 170bp product bands of the bar gene, 478bp product bands of CBF gene suggesting the integration of the bar and CBF transgenes cassettes in the maize genome of the R₀ generation progeny. Screening of R₁ generation progeny plants of transformed events for expression of the CBF, gus and bar transgenes also confirmed the stable integration of the transgenes in plant genome. A similar detection of transfer the bar and gus gene into the maize cells by Agrobacterium mediated transformation technique to infected immature zygotic embryos was confirmed by the PCR amplification (Ombori et al. 2013). Also, the T-DNA integration into plant genome using Agrobacterium-mediated transformation method was molecularly confirmed by the PCR amplification (Bundock et al. 1995; Belarmino and Mii 2000; Zeilinger 2004).
The real-time PCR confirmed stable integration of CBF and bar transgenes into maize genome of R₁ progeny. The estimated copy number of the transgenes obtained by quantitative real-time PCR (qRT-PCR) has been previously shown to yield identical results to the more established Southern blot analysis (Song et al. 2002) and so this technique was used here. Furthermore, a TaqMan quantitative real-time PCR detection system is suitable for efficient early screening of transgenic clones. Identifying transgene copy number by this system has been found to be more accurate than genomic Southern blot hybridization (Mason et al. 2002) and it is suitable for the determination of transgene homozygotes in segregation populations effectively and reduces the cost and intensive labour requirements (Yi et al. 2008). In addition this method is amenable to identifying transgene copy number for large numbers of transgenic events rapidly while requiring very little tissue (Song et al. 2002). Thus, qRT-PCR represents an efficient means for determining transgene copy numbers in transgenic maize plants using Applied Biosystem, StepOne Plus Real-Time PCR Software v2.3. Results demonstrated that maize transformation by the Agrobacterium tumefaciens containing a binary vector with a selectable marker gene cassette containing transcription factor resulted in a low copy of transgene inserted in transgenic maize plants and this results was compatible with Sivamani et al. (2015).
6.11 Conclusion

Integration of transgenes *bar*, *gus* and CBF in the transgenic genome of R₀ and R₁ progenies was investigated. The histochemical GUS assay confirmed the integration and stable transformation of the reporter gene *gus* in the genome of transgenic maize plants that had been transformed with the pTF102 plasmid construct containing the reporter gene and the selectable marker gene *bar*. The stable expression of the selectable marker gene *bar* was also confirmed by the herbicide bialaphos (glufosinate) leaf-spray test according to Brettschneider *et al.* (1997). The *bar* gene-expressing plants showed bialaphos resistance compared with the control plants that were *bar* gene non-expressing. These putative transgenic plants were identified and the stable transgene integration was confirmed by the PCR amplification using the specific-gene primers of CBF and *bar* genes. The insertion and integration of specific DNA fragments in the transgenic genome of R₀ and R₁ progeny of maize plants transformed with the pTF102 construct was investigated by PCR amplification. The presence of the CBF gene was also confirmed in transgenic plants transformed with the pBINPLUSARS/PpCBF1 plasmid contained in the *Agrobacterium tumefaciens* strains EHA105 using the amplification of specific fragments of genomic DNA by PCR amplification.
Chapter 7:

Characterisation of abiotic stress resistance of transformed maize plants by physiological assays
Abiotic stress assessment techniques

Physiological assay of salt stress resistance of transgenic plants

7.1 Introduction

Plants respond to numerous external stimuli and environmental stresses to regulate its growth and development (Wolters and Jürgens 2009). Several physiological changes possibly occur when plants are subjected to harmful stress conditions like drought and salinity. It has been observed that several physiological pathways like photosynthesis, nitrogen fixation, respiration and carbohydrate metabolism can be affected by high salinity (Chen et al. 2008).

Salinity affects photosynthetic components such as chlorophylls and enzymes causing changes in the photosynthetic parameters. These changes in photosynthetic parameters depend on the severity and duration of salt stress (Misra et al. 1997), and on plant species (Dubey and Pessarakli 2002).

Chlorosis is a common response of salinity stress (Parida and Das 2005; Jamil et al. 2007). It has been found that salinity reduces chlorophyll content due to the accumulation of sodium ions during the biosynthesis of the different chlorophyll fractions (Reddy and Vora 1986; Ali et al. 2004).

The main intention of this chapter is to investigate maize responses to salinity stress, and how the transgenic plants modify this response. Comparison between transgenic and non-transgenic plants photosynthetic efficiency, the accumulation of dry matter, leaf area index, plant height, stem diameter and the production were carried out.
7.2 Aims and objectives

The aim of the physiological assessment of genetically modified maize plants was to confirm the stability of abiotic stress tolerance/resistance of the transformed plants under salt stress. The physiological assay was a very important indicator to identify the response of the transgenic plants under in-vivo conditions.

The objectives of these experiments are summarised as:

1- To investigate the effect of salt treatments on in-vivo maize plants growing in the lab.
2- To estimate of the expression and nature of the resistance trait of abiotic stress of altered plants under salt stress in the lab.
3- To determine whether transgenic Syrian maize cultivar varieties have appreciable tolerance ability of salinity to potentially improve crop productivity in salty lands.

7.3 Materials and methods

7.3.1 Plant materials

R₁ progeny of altered Syrian maize var. Ghota.1 that showed positive PCR analysis indicating the presence of the gene of interest (CBF) were used in this study and compared with R₁ plants of non-transgenic var. Ghota.1 used as a control.
7.4 Methodology

7.4.1 Experimental design

Laboratory experiments were conducted to monitor the response of transformed maize plants to salt stress conditions during the whole plant life-cycle up to and following tasselling, and to compare several indicators of salt resistance or plant tolerance. Many physiological measurements of control and transformed maize plants were carried out.

This experiment consisted of 9 treatments of salinity with different concentrations of NaCl (0 mM (control), 50, 100, 150, 200, 250, 300, 350 and 500 mM), the approximate concentration of seawater being 500-600 mM NaCl. These different concentrations of salt were prepared from a Stock watering solution of 500 mM NaCl and mixing with distilled water in proportions of 0, 10, 20, 30, 40, 50, 60, 70, and 100% for the salinity treatments respectively. Each treatment contained three transformed maize plants of R₁ progeny, and three non-transformed plants of R₁ progeny of Ghota.1 genotype. The total experimental design was as follows: 2 genotypes (transformed and non-transformed plants) x 9 treatments x 3 replications. Every replication was based on one maize plant growing in a 20 cm diameter plastic plant pot, containing a substrate mix consisting of a 1:2:1 volumetric mixture of John Innes No.2 compost + multi-purpose compost (manufactured by Westland Ltd) + sand with 35g Osmocote Pro per pot for fertilization.

Initially, seedlings were grown in growth chambers (Sanyo Phytotron) in 10 cm square (0.70 L) pots containing John Innes seed compost under a 13 hour photoperiod (170 μmol s⁻¹ m⁻² photon flux density) at 22/20°C day/night for three weeks until full development of the third leaf. Doses of a nutrient solution (Calcium...
nitrate and Magnesium sulphate) were added to the plants as required. Then, the plantlets were transplanted into the 20 cm pots located in a walk-in growth room and grown to full maturity.

7.4.2 Screening for salt stress resistance

After 5 weeks of planting, plants for each treatment were irrigated using the saline solutions described above. The irrigation was repeated as necessary at the same time to all treatments. Eight irrigations were applied. Irrigation amounts ranged between 250 to 350 mL of saline water, but every time the same amount of watering solution was added to each pot.

Evaluation of plant resistance of salt stress was observed using physiological and productivity indicators including: Chlorophyll Fluorescence (Fv/Fm), dry weight, plant height, plant stem diameter, leaf area index, specific leaf area, size of tassels and the yield (number of ears and number of grains/ear). These parameters were recorded for each individual plant separately.

7.4.3 Chlorophyll fluorescence measurements

The parameter Fv/Fm; ratio of variable fluorescence Fv to maximum fluorescence Fm (Optisci.com. 2011) was measured as an indicator of the maximum efficiency of Photosystem II using the Pocket PEA Plus Chlorophyll Fluorimeter, Version: 1.10 (Hansatech Instruments Ltd) (Plate 7.1).

The first step of the measurement process of chlorophyll fluorescence was a dark adaptation period. The dark adaptation was carried out using custom built small lightweight leaf clips. The leaf clips were attached to three leaves per plant, and small shutter plates of the clips were closed over the plant leaves to exclude light for 30 minutes (Plate 7.2).
Fv/Fm measurement was achieved at a light intensity level of 3500 μmol m⁻² s⁻¹ for 1 second of saturation as the measurement period. Fv/Fm was observed after 30 minutes of a dark adaptation for all samples at the same time of measurement. The measurements were taken for all samples before the application of saline irrigation and subsequently once every 10 days for up to 1 month and then were observed every 7 days. Nine readings of chlorophyll fluorescence were taken, and one accompanied the time of leaf area index measurement. All the Fv/Fm readings were done at the same time of the day at each monitoring in order to mitigate against any diurnal fluctuations.
Plate 7. 1: The Pocket PEA Plus Chlorophyll Fluorimeter used for measurement of chlorophyll fluorescence.

Plate 7. 2: Dark Adaptation leaf clips placed on samples of maize plants with shutters in closed position prior to PEA measurements.
7.4.4 Dry weight (g)

Shoot dry weight, g (Wt) of maize plants was obtained by harvesting the whole plants individually after 14 weeks of planting. Fresh shoot weight of plants was taken and subsequently the dry weight was determined after wrapping in aluminium foil and drying in a forced-air oven at 70°C for 72h.

7.4.5 Leaf area index, LAI

Leaf area index (LAI) is the ratio of the leaf surface area to the ground area occupied by a plant stand. Leaf area per pot was measured non-destructively 12 weeks after planting. The lengths and maximum widths of three leaves per plant, the flag leaf and the two subtending leaves, were measured. The total leaf area per plant $L_A$ (cm$^2$) was calculated by multiplying the number of leaves on the plant by the leaf length, the leaf width and a correction factor (0.75) to account for the shape of the leaves. Subsequently, the LAI was calculated as the ratio of the total leaf area to the surface area of the pot:

$\frac{\text{Total leaf area cm}^2}{\text{Growth area cm}^2} = \frac{[\text{Leaf length x leaf width x No. of leaves per plant x 0.75}]}{\text{growth area}^*}$

* Growth area (A): the area of the pot surface; $d=20$ cm. $A = \pi r^2$

0.75: Correction factor

7.4.6 Specific leaf area SLA, cm$^2$ g$^{-1}$

Specific leaf area is the ratio between the total leaf area per plant $L_A$ and total leaf dry weight per plant $L_W$. This index of the leaf thickness involves an assessment of the leaf area in relation to its dry weight to reflect the area per mass cm$^2$ g$^{-1}$.

Specific leaf area was calculated by division of the total leaf area per plant by the dry weight of the leaves $L_A/L_W$ cm$^2$ g$^{-1}$ (Pérez-Harguindeguy et al. 2013).
7.4.7 Plant height and plant stem diameter (cm)

Plant height was defined as the maximum height between the ground level and the upper boundary of the photosynthetic shoots at the point of tassel shed. Plant height is associated with growth vigour, reproductive size, competitive ability and whole-plant fertility (Pérez-Harguindeguy *et al.* 2013).

It was reported that there is a consistent relationship between relative height of maize plant and biomass within a genotype (Wilhelm *et al.* 2010).

The plant stem diameter (d) around the ear position for each plant was calculated using the following formula:

\[
C = 2\pi r^*
\]

c: The circumference of the stem (cm) which measured using a measuring tape (Soft Tape Measure Sewing Tailor Ruler).

*r: radius, d= 2r.

7.5 Statistical analysis

The multiple comparison post hoc test Fisher’s least significant difference (LSD) was used to compare the means. Data in tables and figures are presented as means +/- standard error (SE) and the probability p< 0.05 was considered significant using probability tables (Fisher and Yates 1957) at the significance level 5% of the t-distribution table (two-tailed).
7.6 Results

Physiological assay of salt stress resistance for assessing transgenic maize plants

7.6.1 Chlorophyll Fluorescence monitoring Fv/Fm

Results showed significant differences of the maximum quantum efficiency of PSII photochemistry Fv/Fm, (Table 7. 1) between the transgenic and control genotypes in their response to the salt stress. The photosynthesis of both genotypes was strongly affected by salt concentrations and there were highly significant differences among the treatments. The interaction between the treatments and genotypes was significant (Table 7. 1).

The overall response of transformed plants to salt treatments was better than the response of non-transformed plants with a significant difference of the maximum yield of Fv/Fm (Figure 7. 1). When the salt concentration was increased above 100 mM up to 200 mM, the Fv/Fm values of both transformed and non-transformed plants decreased (Figure 7. 2) indicating that salt stress was having a detrimental effect on the physiology of the photosynthetic apparatus. The transformed plants however were characterized by a higher chlorophyll fluorescence value than the non-transformed plants, particularly for the salinity treatments of 150 and 200 mM indicating that they coped better with the salt stress than the control plants (Figure 7. 2).
Table 7.1: Fishers significance test (p value) and Least Significant Difference (L.S.D) values of Fv/Fm and dry weight Wt at 5 and 1% level of significance (two-tailed).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>P value</th>
<th>L.S.D (0.05)</th>
<th>L.S.D (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fv/Fm</td>
<td>Wt</td>
<td>Fv/Fm</td>
</tr>
<tr>
<td>Treatments (salt)</td>
<td>0.000***</td>
<td>0.000***</td>
<td>0.11</td>
</tr>
<tr>
<td>Genotypes</td>
<td>0.015*</td>
<td>0.008**</td>
<td>0.05</td>
</tr>
<tr>
<td>Treatments x Genotypes</td>
<td>0.039*</td>
<td>0.172</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Significant - *  Very significant - **  Highly significant - ***  N.S. Non-significant

Figure 7.1: The effect of salinity on overall mean chlorophyll fluorescence of maize genotypes leaves. Values are means of nine treatments containing 54 plants.
Figure 7. 2: The response of PS II of transformed and non-transformed plants to irrigation with increasing salt concentrations.
7.6.2 Combined Fv/Fm monitoring over time

The total Fv/Fm was accumulated and averaged over time that is the readings for 1, 10, 20, 30, 40, 47, 54, 61 and 68 days were combined and this analysis showed highly significant differences between the two genotypes (p< 0.001) with the transformed plants being better than the controls (Figure 7. 3).

Figure 7. 3: The overall effect of salinity on maize genotypes performance chlorophyll fluorescence parameter of leaves over time. Values are means of eight readings of Fv/Fm for nine treatments containing 54 plants. SE: 0.020- 0.017.

There was a significant negative relationship between the salt stress resistance of genotypes and the plant exposure time of salinity through treatments (p< 0.001 for Genotypes x Time). As the exposure time of salt stress increased, the chlorophyll fluorescence efficiency decreased indicating the incremental increase in salt stress as salinity irrigations increased over time.

The response of transformed maize plants of salt stress was better than the response of non-transformed plants (Figure 7. 4). However, the Fv/Fm values were
decreased significantly in both genotypes over time by the salinity treatments. Results demonstrated that the response of transformed plant differed significantly from non-transformed plants and the salt-stress resistance of transformed plants was higher than the resistance of non-transformed plants (p< 0.001).

**Figure 7.4: The response of transgenic and non-transgenic maize plants to salt stress over time.**

The interaction between the treatments and the time of treatment was highly significant (p< 0.001). Plants were affected gradually by the time of salinity treatments. After 61 days of salt stress, transformed plants showed decreasing in the chlorophyll fluorescence response (Figure 7. 5. a). However, the transformed plants response of salt stress was better than the response of non-transformed plants which showed a greater decrease in the Fv/Fm values at an earlier time 47 days of treatment (Figure 7. 5. b).
Figure 7.5.a. The response of transformed plants to salinity treatments over time.

Figure 7.5.b. 1: The response of non-transformed maize plants to salinity over time.
7.6.3 Dry weight (g)

Results showed highly significant differences within treatments regarding dry matter accumulation under different concentrations of salt \( p<0.001 \) (Table 7.1). At concentrations of salt irrigation above 250 mM the transformed plants ability to accumulate dry matter was higher than the ability of non-transformed plants of dry matter accumulation (Figure 7.6). There were also significant differences between the transformed and non-transformed plants (at 5% and 1% level of significance, see Table 2, appendix 3) at concentrations of 50, 100, 150, 200 mM (Figure 7.6).

![Figure 7.6: Dry matter accumulation of maize plants at different concentrations of salt irrigation.](image)

There was an overall significant difference between the genotypes according ANOVA analysis \( p=0.008 \) (Table 7.1) with the transformed plants being significantly superior to the non-transformed plants in their ability to accumulate the dry matter under the salt stress (Figure 7.7).
Dry matter accumulation was significantly affected by the salt concentrations. There was a significant regression in the dry weight associated with the increase of salinity concentrations (Figure 7.8).

Figure 7.8: Dry matter accumulation of non-transgenic and transgenic maize plants influenced by different applications of salt concentrations.
7.6.4 Leaf area index (LAI)

There were highly significant differences within treatments regarding the leaf area index (Table 7.2) with significant differences between the two genotypes. Salt treatments interacted with genotypes, so the interaction between the treatments and genotypes was significant (Table 7.2).

The ability of the transformed plants to form a leaf surface under the salt stress was better than the ability of non-transformed plants (Figure 7.9) and LAI was higher for transformed plants under all salinity treatments except that of 250 mM.

Table 7.2: Fisher significance test (p value) and Least Significant Difference (L.S.D) values of leaf area index, LAI and specific leaf area, SLA at 5 and 1% level of significance (two-tailed).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>P value</th>
<th>L.S.D (0.05)</th>
<th>L.S.D (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traits</td>
<td></td>
<td>LAI</td>
<td>LAI</td>
</tr>
<tr>
<td>Treatments</td>
<td>0.000***</td>
<td>0.012*</td>
<td>1.38</td>
</tr>
<tr>
<td>Genotypes</td>
<td>0.028*</td>
<td>0.049*</td>
<td>0.65</td>
</tr>
<tr>
<td>Treatments x Genotypes</td>
<td>0.041*</td>
<td>0.804</td>
<td>1.96</td>
</tr>
</tbody>
</table>

Significant -* Very significant - *** Highly significant -**** N.S. Non-significant
Characterisation of abiotic-stress resistance of transformed plants

Figure 7.9: Leaf area index of genotypes under the salt stress.

ANOVA analysis showed that overall differences between genotypes was significant (p= 0.028) with transformed plants showing LAI values higher than non-transformed plants (Figure 7.10).

Figure 7.10: Overall leaf area index of transformed and non-transformed plants. Values are the means of LAI of 54 plants of transformed and non-transformed plants (27 plants each). SE: 0.62-0.71.
The leaf area decreased in association with the increase of salinity concentrations for most treatments and the relationship between the LAI and the salinity concentrations was negative in a linear model (Figure 7.11).

![Graph showing the relationship between leaf area index (LAI) and NaCl concentrations (mM). The equation is y = -0.0106x + 6.7232 and R² = 0.7067.]

**Figure 7.11: Overall leaf area index under different salt concentrations.**

### 7.6.5 Specific leaf area, SLA (cm² g⁻¹)

Analysis of variance revealed significant differences between the genotypes and within salt treatments for the trait of specific leaf area (Table 7.2) with significant differences between the transformed and non-transformed plants regarding its specific leaf area (Figure 7.12). SLA values of non-transformed plants were higher than the transformed plants values in the treatments 150, 200, 250 and 500 mM. The differences between the genotypes were significant in the 50 mM treatment and 200, 250 and 500 mM treatment (Table 3, appendix 3). This is an index that the response of transformed plants to salt stress was better than non-transformed plants.
Characterisation of abiotic-stress resistance of transformed plants

Figure 7. 12: The effect of salt application on Specific Leaf Area.

In general, for specific leaf area index of all treatments, results derived from balanced ANOVA analysis showed significant differences between the genotypes (Figure 7. 13). It is clear that the decline of SLA values from 338 cm$^2$ g$^{-1}$ for non-transformed plants to 290 cm$^2$ g$^{-1}$ for transformed plants was a significant index that transformed plants were more efficient in their ability to accumulate dry weight.

Figure 7. 13: Overall Specific leaf area of transformed and non-transformed genotypes for all salinity treatments.
The density of leaf or thinness of the leaf was affected by salinity concentrations. There were significant differences between genotypes for the 50mM treatment and the treatments of 200, 250, 300, 350 and 500mM (Table 3, appendix 3). However, the relationship between the SLA and the salt concentrations was positive. Findings reflected the sensitivity of plants under salt stress. The decreasing SLA values were correlated with the decrease in concentrations of salt in a linear trend ($R^2 = 0.87$) (Figure 7.14).

**Figure 7.14:** The effects of salinity treatments on the specific leaf area (cm² g⁻¹).
7.6.6 Plant height (cm)

There were highly significant differences between treatments in terms of plant height (Table 7.3) but there were no overall significant differences between transformed and non-transformed plants (Figure 7.15). However there were significant differences between the transformed and non-transformed plants under salt stress of 50, 200 and 250mM (Figure 7.16 and (Table 5, appendix 3)).

Table 7.3: Fisher significance test (p value) and Least Significant Difference (L.S.D) values of plant height and stem diameter (cm) at 5 and 1% level of significance (two-tailed).

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Traits</th>
<th>P value</th>
<th>L.S.D (0.05)</th>
<th>L.S.D (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plant height</td>
<td>Stem diameter</td>
<td>Plant height</td>
<td>Stem diameter</td>
</tr>
<tr>
<td>Treatments</td>
<td>0.000***</td>
<td>0.000***</td>
<td>20.37</td>
<td>0.15</td>
</tr>
<tr>
<td>Genotypes</td>
<td>0.267</td>
<td>0.009**</td>
<td>N.S</td>
<td>0.07</td>
</tr>
<tr>
<td>Treatments x Genotypes</td>
<td>0.654</td>
<td>0.101</td>
<td>N.S</td>
<td>N.S</td>
</tr>
</tbody>
</table>

Significant -* Very significant - ** Highly significant - *** N.S. Non-significant
Figure 7. 15: The height of transformed and non-transformed plants. Values are the means of plant height of 27 plants of each genotype.

Figure 7. 16: Assessment of plant height (cm) of genotypes under salt applications.
7.6.7 Plant stem diameter

There were highly significant differences in plant stem diameter within salt treatments with very significant differences between genotypes (Table 7.3). There were significant differences between transformed and non-transformed plants in treatments of 50, 100, 150 and 200mM (Figure 7.17). The transformed plants response under salt stress was better than the response of non-transformed plants for most treatments. There was a significant difference (p= 0.009) between the overall results for genotypes (Figure 7.18) with transformed plants having a bigger diameter than non-transformed plants (LSD= 0.07) (Table 4, appendix 3).

![Figure 7.17: Stem diameter of transformed and non-transformed plants in response to increasing salt stress.](image-url)
Figure 7.18: The overall effect of salt stress on stem diameter of genotypes, (LSD 5% = 0.07).

### 7.6.8 Fertility and productivity of salt-stressed plants

The fertility of maize plants was tested under salt stress by monitoring both male and female flowering and the ability to produce seeds. Seeds were produced from 3 of the transgenic plants under the salt stress while the non-transgenic plants failed to produce seeds under the treatments of salinity higher than 50mM (Table 7.4). The average number of seeds harvested from the transgenic plants grown in at 50mM NaCl was higher than that kernels harvested per ear of non-transgenic plants.
Table 7.4: Fertility of R₁ progeny of transgenic Ghota.1 plants transformed by EHA105/PpCBF1 in comparison with non-transgenic plants under salt stress.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>NaCl concentrations mM</th>
<th>R₁ Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transgenic plants</td>
<td>Non-transgenic plants</td>
</tr>
<tr>
<td></td>
<td>No. of Plant harvested</td>
<td>No. of seeds/cob</td>
</tr>
<tr>
<td>Variety</td>
<td>Control (0)</td>
<td>1</td>
</tr>
<tr>
<td>Ghota.1</td>
<td>50 mM</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>200 mM</td>
<td>1</td>
</tr>
</tbody>
</table>

a: The female plant was crossed with another non-transformed plant (control).
b: Harvested seeds were dried at room temperature before the weight.

7.7 Discussion

7.7.1 Effects of salt stress on transformed and non-transformed maize plants

The experimental design of this investigation was to gradually increase the salt stress to maize plants by irrigation with salty water thereby increasing the sodicity with time. By using different concentrations of salt in the irrigation water, the sodicity was increased at different rates according to the treatments. The objective was to assess whether the plants would be affected physiologically as the sodicity increased and whether these physiological effects were manifested in productivity decline traits and to see if the transgenic plants were in any way different from the control non-transformed plants. Non-significant differences were registered between genotypes for the efficiency of PSII at concentrations of salinity that less than 150mM but non-transformed plants were negatively affected by salt stress at concentrations of 150 and 200mM. Transformed plants also responded to salt stress significantly and their utilization of light (Fv/Fm values) was better than non-
transformed plants (Figure 7. 2). The effects of salinity on plant growth were accumulative over time and both genotypes were affected by salt stress significantly in association with the time of treatments. The negative effects of salt stress appeared clearly after 47 days of treatment. Transgenic plants were characterized with a better response to salinity, where its photosynthetic performance was better resulting in better growth than the non-transformed plants over the period of the experiment (Figure 7. 4). Low Fv/Fm values indicated the occurrence of photoinhibition related to an injury caused to the PSII complexes, or that this reduction was caused due to fluorescence emission from PSI (Krause and Jahns 2004; Papageorgiou 2004; Roháček et al. 2008).

Transgenic plants responded better to salt stress and accumulated dry matter better than the non-transformed plants up to 200mM salinity level. This increase in the dry weight was associated with both increased LAI and decreased SLA thickness of leaves. Thickening of leaves is a common observation of plants under stress. Specific leaf area of transformed plants was higher than non-transformed plant’s SLA under control. However, SLA values of transformed plants were lower than those of non-transformed plants under salt stress with significant differences in most treatments. This reveals the relative thinness of transgenic plant leaf, which is considered to be a more efficient anatomy than that shown by the non-transformed plants.

Increasing salt stress also affected plant height and stem diameter of the plants and transformed plants succeeded in forming a better plant height and thicker stem diameter until the salinity level of 200mM.
7.7.2 Transgenic plants resistance to salt stress

This experiment clearly demonstrated that the tolerance of transformed plants to salt stress was better than non-transformed plants. The photoinhibition was lower than the non-transformed plants suggesting that the transgenic plants had a better efficiency of light use under salt stress.

The transformed plants had a good density or relative thinness of leaf based on the leaf’s area in relation to its dry weight leading to a greater efficiency of transformed plants to accumulate the dry matter in relation to its leaf thickness compared to non-transformed plants (Figures 7.6 and 7.12).

Transgenic maize plants were superior to non-transformed plants for traits of plant height and stem diameter although transgenic plants trended to form thick stems with short plants under the higher saline concentrations above 300 mM. These results consistent with (Murchie and Lawson 2013).
7.8 Conclusion

Transgenic maize plants had a significantly greater stress tolerance than the control plants.

Findings reflected that the photosynthetic apparatus ability to utilize of light can affected by abiotic stress but the transformed plant response to salt stress was better than the response of non-transformed plants. The results were a significant indicator of how transformed plants respond to abiotic stress. It was demonstrated that transformed plants respond to salt stress up to a concentration of 250mM. Transformed plants were superior to non-transformed plants for the following traits: Fv/Fm, SLA, Wt and stem diameter. This superiority confirms the expression of the transgene and its physiological effectiveness.

Lower photoinhibition, lower leaf thickness and thicker stem diameter of transformed maize plants under saline stress might be a mechanism of resistance in maintaining good growth. This resistance to salinity is attributed the genetic variation between transformed and non-transformed plants. This genetic variation was derived from the action of the transgene as this is the only genetic difference between the genotypes tested. The successful transformation of a Syrian maize genotype with the stress gene CBF using A. tumefaciens confers improved stress resistance.

This study opens the possibility for maize plant growers to continue growing maize in saline soils or under irrigation with mildly saline water. Furthermore, a better understanding of the mechanisms of action of the transgene and the plant stress-tolerance is expected to utilize effectively in the production of maize plant abiotic stress resistance in arid and semiarid areas.
Chapter 8:

General discussion
General discussion

8.1 Genotype amenability to transformation

8.1.1 Stable transformation frequency

In this study 4 Syrian genotypes (2 varieties and 2 hybrids) were evaluated and genotypes varied somewhat in their amenability to transformation with the Agrobacterium tumefaciens strains EHA101 and EHA105 through immature embryos transformation. It was found that the transformation frequency of the two varieties (4.2%) was higher than the frequency of hybrids (3.7%) averaged over both Agrobacterium strains (Table 11, Chapter 5). Even, in previous studies, it was confirmed that callus induction was affected by genotype and callus induction medium with different concentrations of auxin (Rakshit et al. 2010). There were no significant differences between Syrian genotypes regarding callus induction. But, significant differences were found between genotypes regarding the callus weight. The callus weight was significantly affected by the initial embryo (explant) size and the genotype. The callus derived from varieties weighed significantly more than those initiated from the hybrids. The Syrian varieties developed callus successfully more than the hybrids with a higher percentage of transformation frequency indicating that the transformation frequency and callus differentiation was influenced by the genotype and these results correspond with the results of Wei, (2009) who demonstrated that transformation efficiency was influenced by genotype, and is one of the most important factors affecting the efficiency of transformation through the Agrobacterium tumefaciens-mediated plant transformation (Cao et al. 2014). The successful interaction between the Agrobacterium and plant genotype which involves numerous genes function from both Agrobacterium and plants was an essential
factor to ensure the efficiency of *Agrobacterium*-mediated plant transformation (Gelvin 2000).

Callus induction and callus weight were both significantly influenced by the size of the initial embryo (explant) infected with the *Agrobacterium* inoculation. This result confirmed that the embryo size is one of the most significant factors that influence the callus induction and callus weight as stated in Wu *et al.* (2003) and Shrawat *et al.* (2007). Similar results also demonstrated that the embryo size produced significant differences in T-DNA delivery and regeneration of wheat transformed by *Agrobacterium*-mediated transformation in addition to other factors affecting T-DNA delivery and plant regeneration such as duration of pre-culture, inoculation and co-cultivation, and the presence of acetylsyringone and Silwet-L77, a plant growth regulator, in the media (Wu *et al.* 2003). The callus induction frequency and somatic embryo-derived plantlet formation and development into fertile plants were influenced by plant media and the age of the immature embryos of maize genotypes from the tropics infected by *Agrobacterium* (Vega *et al.* 2008; Anami *et al.* 2010).

The Syrian varieties tested here responded positively to callus induction, but the percentage of callus induction was slightly less than that of the Syrian hybrids. However, the varieties had a transformation frequency higher than the hybrids indicating that the callus induction and transformation ability can be independently genotype dependent and these results correspond with the results of (Frame *et al.* 2011).
8.1.2 Transgenic plants production; Regeneration frequency and plant acclimatization

Results showed significant differences between genotypes regarding the regeneration of mature somatic embryos to produce transgenic plants. Syrian varieties were superior to the hybrids and showed a high ability of regeneration which was again more than that with the hybrids. The regeneration frequency of the two studied varieties (59.2%) was higher than the regeneration frequency of the hybrids (17%). Of the 49 transformed events with mature somatic embryos for which regeneration was attempted, 29 transgenic events of Syrian varieties regenerated to transgenic plants successfully but only 8 transgenic events of hybrids were regenerated out of 47 transformed survived events for which regeneration was attempted. Thereby, genotypic variation for regeneration is clearly present in the Syrian germplasm and confirms that the regeneration frequency is genotype dependent (Huang and Wei 2004; Hensel et al. 2009; Frame et al. 2011). This agrees with Rakshit et al. (2010) who confirmed that the percentage of shoot formation and root formation were influenced by regeneration media, source and concentrations of auxin and the genotype of maize. Results revealed that Syrian maize genotypes varied in their ability for regeneration in-vitro, which is a necessary pre-requisite to ensure success of transformation by Agrobacterium (Tzfira et al. 2002). The selection of immature embryo explants to transfer the genes of interest using Agrobacterium-mediated transformation led to successful transformation of maize with an efficient regeneration frequency agreeing with the literature (Shrawat and Lörz 2006). Furthermore, transformed cells of Syrian varieties that integrated the T-DNA into their chromosomes were recovered and regenerated into plants thus
overcoming the main hurdle in *A. tumefaciens*-mediated maize transformation after amenability of infection as stated by Komari and Kubo (1999).

The average regeneration frequency of transformed callus of Syrian varieties was lower (59.2%) compared with that from control non-transformed callus (67.8%) but, the regeneration frequency of the control hybrids (16.3%) was lower than the frequency of regeneration of transformed events of Syrian hybrids (17%). These slight differences between the transformed and non-transformed events regarding the regeneration ability are an indication that the regeneration frequency did not significantly influenced by the *Agrobacterium*-mediated transformation.

Syrian genotypes showed a good response to regenerate from explants of transgenic clones, 54 explants were regenerated from 32 transgenic clones of varieties transformed by both *Agrobacterium* constructs pTF102 and PpCBF1, 17 explants were, also, regenerated successfully from 8 transgenic clones of hybrids. This result revealed that in agreement with similar previous work on other maize genotypes (Frame et al. 2006 and 2011) an efficient protocol for Syrian maize regeneration and transformation using the *A. tumefaciens* standard binary vector system was established by empirical iteration of *in vitro* growing conditions and transformation conditions.

Moreover, Syrian maize genotypes responded to acclimatization in the growth chamber and succeeded to produce mature transgenic plants in the growth room. Of the 54 transgenic explants of varieties subjected to acclimatization, 41 (76%) grew successfully but only 11 (65%) transgenic explants of Syrian hybrids succeeded to produce mature transformed plants from 17 explants during acclimatization. Almost all transgenic plants successfully acclimatized in the growth room were normal in
morphology (98%). Recovery of morphologically normal transgenic maize plants from immature somatic embryos transformed with *Agrobacterium* was emphasized by (Ishida *et al.* 1996). Similar results were also reported in wheat and rice (Rashid *et al.* 1996; Cheng *et al.* 1997; Wu *et al.* 2003) and in dicots species (*Prunus subhirtella autumno rosa*) transformed by *A. tumefaciens* (da Camara Machado *et al.* 1995). However, a few events of abnormal morphologies of transgenic plants were registered in which abundant vegetation was formed containing meristems and leaf primordia which did not elongate to form shoots. In some cases, transgenic plants had a less severe abnormal phenotype which formed elongated shoots, but had abnormally shaped leaves i.e. severely lobed leaves or colourless leaf. Abnormal morphologies were attributed to the biological function of specific genes that encoding of products caused changes in the normal phenotype during the plant development process. The findings of Matsuoka *et al.* (1993) indicated that the expression of the *Oryza sativa* homeobox 1 (OSH1) gene, which is homologous to the maize morphological mutant Knotted-1 (Kn1) gene, introduced into rice resulted in altered leaf morphology during the plant development process. Moreover, it was demonstrated that abnormal leaf morphology that included severely lobed leaves in transgenic *Arabidopsis* plants caused by the over-expression of KNAT1 and kn1 supporting the hypothesis that kn1-like genes play a role in morphogenesis (Lincoln *et al.* 1994).

More importantly, seeds were harvested from emasculated and outcrossed (with control plants, transgenic plants as female) of 42 transgenic plants of R₀ progeny representing 31 plants of Syrian varieties with an average of 99 kernels per ear, and 11 of these events for Syrian hybrids with an average of 116 kernels per ear. Most of these transgenic events (74%) for both constructs PpCBF1 and pTF102 produced
seeds. Eighteen of these transgenic events were derived from *A. tumefaciens* infected maize with pTF102, whereas the remaining twenty four transgenic plants were recovered from transformation events using the PpCBF1 construct. However, the majority of non-transformed R₀ plants were produced seeds harvested from Syrian varieties and hybrids control plants (75, 63)% respectively. In contrast, transgenic seeds were produced from 3 outcrosses (60%) of R₁ Syrian Gh.1 variety transformed by *Agrobacterium*-PpCBF1 infection with an average of 83 kernels per transgenic ear. The majority (83%) of R₁ plants of Syrian genotypes transformed with pTF102 produced as many seeds as the seed-derived control hybrids plants (75%). These results demonstrate the fertility of transgenic plants produced by the *A. tumefaciens* agreeing with (Frame *et al.* 2002). Moreover, the reproducibility of this method of maize transformation using both vector systems was confirmed in this study through the production of fertile transgenic maize plants for R₀ and R₁ progenies and this result is consistent with the finding of Ishida *et al.* (1996) and Negrotto *et al.* (2000) in infection of immature zygotic embryos of the inbred line A188 or the hybrid line Hi II (Frame *et al.* 2002; Zhao *et al.* 2004).

### 8.2 *Agrobacterium* strain’s ability to transform Syrian maize genotypes

There were significant differences between the *Agrobacterium* strains used in this study. The *Agrobacterium* strain EHA101 had greater efficiency (6.4%) than the *Agrobacterium* strain EHA105 (3.3%) in the transformation of Syrian maize varieties. For the transformation of Syrian hybrids, also, the frequency of transformation using the strain EHA101 was higher (5.1%) than the transformation frequency resulted the *Agrobacterium* strain EHA105 (3.0%). These results agree with previous studies

8.3 Analysis of gene expression in transgenic progenies

8.3.1 Integration of transgenes in \( R_0 \) progeny of transgenic plants

Transient \( gus \) gene expression and distribution of blue foci was confirmed in putative transformed callus, root, stem and leaf of \( R_0 \) progeny derived from immature zygotic embryos of maize infected with \( A.\ tumefaciens \) EHA101 harbouring the pTF102 plasmid. The activity of the reporter \( gus \) gene in the transformed \( R_0 \) plants was detected histochemically using the histochemical GUS assay (Jefferson 1987; Wilson et al. 1995) agreeing with the literature Jefferson (1989) and Gallagher (2012).

Results revealed that the majority (about 92%) of bialaphos-resistant callus recovered from Syrian hybrids showed positive-GUS expression (24 of 26 survived Calli) with an average of transformation frequency of 4.2% based on the events evaluated using the GUS assay. The GUS assay also confirmed that of the 21 bialaphos-resistant recovered callus derived from Syrian varieties, 17 (81%) transformed calli expressed positive-GUS expression, and the frequency for varieties transformation averaged 4.7%. These results confirmed the integration of transgenes in \( R_0 \) progeny of Syrian maize genotypes transformed by the \( A.\ tumefaciens \) standard binary vector system confirming the efficiency of this method for maize transformation and agreeing with the literature in terms of efficiency of transformation (Frame et al. 2002; Kelly and Kado 2002; Ishida et al. 2007; Hensel et al. 2009; Cao et al. 2014). Moreover, it was shown, in this study, that the use of the Gus reporter gene in maize transformation, was an easy utilized method to confirm the integration
of transgenes and thereby to assess the stable transformation by *A. tumefaciens* as reported by Gallagher (2012) and Jefferson (1989). This utility of the histochemical GUS assay results from the catalytic activity of β-glucuronidase (GUS gene) and can easily be detected with commercially available synthetic substrates containing X-Gluc (Geddie and Matsumura 2007).

Results demonstrated that the GUS staining patterns of the transgenic calli varied quantitatively and qualitatively. Samples ranged from those which strongly expressed GUS which often exhibited a distribution of blue colour visually in all of the cells to those with only a few blue staining cells.

The variation in the GUS expression may have been the result of differences in the concentrations of potassium ferricyanide and potassium ferrocyanide. Therefore, transgenic calli that mostly expressed GUS expression in all of the cells or as a 'patchy' distribution of blue colour exhibited with densely stained aggregates of cells interspersed with aggregates that faintly stained accompanied with the concentrations of 1.0 mM potassium ferricyanide using 2.0 mM X-Gluc. Also, the number of calli that expressed the positive-GUS gene expression was best using the above concentrations of potassium ferricyanide and X-Gluc. However, the highly significant differences (p < 0.001) between samples regarding the GUS activity using different concentrations of ferri/ferrocyanide confirmed that the concentration of ferri- and ferrocyanide potassium in the staining solution was a critical point for the visualization of GUS activity as discussed by Vitha (2012). It was found that the presence of the ferri/and ferrocyanide in the GUS staining solution accelerated the tissues staining and protected the final reaction product from further oxidation that may convert the blue colour to a yellowish colour. On the other hand, while the optimal concentration of the ferri/ferrocyanide provides more precise localization of
blue colour by minimization of the diffusion of the primary reaction product in tissues, it may at the same time inhibit the β-glucuronidase enzyme activity (Vitha 2012). Therefore, the concentrations of ferricyanide and ferrocyanide are very important for the precision of the tissue staining in relation to the intensity of staining and the precision of localization. It is recommended; according this study, that the concentration of 1.0mM potassium ferricyanide is the optimal concentration in comparison with the absence of the ferri and ferrocyanide or use 1.0mM of ferri/and ferrocyanide each that led to low GUS activity. Empirical results indicated that the staining of leaf tissues was highly significantly affected by the vacuum infiltration of GUS staining solution (p< 0.001).

It was found that even when Triton X-100 (a wetting agent) was present in the GUS solution to facilitate the penetration of X-Gluc into the cell membrane, the GUS staining was not sufficient to stain leaf tissues without vacuum infiltration. Non-vacuum treated leaf tissues showed negligible GUS activity. In contrast, the leaf tissues that were subjected to vacuum infiltration showed strong GUS activity (positive-GUS expression) in transformed progenies. GUS activity was never observed in tissues of non-transformed control callus or plants (negative-GUS expression). Levels of the GUS expression in leaf tissues, as assayed with the vacuum infiltration, generally corresponded with the degree of staining observed with the histochemical GUS assay of calli without vacuum infiltration indicating that the low levels of GUS expression or the negative-GUS expression as detected by the histochemical assay of leaf tissues were caused by a lack of penetration of the GUS substrate into the leaf cells.

The assumption that transgenes were not efficiently expressed without using the vacuum infiltration was also the basis for accepting vacuum infiltration to detect the
activity of the GUS gene and relatively high levels of activity when compared to non-vacuum infiltration of transgenic materials.

Results showed that there were no differences between samples after incubation at the room temperature or at 37 °C (p= 0.761) agreeing with (Witcher et al. 1998; Vitha 2012).

8.3.2 Stable expression of transgenes in the R_1 progeny of transgenic maize plants

The bialaphos-resistant plants of R_1 progeny confirmed the stability of the transgene integrated in transgenic plants agreeing with previous studies (Frame et al. 2002; Gao et al. 2009; Zheng et al. 2009). Furthermore, the bialaphos-resistant plants that expressed positive bar gene expression in R_1 progeny were also monitored by the histochemical GUS assay to confirm the stability of transient genes since in addition to the selective bar gene; the binary vector carried the reporter GUS gene.

The GUS gene used in this study contained a portable intron at the start of the coding sequence (Vancanneyt et al. 1990) to prevent the expression of GUS activity in bacterial cells (Broothaerts et al. 2005). Thus, the results reported here (using the intron-GUS) restricted the GUS activity to transgenic plant cells that had incorporated and expressed the gene in transgenic clones more than the expression of bacteria indicating that the GUS reporter gene in the presence of an intron was useful to detect the expression of transgenes transferred into plant cells by the Agrobacterium agreeing with Ohta et al. (1990) and Broothaerts et al. (2005).

Results also showed that the GUS assay can be used in the R_1 to visually screen bialaphos-resistant plants to verify their transformation. The presence of the
GUS gene was detected in various parts of transformed plants such as leaf, male and female flowers of R₁ progeny that underwent screening. All bialaphos-resistant plants had at least some GUS-expressing cells while blue cells were never observed in the control (non-resistant plants of bialaphos).

The bialaphos-resistant samples observed by the GUS staining assay to detect the development of the blue colour were indicative of GUS expression and thereby for the stable gene expression in transgenic plants as stated by Frame et al. (2002) and Jefferson et al. (1987).

Putative transgenic clones that yield a positive-GUS expression were subsequently verified by PCR amplification of the bar coding region of the pTF102 plasmid and by the leaf spray test. Results, also, confirmed the stable transformation in putative transformants derived from R₀ and R₁ progenies by the qRT-PCR and standard PCR amplification of the CBF and bar gene transferred using the pBINPLUSARS/ PpCBF1 and pTF102 vectors respectively. These results, in addition to the expression of GUS activity visualized by the histochemical GUS assays, provided further evidence for the stable expression in the both progenies and that transformants were stable transgenic events in agreement with previous studies (Bundock et al. 1995; Ishida et al. 1996; Zeilinger 2004; Frame et al. 2011). It has been demonstrated in many plant transformation studies, that the transgenic plants of many species were molecularly confirmed by the PCR and qRT-PCR as stated in the literature (Falcone Ferreyra et al. 2010; Zhang et al. 2011; Ombori et al. 2013).
8.4 Physiological assays to characterize abiotic stress resistance in transgenic Syrian maize plants

8.4.1 Genotype response

The R₁ progeny of plants identified as transgenic were evaluated physiologically and for fertility under salinity stress as an easily applied abiotic stress.

Plants strongly varied in their response to the salt stress. Results showed significant differences of the maximum quantum efficiency of PSII photochemistry Fv/Fm, dry weight, leaf area index (LAI), specific leaf area (SLA) and stem diameter between the transgenic and non-transgenic plants in their response to the salt stress. The transgenic plants were characterized by a statistically higher maximum yield of Fv/Fm and dry matter than non-transgenic plants indicating that salt stress affected the physiology of the photosynthetic apparatus as stated by (Flexas et al. 2004; Qu et al. 2012; Athar et al. 2015). In addition according ANOVA analysis, there were overall significant differences between the genotypes with the transformed plants being significantly superior to the non-transformed plants in their ability to accumulate dry matter derived from less specific leaf area values (SLA) indicating that the efficiency of photosynthetic apparatus was better than non-transgenic plants to produce dry matter agreeing with (Zhou et al. 2008). The transgenic plants were less affected by salt stress and significantly superior to the non-transgenic plants in stem diameter and LAI agreeing with (Lindsey 2015).

The increase in dry matter accumulation affected by the leaf photosynthetic rate of green leaf area as reported by (Tollenaar et al. 2000; Tollenaar and Lee 2011). The transgenic plants succeeded to form a comparatively efficient leaf surface with a thick stem under the salt stress more than the non-transgenic plants confirming more
tolerance to salinity than the control. Thus, results revealed that the response of the transgenic plants to salt stress was much more effective than the control plants.

8.4.2 Effect of degree of salt stress

Results showed that there were highly significant differences between the salinity treatments regarding chlorophyll fluorescence, dry weight, leaf area index, specific leaf area, stem diameter and plant height. Findings reflected the sensitivity of plants under increasing salt stress. All traits monitored in this study for both genotypes (transgenic and non-transgenic) were strongly affected by salt concentrations. In general there was a systematic decrease in positive growth trait values recorded in association with increasing salt concentrations with a negative relationship between the treatments and the studied traits in a linear fashion.

It has been found that in transgenic plants carrying CBF that the Arabidopsis CBF/DREB1A gene activated as many as 12 target genes which regulated and induced with an additional 13 genes after exposure to drought stress leading to the transgenic plant’s tolerance to stress conditions (Oh et al. 2005). Overexpression of transcription factors DREB/CBF (TFs), which are the main regulators of abiotic stress gene expression, have been reported to enhance drought tolerance in rice (Datta et al. 2012), in wheat (Saint Pierre et al. 2012) and in maize (Liu et al. 2013). Furthermore, improvements in tolerance to abiotic stress have also been reported in many plant species such as maize, wheat, barley and rice which induced DREB2-type proteins under stress (Mizoi et al. 2012).

It was likely, according to this study, that the CBF gene inserted in the salt-tolerant transgenic plants controlled the transport of salt ions across membranes preventing their build-up in cytoplasm or cell walls and thus avoided salt toxicity. It
was confirmed that the CBF3 gene function in abscisic acid-independent stress-response pathways in transgenic plants enhanced the tolerance to salt stress agreeing with Oh et al. (2005), and Xu et al. (2011) who reported that CBF3 increased the tolerance of transgenic rice plants to high salinity and drought, and elevated relatively tolerance to low-temperature stresses. Shi et al. (2015) reported that AtCBF-mediated signalling pathway to accumulate soluble sugars such as sucrose in Arabidopsis thaliana which conferred resistance to abiotic and biotic stresses.

8.4.3 Fertility of salt-stressed maize plants

Results obtained in the current study emphasized that transgenic plants carrying the inserted anti-stress CBF gene were able to cope better with the salt stress than the control plants and to go on to produce seeds under the salt concentration of 200 mM whilst control plants were completely infertile.

Seeds were produced from outcrossing (pollen derived from non-transformed plants for bio-security reasons) R1; transgenic plants exposed to treatments of salinity at 50, 100, and 200mM of NaCl in contrast to the controls. While the transgenic plants produced seeds under the treatment of 200mM NaCl with an average of 23 kernels per ear, non-transgenic maize plants were infertile under the treatments of salinity higher than 50mM. Non-transgenic plants grown in soil irrigated with 50mM NaCl produced 22 kernels per ear compared to 49 kernels per ear of transgenic plants. At the control level (without salt addition), the average number of seeds harvested from the transgenic and non-transgenic plants was 43 and 49 kernels per ear respectively. These results revealed that the transgenic plants expressed the anti-stress gene under the salt stress and the transgenic Syrian maize varieties had appreciable tolerance. This tolerance to salinity has the potential to improve crop
productivity in salty lands. These results demonstrated that the genetic modification by modulation of the expression of the anti-stress genes that encode for enzymes involved in the biosynthesis of proteins and transcription factors, could possibly be used to produce abiotic stress-tolerant crops as referred to in previous studies (Xu et al. 1996; Sivamani et al. 2000; Zhang and Blumwald 2001; Quan et al. 2004a; Oh et al. 2005; Huang et al. 2008; Marco et al. 2015).
8.5 Conclusion

Despite intensive efforts in the plant genetic transformation, which is an important tool to produce abiotic stress-resistant plants, many agronomically important plant species such as maize still remain recalcitrant to Agrobacterium tumefaciens-mediated transformation, one of the most important methods of plant transformation. In this study, an efficient protocol of transformation by A. tumefaciens-mediated maize transformation was empirically developed to transform Syrian genotypes (varieties and hybrids) in an attempt to verify whether the Syrian genotypes have any suitability for transformation by Agrobacterium-mediated transformation or not. It was found that the transformation and generation of transgenic plants can be genotype dependent and it can be difficult to generate transgenic plants from genotypes that are recalcitrant to Agrobacterium-mediated transformation in agreement with Zheng et al. (2009). The approach of co-infection of immature maize embryos via the both Agrobacterium strains EHA101, harbouring the standard binary vector pTF102, and by the strain EHA105, containing the pBINPLUSARS/PpCBF1 vector, succeeded in the efficient transformation and generation of Syrian maize genotypes in this study and this is the first recorded success in the literature with such genotypes.

In order to maintain and care for the recombinant Agrobacterium strains used in this study containing the introduced plasmid, which have a tendency to be lost their plasmid and convert to wild type; glycerol stocks were prepared and subsequently Agrobacterium maintained with appropriate antibiotics in the freezer for future use as a pure transgenic culture. Moreover, the optimum time of incubation, pH,
temperature and the composition of the medium containing the appropriate antibiotics were investigated to initiate the transformation experiments with the maximum of harvest bacteria cells. Such maintenance of *Agrobacterium* strains needs then to be combined with precise techniques of co-cultivation of *A. tumefaciens* and immature embryos maize tissue cultures and the inoculation with active cells of *Agrobacterium* grown within the optimized conditions. Care and precision with the protocol is an absolute requirement for the repetition of the transformation efficiencies obtained in this study.

Transformants of Syrian genotypes were efficiently produced using callus derived from immature somatic embryos transformed by *A. tumefaciens* strains EHA101 and EHA105. Transformation frequencies (independent transgenic calli/infected embryos (x100)) were between 5.1 and 6.4% for hybrid and varieties using the *Agrobacterium* EHA101/pTF102, and averaged between 3.0 and 3.3% in the transformation of hybrids and varieties using the *Agrobacterium* EHA105/CFB respectively. Thus, the efficiency of *Agrobacterium*-mediated maize transformation varied in transformation of the Syrian genotypes. The *A. tumefaciens* strain EHA101 containing the pTF102 plasmid was almost up to 2-fold more efficient than the *A. tumefaciens* strain EHA105 containing the construct PpCBF1 in transformation of Syrian genotypes. This variation in the *Agrobacterium* efficiency is either caused by the susceptibility of the plant cells to transfer the transgenes, or by the virulence of the *Agrobacterium* strain resulted from virulence gene containing in the *Agrobacterium* (Gelvin 2000, 2003; Hwang and Gelvin 2004). However, results showed that the efficiency of transformation was genotype-dependent. Syrian varieties showed more transformation amenability by *A. tumefaciens* than hybrids that were more difficult to be transformed.
Co-infection of immature embryos sized 1.5-2.0 mm with *Agrobacterium* significantly increased the percentage of callus induction to 76%. Meanwhile, the percentage of callus weight was decreased from 11.7 g of calli derived from IEs sized >2.00 mm to 8.2 g in comparison with the callus weight derived from immature embryos 1.5 - 2.0 mm.

Despite all of these variations, the efficiencies obtained were equivalent to those quoted in the literature for many species including previously tested “efficient” maize cultivars such as Hi II, and encouragement is drawn from these results.

The selected putative transgenic callus events were subsequently regenerated to plants and successfully transferred to the growth room growing in soil. The regeneration frequency of Syrian genotypes ranged between 17 and 59.2% for hybrids and varieties respectively. These regeneration frequencies provide a basis for the success of Syrian maize transformation.

Stable transformation in $R_0$ and $R_1$ progenies was confirmed by the histochemical GUS assay demonstrating that the application of the GUS assay technique for an identification of transgenic clones by detection of the transgenes expression in maize plants was useful. The histochemical GUS staining of transgenic callus, shoots, leaves, root and floral parts which expressing the $\beta$-glucuronidase (GUS) reporter gene containing an intron under the transcriptional control of the Cauliflower Mosaic Virus (CaMV) 35S promoter, confirmed the transient expression gene in the $R_0$ and $R_1$ progenies. Moreover, results showed that the staining of leaf tissues was significantly affected by vacuum infiltration. A positive
correlation between the vacuum infiltration of leaf samples and a detection of GUS-positive expression in transgenic plants was also confirmed.

Molecular analysis of the R₀ and R₁ progenies provided strong evidence of the incorporation of transgenes into the maize genome. Integration of the bar and CBF genes in the R₀ progeny was confirmed by the PCR amplification of genomic DNA of transgenic events. Furthermore, the data from PCR analysis of R₁ progeny supported the findings of the GUS assay analyses confirming the stable transformation in R₁ plants, and revealing that the transgenes were inherited to the R₁ progeny. Similar results of qRT-PCR also confirmed the presence and stable expression of the CBF and bar transgenes in the maize genome of R₁ progeny of transgenic plants compared with control plants.

In summary, this research programme has produced transgenic maize plants of Syrian genotypes using A. tumefaciens-mediated transformation. Transgenic plants were regenerated from mature somatic embryos and plantlets transferred to soil approximately 17 to 25 weeks after inoculation of immature somatic embryos. Moreover, transgenic seeds of R₀ and R₁ progenies were harvested from outcrossing of transgenic plants (as female) with non-transformed plants (as male). Most of these outcrosses (74%) produced seeds and more than 98% of transgenic plants were normal in morphology.

Finally, it has been demonstrated in this study that A. tumefaciens-mediated maize transformation using a standard binary vector system is reproducible although genotype variability in transformation efficiency existed. It was demonstrated that this transformation system can be used to introduce genes of interest (anti-stress genes) into Syrian maize genomes for genetic improvement to abiotic-stress. Transgenic
CBF maize plants were produced using the *Agrobacterium* EHA105/PpCBF1. The transgenic CBF Syrian maize varieties expressed more tolerance ability of salinity than control plants.

Since *Agrobacterium*-mediated transformation using the protocol described in this study succeeded in the transformation of Syrian maize genotypes, it is possible to recommend this approach to transform other Syrian maize genotypes. This study constitutes an essential fundament for Syrian maize breeding programmes and applied research aiming to improve Syrian maize against drought, salinity and cold stresses.
Chapter 9:

Future perspectives and recommendations
Future perspectives and recommendations

9.1 Evaluation and selection of abiotic stress-resistant transgenic maize plants

The physiology and agronomic management of transgenic salt-tolerant Syrian maize genotypes produced in this study are required. Thereby, field studies containing the physiological and productivity traits should be conducted for 3 to 7 years in Syria, where the new transgenic genotypes would be adopted, before the full acceptance of these transgenic plants into the maize breeding programmes. The morphology or architecture of the transgenic plants such as: plant height, length and width of leaves, number of leaves, number of ears per plant, ear position, maturation cycles, number of rows/cob, number of grains per row and 1000 kernel weight should be considered in comparison with the non-transgenic local varieties.

9.2 Future recommendations for conservation of transgenic Syrian genotypes and yield improvement

- Provide training courses in maize breeding and crop management research to help the local farmers exploit the full potential of improved genotypes while conserving soil and water resources by providing them the information needed for optimum utilization of transgenic maize varieties.

- Collaborations between national (government), non-government organizations and international agricultural research institutions should be conducted to
tackle the problem either on a regional or global scale by providing farmers diverse, high-yielding maize varieties that withstand abiotic stress.

- The specific genetic characteristics of Syrian genotypes, which are adapted locally to specific ecological zones and so developed morphological characteristics, can be attained by backcrossing with transgenic genotypes through breeding programmes.

9.3 Studying the ability of Syrian maize transformation with anti-stress genes

Genetic modification of maize with anti-stress genes is very important, and by this technology, the demand for production of abiotic stress-resistant genotypes can be met. However, a range of transformation methods is available to help achieve the efficient transformation and specific pattern of transgene expression required, but there are still some challenges in this technology. A key remaining challenge is the genotype dependence of Syrian maize to be transformed and regenerated by this technology. This reluctance of regeneration continues to restrict the application of transformation technologies. It is necessary to address the issue of genotype-dependent transformation and to improve the efficiency of transformation by understanding and manipulating maize plant genes in both the genotype susceptibility to transformation via *Agrobacterium tumefaciens* and in the plant regeneration process (Harwood 2012).

The utility of *Agrobacterium*-mediated transformation as a “tool” for plant genetic engineering may help to produce abiotic stress-resistant genotypes of Syrian maize but further improvements in transformation technology of maize will necessarily involve the manipulation of fundamental biological processes of transforming and
regeneration of the specific genotypes that are locally adapted to Syrian agricultural systems within the environment conditions.

- More research is needed to determine whether inbred lines of Syrian maize (a cornerstone of maize breeding programmes) may have any possibility to be transformed by the Agrobacterium-mediated transformation and if so, do they have optimal regeneration frequencies or not? It is also important to investigate the ability of other explant of maize like pollen to be transformed via Agrobacterium-mediated transformation.

Use the Agrobacterium-mediated transformation to introduce other anti-stress genes such as: APX, a gene for ascorbate peroxidase (APX) contained in an expression vector (pCGN1578) in EHA101, and SOD; a gene for superoxide dismutase contained in A. tumefaciens strain EHA105 harbouring the pBINPLUS/ARS vector.

Furthermore, as maize is highly vulnerable to high temperature and it is mostly cultured during the spring in warm areas of Syria, a heat shock protein (HSP) gene could be selected as the gene of interest in the construction of a binary vector to enhance heat tolerance characteristic. HSPs, HSP101 has been reported to have a specific effect on increase of thermos-tolerance in several different organisms (Hong and Vierling 2000; Katiyar-Agarwal et al. 2003; Abdeeva et al. 2012; Ravanfar and Aziz 2015).
References:

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329


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Appendices
Appendices

Appendix 1:

Publications, presentation and conferences attended

Publications


Presentation and Conferences Attended

Oral Presentations

- **Almerei A, Lane S and Fuller MP.** “Genetic transformation of maize immature embryos using Agrobacterium tumefaciens”. School of Biological Sciences, University of Plymouth, 23rd January 2013.
- **Almerei A, Lane S and Fuller MP.** “Callus Induction Using Maize Embryos for Agrobacterium Tumefaciens-Mediated Transformation”. The 8th International Symposium on In Vitro Culture and Horticultural Breeding, Department of Life Sciences, University of Coimbra, Portugal, 2nd -7th June 2013.
- **Almerei A, Lane S and Fuller MP.** “Exploiting genetic transformation for abiotic stress-tolerant maize breeding”. SCI Young Researchers in
Agrisciences conference 2013; Crop production, protection and utilisation, University of Reading, 2\textsuperscript{nd} July 2013.

- Almerei A, Lane S and Fuller MP. “Agrobacterium mediated genetic modification of Syrian maize varieties with anti-stress genes”. The Centre for Agricultural and Rural Sustainability (CARS) 4\textsuperscript{th} Postgraduate Symposium 2013, Duchy College Rosewarne, Cornwall, UK, 11 November 2013.

Poster presentations

- Almerei A, Lane S and Fuller MP. The SEB Conference Glasgow, UK, 02\textsuperscript{nd} July 2011.
- Almerei A, Lane S and Fuller MP. “Genetic transformation using Syrian maize immature zygotic embryos”. The Centre for Agricultural and Rural Sustainability (CARS) PG Symposium, 10th December 2012.
- Almerei A, Lane S and Fuller MP. The second annual ISSR Sustainability Research Event, institute for sustainability solutions research, university of Plymouth, 29 April 2013.
- Almerei A, Lane S and Fuller MP. The SEB Main Meeting 2013, Valencia, Spain, 3\textsuperscript{rd} – 6\textsuperscript{th} July 2013.
- Almerei A, Lane S and Fuller MP. A short flash presentation. The SEB Main Meeting 2013, Valencia, Spain, 3\textsuperscript{rd} - 6\textsuperscript{th} July 2013. “Exploiting genetic transformation for abiotic stress-tolerant maize breeding”.
- Almerei A, Lane S and Fuller MP. “Biocontrol and challenges of maize production in glasshouses”. The Postgraduate Society Conference, 27\textsuperscript{th} November 2013.
- Almerei A, Lane S and Fuller MP. The Challenge Accepted! Creating Solutions for Horizon 2020 - the ISSR 3rd Annual Research Event, University of Plymouth, 02\textsuperscript{nd} May 2014.
• **Almerei A, Lane S and Fuller MP.** The Postgraduate Society Conference 27th November 2013. “Biocontrol and challenges of maize production in glasshouses”.

**Training Courses & Workshops**

• General Teaching Associate (GTA), University of Plymouth, April 2011.
• The conference: (GM Crops: From Basic Research to Application), Rothamsted research, Harpenden, UK, 28-29 June 2011.
• Pipetting Academy workshop, Alpha Laboratories, University of Plymouth. 03 May, 2012.
• Cryogenic Gases Safety Awareness Workshop. 25 June 2012.
• Vice-Chancellor’s teaching and learning conference 2012, 6th July 2012 at Plymouth University.
• The Faculty’s Science Showcase event on the Hoe on September 11th 2012.
• PG Society conference, 17th June 2014.
• A workshop on the new GE gel documentation system (using ImageQuant LAS 4000) at Plymouth University was attended, on Tuesday 3rd December 2014.

**Grant and award applications**

• The 1st place Oral Presentation Presenter was awarded, The Postgraduate Society conference, University of Plymouth, 14th March 2012
• Travel bursary awards from: SCI, this was published at [http://www.soci.org/News/Awards/Messel/Ayman-Almerei](http://www.soci.org/News/Awards/Messel/Ayman-Almerei) in Sep 2013. And from the Society for Experimental Biology SEB, 2011 and 2013. And from the Association of Applied Biologists AAB.
Appendix 2:

Data relevant to chapter 6, sequencing of genomic DNA and identification of transgenes

Determination the quality and quantity of genomic DNA

Figure 1. The quality and concentration (ng μl⁻¹) of purified genomic DNA products extracted from Hi II measured by Nano-Drop® UV spectrophotometer (Chapter 6).

Figure 2. The quality and concentration (ng μl⁻¹) of purified DNA plasmid pTF102 measured by Nano-Drop® UV spectrophotometer (Chapter 6).
Figure 3. The concentration (ng \(\mu l^{-1}\)) and quality of purified genomic DNA, and DNA plasmid construct (PpCBF1) which measured by Nano-Drop® UV spectrophotometer.

DNA agarose gel electrophoresis

Prepare a Stock Solution of Tris-acetate-EDTA (TAE) buffer used for agarose gel electrophoresis in the analyses of DNA products.

**TAE Buffer:** 50x stock solution, can be stored at room temperature.

- Tris-base (FW= 121.14) \(242g\)
- Glacial acetic acid \(57.1mL\)
- EDTA (0.5M) \(100mL\) (Ph 8.0).

The final volume of solution was adjusted to 1 liter with water.
Figure 4. Detection of the DNA quality of non-amplification genomic DNA, and DNA plasmids visualized by the gel electrophoresis.

**Identification of transgene by DNA sequencing**

**Genomic DNA sequencing of transient gene CBF using gene-specific primer CBF-r by GATC-Biotech at [http://www.gatc-biotech.com](http://www.gatc-biotech.com):**

ttgctcaTcactngCaTaTaAACATTtCCTCCCTATCCAAATAAAACAAGTtTaATctgctgCAGCTTTTTCCCATATC
CACAtaAGCCTgCCTTTCTCTCTCTCCACCACAAACCATTCTCTCTCTCTCTCATCActgCTGCGGctgGAACCCCCC
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TCAACTTTGCTGACTCGGGTTGGCGGCTGCCGGTCGCGCATCCTTGGATTCCATGGATATCCAGGGCAGGAGGGCAGG

**DNA sequencing of CBF-plasmid (PpCBF1) using gene-specific primer CBF-f by GATC-Biotech at [http://www.gatc-biotech.com](http://www.gatc-biotech.com):**


gccngGTCGaGTTCGtCATTCCAGGAcAACGGTGAGcaCCTTACGCACCTTGACGACTTACGAGGAGCCCTTTTAAAAGGGAAGCTTGC
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342
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**DNA sequencing of the pTF102 plasmid using bar gene primer, partial sequencing obtained from GATC-Biotech at** [http://www.gatc-biotech.com](http://www.gatc-biotech.com).

ganCgtGgTgcTGTcaTCGGGCTGCCcAGCACCGAGCGCGCATGACGAGGCCGCTCCGATATGCCGGCCTAGGGGCCC

Genomic DNA sequencing of the **bar** gene extracted from Hi II transgenic maize plants, partial sequencing obtained from GATC-Biotech at [http://www.gatc-biotech.com](http://www.gatc-biotech.com).

CGAgCgcCtcgagtATGCCCCCGcCGcATGCTGCGGgCGGcCCTAAGCagggACTGgCATGAcgngnTt

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GenBank: JQ293091.1

FASTA Graphics

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DEFINITION Streptomyces hygroscopicus strain A10 phosphinothricin acetyl transferase (bar) gene, complete cds.

ACCESSION JQ293091

VERSION JQ293091.1  GI:375153555

KEYWORDS .

SOURCE Streptomyces hygroscopicus

ORGANISM Streptomyces hygroscopicus

Bacteria; Actinobacteria; Streptomycetes; Streptomycetaceae; Streptomyces.

REFERENCE 1  (bases 1 to 555)

AUTHORS Kumar,A., Jolly,M. and Sachdev,A.

TITLE Bar gene: A selectable marker for herbicide resistance selection in plant transformation

JOURNAL Unpublished

REFERENCE 2  (bases 1 to 555)

AUTHORS Kumar,A., Jolly,M. and Sachdev,A.

TITLE Direct Submission

JOURNAL Submitted (16-DEC-2011) Division of Biochemistry, IARI, Pusa Campus, West Patel Nagar, New Delhi, Delhi 110012, India

FEATURES Location/Qualifiers

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ORIGIN

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gene
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gene
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gene
241  tagcctacgc actgagcggc cgagtgccac gttagctct ccgccggcga ccagcgacgc
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function
361  gcagtcgctcg tgtcctacgc gcggcccagcg tcgccggcgt gcgccggcgt
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Amplification plots of transgenes using (qRT-PCR)

Figure 5. Amplification plots of endogenous single copy gene chi gene (a), and transgenes; *cbf* gene (b) and the *bar* gene (c).

GUS assay solutions:
1. GUS\textsuperscript{*} buffer staining solution, (made fresh immediately prior to use).

2. Prepare a stock solution of 0.1M X-Gluc, (stored at -80 °C).

\begin{center}
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 & Stock & Want & Per 1 ml final volume \\
\hline
X-Gluc & 521.8 FW & 0.1M & 52 mg \\
Methanol or Dimethyl Sulfoxide (DMSO) & & & 1.0 ml \\
\hline
\end{tabular}
\end{center}

3. Prepare stocks of 50 mM Potassium Ferri/ferroCyanide. The stock was stored at RT but for long term; storage at -20 °C was recommended.

\begin{center}
\begin{tabular}{lccc}
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 & Stock & Want & Per 5 ml final volume \\
\hline
Potassium ferrocyanide & 422.39 FW & 50 mM & 105.6 mg \\
H\textsubscript{2}O & & & added to 5 ml \\
Potassium FerriCyanide (K\textsubscript{3}Fe(CN)\textsubscript{6}) & 329.26 FW & 50 mM & 82.3 mg \\
\hline
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Appendix 3:

Data relevant to chapter 7, characterisation of abiotic-stress resistance of transformed plants

Table 1. Compare 5% and 1% LSD with the differences between the pairs of means of Fv/Fm, and make a decision as to which pairs are significantly different.

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<th>Between</th>
<th>Difference</th>
<th>&gt;0.07</th>
<th>signif @ 1% ?</th>
</tr>
</thead>
<tbody>
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<td>Non- Trans. &amp; Trans. Plants</td>
<td>50</td>
<td>Salinity 10%</td>
<td>0.01</td>
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<td>50</td>
<td>Salinity 10%</td>
<td>0.01</td>
<td>No</td>
</tr>
<tr>
<td>100</td>
<td>Salinity 20%</td>
<td>-0.01</td>
<td>No</td>
<td></td>
<td>100</td>
<td>Salinity 20%</td>
<td>-0.01</td>
<td>No</td>
</tr>
<tr>
<td>150</td>
<td>Salinity 30%</td>
<td>0.32</td>
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<td></td>
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<td>Salinity 30%</td>
<td>0.32</td>
<td>Yes</td>
</tr>
<tr>
<td>200</td>
<td>Salinity 40%</td>
<td>0.18</td>
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<td></td>
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<td>Salinity 40%</td>
<td>0.18</td>
<td>Yes</td>
</tr>
<tr>
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<td>Salinity 50%</td>
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<td>Yes</td>
<td></td>
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<td>Salinity 50%</td>
<td>0.15</td>
<td>Yes</td>
</tr>
<tr>
<td>300</td>
<td>Salinity 60%</td>
<td>-0.03</td>
<td>No</td>
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<td>Salinity 60%</td>
<td>-0.03</td>
<td>No</td>
</tr>
<tr>
<td>350</td>
<td>Salinity 70%</td>
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<td>No</td>
<td></td>
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<td>Salinity 70%</td>
<td>0.04</td>
<td>No</td>
</tr>
<tr>
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<td>Salinity 100%</td>
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<td>No</td>
</tr>
<tr>
<td>control</td>
<td>-0.01</td>
<td>No</td>
<td></td>
<td></td>
<td>control</td>
<td>-0.01</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

*The salinity concentrations are compatible with the percentage of sea water adjusted with distilled water. (Salinity concentration 10% of saline equals 50mM).

Table 2. Compare 5% and 1% LSD with the differences between the pairs of means of dry weight and make a decision as to which pairs are significantly different.

<table>
<thead>
<tr>
<th>Between</th>
<th>Difference</th>
<th>&gt;2.96</th>
<th>signif @ 5% ?</th>
<th>Between</th>
<th>Difference</th>
<th>&gt;3.97</th>
<th>signif @ 1% ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non- Trans. &amp; Trans. Plants</td>
<td>Salinity 10%</td>
<td>13.12</td>
<td>Yes</td>
<td>Salinity 10%</td>
<td>13.117</td>
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<td></td>
</tr>
<tr>
<td>50</td>
<td>Salinity 20%</td>
<td>8.05</td>
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<td>Salinity 20%</td>
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<td>Yes</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Salinity 30%</td>
<td>11.7</td>
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<td>Salinity 30%</td>
<td>11.7</td>
<td>Yes</td>
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</tr>
<tr>
<td>150</td>
<td>Salinity 40%</td>
<td>4.05</td>
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<td>Salinity 40%</td>
<td>4.05</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>Salinity 50%</td>
<td>0.05</td>
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<td>Salinity 50%</td>
<td>0.05</td>
<td>No</td>
<td></td>
</tr>
<tr>
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<td>Salinity 60%</td>
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<td>Salinity 60%</td>
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<td></td>
</tr>
<tr>
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<td>Salinity 70%</td>
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<td>Salinity 70%</td>
<td>1.533</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>Salinity 100%</td>
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<td>-0.45</td>
<td>No</td>
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</tbody>
</table>
### Table 3. Compare (5 and 1)% LSD with the differences between the pairs of means of specific leaf area and make a decision as to which pairs are significantly different

<table>
<thead>
<tr>
<th>Between</th>
<th>Difference</th>
<th>&gt; 47.09</th>
<th>signif @ 5% ?</th>
<th>Between</th>
<th>Difference</th>
<th>&gt; 63.14</th>
<th>signif @ 1% ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non- Trans. &amp; Trans. Plants</td>
<td>Salinity 10%</td>
<td>-58.26</td>
<td>Yes</td>
<td>✓</td>
<td>Slinity 10%</td>
<td>-58.26</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Salinity 20%</td>
<td>-4.91</td>
<td>No</td>
<td>–</td>
<td>Salinity 20%</td>
<td>-60.65</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 30%</td>
<td>-39.20</td>
<td>No</td>
<td>–</td>
<td>Salinity 30%</td>
<td>-100.15</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 40%</td>
<td>-60.65</td>
<td>Yes</td>
<td>✓</td>
<td>Salinity 40%</td>
<td>-100.15</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 50%</td>
<td>-22.37</td>
<td>No</td>
<td>–</td>
<td>Salinity 50%</td>
<td>-150.06</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 60%</td>
<td>-1.50</td>
<td>No</td>
<td>–</td>
<td>Salinity 60%</td>
<td>-150.06</td>
<td>Yes</td>
</tr>
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<td></td>
<td>Salinity 70%</td>
<td>-1.50</td>
<td>No</td>
<td>–</td>
<td>Salinity 70%</td>
<td>-150.06</td>
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</tr>
<tr>
<td></td>
<td>Salinity 100%</td>
<td>-150.06</td>
<td>Yes</td>
<td>✓</td>
<td>Salinity 100%</td>
<td>-150.06</td>
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</tr>
<tr>
<td></td>
<td>control</td>
<td>11.33</td>
<td>No</td>
<td>–</td>
<td>control</td>
<td>11.33</td>
<td>No</td>
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</tbody>
</table>

### Table 4. Compare (5 and 1)% LSD with the differences between the pairs of means of plant height and make a decision as to which pairs are significantly different

<table>
<thead>
<tr>
<th>Between</th>
<th>Difference</th>
<th>&gt; 0.07</th>
<th>signif @ 5% ?</th>
<th>Between</th>
<th>Difference</th>
<th>&gt; 0.09</th>
<th>signif @ 1% ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non- Trans. &amp; Trans. Plants</td>
<td>Salinity 10%</td>
<td>0.37</td>
<td>Yes</td>
<td>✓</td>
<td>Slinity 10%</td>
<td>0.37</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 20%</td>
<td>0.13</td>
<td>Yes</td>
<td>✓</td>
<td>Salinity 20%</td>
<td>0.13</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 30%</td>
<td>0.20</td>
<td>Yes</td>
<td>✓</td>
<td>Salinity 30%</td>
<td>0.20</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 40%</td>
<td>0.16</td>
<td>Yes</td>
<td>✓</td>
<td>Salinity 40%</td>
<td>0.16</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 50%</td>
<td>-0.12</td>
<td>No</td>
<td>–</td>
<td>Salinity 50%</td>
<td>-0.12</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Salinity 60%</td>
<td>0.02</td>
<td>No</td>
<td>–</td>
<td>control</td>
<td>0.08</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Salinity 70%</td>
<td>0.04</td>
<td>No</td>
<td>–</td>
<td>control</td>
<td>0.08</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Salinity 100%</td>
<td>-0.02</td>
<td>No</td>
<td>–</td>
<td>control</td>
<td>0.08</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### Table 5. Compare (5 and 1)% LSD with the differences between the pairs of means of stem diameter of plant and make a decision as to which pairs are significantly different.
Appendix 4:

Sequence of plasmid constructs

The complete sequence of pTF102 plasmid is:

```
1  AGTACTTTAA AGTACTTTAA AGTACTTTAA AGTACTTTGA TCCAACCCCT
   TCATGAATTT TCATGAATTT TCATGAATTT TCATGAATTT TCATGAAACT
51  CCGCTGCTAT AGTGCAGTCG GCTTCTGACG TTCAGTGCAG CCGTCTTCTG
   GGGCGACGATA TCACGTCAGC CGAAGACTGC AAGTCACGTC GGCAGAAGAC
101  AAAACGACAT GTCGCACAAG TCCTAAGTTA CGCGACAGGC TGCCGCCCTG
   TTTTGCTGTA CAGCGTGTTC AGGATTCAAT GCGCTGTCCG ACGGCGGGAC
151  CCCTTTTCCT GGCGTTTTCT TGTCGCGTGT TTTAGTCGCA T
   AAAGTAGAA GGGAAAAGGA CCGCAAAAGA ACAGCGCACA AAATCAGCGT ATTTCATCTT
201  TACTTGCGAC TAGAACCGGA GACATTACGC CATGAACAAG AGCGCCGCCG
   ATGAACGCTG ATCTTGGCCT CTGTAATGCG GTACTTGTTC TCGCGGCGGC
251  CTGGCCTGCT GGGCTATGCC CGCGTCAGCA CC
   GACGACCA GGACTTGACC GACCGGACGA CCCGATACGG GCGCAGTCGT GGCTGCTGGT CCTGAACTGG
301  AACCAACGGG CCGAACTGCA CGCGGCCGGC TGCACCAAGC TGTTTTCCGA
   TTGGTTGCCC GGCTTGACGT GCGCCGGCCG ACGTGGTTCG ACAAAAGGCT
351  GAAGATCACC GGCACCAGGC GCG
   ACCCGCCC GGAGCTGGCC AGGATGCTTG
401  CTTCTAGTGG CCGTGGTCCG CGCTGGCGGG CCTCGACCGG TCCTACGAAC
451  ACCACCTACG CCCTGGCGAC GTTGTGACAG TGACCAGGCT AGACCGCCTG TGGTGGATGC GGGACCGCTG CAACACTGTC ACTGGTCCGA TCTGGCGGAC
501  GCCCGCAGCA CCCG
   CGACCT ACTGGACATT GCCGAGCGCA TCCAGGAGGC
551  CGGGCGTCGT GGGCGCTGGA TGACCTGTAA CGGCTCGCGT AGGTCCTCCG
   CGGCGCGGGC CTGCGTAGCC TGGCAGAGCC GTGGGCCGAC ACCACCACGC
   GCCGCGCCCG GACGCATCGG ACCGTCTCGG CACCCGGCTG TGGTGGTGCG
   CGGCCGGCCG CATGGTGTTG ACCGTGTTCG CCGGCATTGC CGAGTTCGAG
   GCCGGCCGGC GTACCACAAC TGGCACAAGC GGCCGTAACG GCTCAAGCTC
354
```
601  CGTTCCCTAA TCATCGACCG CACCCGGAGC GGGCGCGAGG CCGCCAAGGC
GCAAGGGATT AGTAGCTGGC GTGGGCCTCG CCCGCGCTCC GGCGGTTCCG
651  CCGAGGCGTG AAGTTTGGCC CCCGCCCTAC CCTCACCCCG GCACAGATCG
GGCTCCGCAC TTCAAACCGG GGGCGGGATG GGAGTGGGGC CGTGTCTAGC
701  CGCACGCCCG CGAGCTGATC GACCAGGAAG GCCGCACCGT GAAAGAGGCG
GCGTGCGGGC GCTCGACTAG CTGGTCCTTC CGGCGTGGCA CTTTCTCAGC
751  GCTGCACTGC TTGGCGTGCA TCGCTCGACC CTGTACCGCG CACTTGAGCG
CGACGTGACG AACCGCACGT AGCGAGCTGG GACATGGCGC GTGAACTCGC
801  CAGCGAGGAA GTGACGCCCA CCGAGGCCAG GCGGCGCGGT GCCTTCCGTG
GTCGCTCCTT CACTGCGGGT GGCTCCGGTC CGCCGCGCCG
A CGGAAGGCAC
384x677 NotI
318x681 ~~~~~~~~~~
851  AGGACGCATT GACCGAGGCC GACGCCCTGG CGCGCGCGCA GAATGAACGC
TCCTGCCTAA CTGGCCTCCG CTGCGGGACC GCGCGCCGCT CTTACTTGCAG
901  CAAGAGGAAC AAGCATGAAA CCGCACCAGG ACGGCCAGGA CGAACCGTTT
GTCTCTCTTG TTCTGTTCTCT GTGGCTTGGC TGCTCCGTCC CTTTTACTCT
951  TGCATTACC AAGAGATCGA GGCAGAGATG ATCGCGCGGG GACGTGGTGT
AAGTAAATCT AGTACTTACAG GCACGCGCTG CTTTTTCTCT CTTTTACTCT
1001 CGAGCGCGCC CGGCACGTCT CACCCCGTGG CCGCGCGCGA GAATGAACGC
TCCTGCCTAA CTGGCCTCCG CTGCGGGACC GCGCGCCGCT CTTACTTGCAG
1051 GTTTTGTCTGA TGCCCAAGCTG GCGGCCTGGC CGGCCAGCTT GGCCGCTGAA
CAAACAGACT CGTGCGGCGA GTGGCGGTGC CCGCGCGCAG CCGCGCGCGA
1101 GAAACCGAGC GCCGCCGTCT AAAAAGGTGA TGTGTATTTG AGATAAACAG
CTTTGGCTCG CGCGCCGCTG CTTTTTCACT ACACATAAAC TCTATTTTCT
1151 CTTCGGFTCAT CGGCGTGCGG CTGCTACGCG CATGCGGCGA GACAGAGCGC
ATGCGTTCCC TTCTGGCTACT CTATTTTACT ACACATAAAC TCTATTTTCT
1201 TACGCAAGGG GAACCGCTAGA AGGTGATCGT GCGGACCTGG CCGCGGTGAC
ATGCGTTCCC TTCTGGCTACT CTATTTTACT ACACATAAAC TCTATTTTCT
1251 GGTGACGCAA GACGACCATC GCAACCCATC TAGCCCGGCC CCTGCAACTC
CGCGCTTGGC GTGCTGGTGG CGCGCTTGGC GTGCTGGTGG CGCGCTTGGC
1301 GCCGCCGCCCG ATGTTTCTGTT AGTGGATTCC GATCCCCCGG CGACCCCTCC
CGCGCGCGCG ATCAGATGGA TCGAGATGGG CGATGGATGG CGATGGATGG
1351 CGATGTTGGCG GCGGTGCTGG CGACGCGAAT CACCGGTGAC CCAGGACGCT
GTCAACCGCC CGCGACCGCC CCTGCTGGTG GTGCTGGTGG CGCGCTTGGC
1401 ACCGGCCGCCG CATGCACGCTG GACGATGAGG CAGCAGCGCC CCCGCGCTTCC
TGCCGCGCGT CTATGCGGAT CTTGTCGCTT CTTGTCGCTT CTTGTCGCTT
1451 GTAGTATGAGG CAGGATCGCC CGAGCGCTCC CGAGCGCTCC CGAGCGCTCC
CATCAGGCGC TGTTGCGCTG CTGGCGCTG CGCGCGCGCG CGCGCGCGCG
1501 CAGGGCGGCC GACCTCATGGA CTGTGCGGCT CGAGGCGGCG GCAGGCGGCG
GTGCGCTGCG CTGGCGCTG CGCGCGCGCG CGCGCGCGCG CGCGCGCGCG
NotI

---

2401  GAATCGTGGC AAGCGGCCGC TGATCGAATC CGCAAAGAAT CCCGGCAACC
2451  GTTGGTATGA CGGCCGCCGC ACCCACTACT GGACCACCTC TTCAACTTCC

2451  GGCAGGAGCC GGGCGCCCGCT CGATTAGGAA GCCGCCAAG GGCGACGAGC
2310  CCTAGTATAG CGCGCGCCGG ACCCACTACT GGACCACCTC TTCAACTTCC
2351  CGCGCGACCG CGCCCGAGCC CAAACGGATCG AGGAGAAAGC ACGCCCCGGT
2251  AACCCCGCAAG CCCGAGGAAT CGGCGTGAGC GGTCGCAAAC CATCCGGCCC
2201  TGGCGCGCG CAGGACGCGC GGGCTCGACG CTGTTGGTAA TGGGCTGGAG
2151  AACCAGGCAC CGACCGCGTC GAGTGGCGCCA GTGGTTGGCGA CCTGGTGCGG
2101  GATAGCATGA ATTGGAGCCG CTAGGCAGTTG TGGCCCGGAG ACCGACGAC
2051  ATATGATGAA ATGGGAGGTC TGGTGGATCG TGGCGCGCAG CACCCGGGAG
2001  CAAGCAGTCG GAGCTGACCC CCAAGGAAAT TTGCTGATCA GACGCGGAG
1951  ACCTGACGTC TACTCCAGAG CCACTGTCAG CTGCTAGTAC ATCGTGTGCT
1901  GTCCGAGGAG GCGCTGGCG CCAAGTAGTT CTGTTAAGAC TGGGCTGGAG
1851  TGAGGTAAA GAGAAAATGA GCAAAAGCAC AAACACGCTA AGTGCCGGCC
1801  TGAGGTAAA GAGAAAATGA GCAAAAGCAC AAACACGCTA AGTGCCGGCC
1751  GCCGCCGGCA CAACCGCTCT TGAATGTGC A CCAAGAGG CAGCTGAGGC
1701  CCATCCTCTGA GTCCCGGCTT ACAGCAGCAG CAGGCTGCAC CAGGCTGCAC
1651  CAGCCGCTATC GCCGCTGCCT CGGCTGTAGT TACCAGGGG TGGCTGGAGA
1601  ACGGATGGAA GGCTACAAGC GGCCTTTGTC GTGTCGCGGG CGATCAAAGG
1551  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
1501  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
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1301  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
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1201  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
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951  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
901  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
851  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
801  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
751  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
701  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
651  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
601  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
551  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
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451  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
401  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
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151  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
101  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
51  TATGGGCCAC CGCGGACCTG GTGGAGCTTC TTAAGCAGCG CATTGAGGTC
2501  AACCAGATTT TTTGCTTCCG ATGCTCTATG ACGTGGGCAC CCGCGATAGT
TTGGTCCTAA AAAGCAAGGC TACGAGATAC TGCACCCGTG GGCGCTATCA
2551  CGCAGCATCA TGGACGTGGC CGTTTTCCGT CTGTCGAAGC GTGACCGACG
GGCTGTAAGT ACCTGCACCG GCAAAAGGCA GACAGCTTCG CACTGGCTCA
2601  CGCAGCATCA TGGACGTGGC CGTTTTCCGT CTGTCGAAGC GTGACCGACG
GCGTCGTAGT ACCTGCACCG GCAAAAGGCA GACAGCTTCG CACTGGCTCA
2651  CGCAGCATCA TGGACGTGGC CGTTTTCCGT CTGTCGAAGC GTGACCGACG
GCGTCGTAGT ACCTGCACCG GCAAAAGGCA GACAGCTTCG CACTGGCTCA
2701  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
2751  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
2801  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
2851  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
2901  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
2951  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3001  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3051  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3101  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3151  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3201  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3251  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
CCGCGGCAGT CGGCAAAAGA GATGGCGGAC CGTGCGGCGC GGCGTCCGTT CCGTCTTCGG
3301  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
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3351  ATGCGGCTTT CTACCGCCTG GCACGCCGCG CCGCAGGCAA GGCAGAAGCC
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BclI
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6151  CCGGAACAGGC TTATGCTCCAC TGGGGTTCGTC CCCGAATTGA TCAACAGGCA
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KpnI

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HindIII       PstI
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PstI    HindIII

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SmaI       SacI

HindIII    PstI    SalI    XbaI    BamHI    KpnI    EcoRI

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EcoRI

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GTTAAGGTGT GTTGTATGCT GCCTCCCTGTT ATTCACATT CGGCCCCCCCA
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PvuII

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366
This sequence of pTF102 construct was obtained from Plant Transformation Facility, Department of Agronomy (College of Agriculture and Life Sciences), Plant Sciences Institute, Iowa State University, USA.

**Binary vector pBINPLUS/ARS, complete sequence is:**

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1861  ggtgcgggtc gtcgggcgttt cggccggttgc cggccggttgc cggccggttgc cggccggttgc
1921  gccaaaccgc cctctgggct cccgggctgg cggccggttgc cggccggttgc cggccggttgc
1981  cctctgggct cccgggctgg cggccggttgc cggccggttgc cggccggttgc cggccggttgc
2041  ggtgcgggtc gtcgggcgttt cggccggttgc cggccggttgc cggccggttgc cggccggttgc
2101  cctctgggct cccgggctgg cggccggttgc cggccggttgc cggccggttgc cggccggttgc
```
Appendices

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12001 ccagacgaac gaagacgat tgaggaagag gcggcggcgg gcggcatgag cctgtcggcc
12061 tacctgctgg ccgctgcccc gggctacaaa atcacgcccgc tcgtgactat cgacacgctc
12121 cgcagctgag cccgcatcaaa tggcgacctg gcgcgcttgg gcggcctgct gaaactctgg
12181 ctcaccgcac acccgccac gcggcgggttc ggtgatgcca cgatccctgc cctgctggcc
12241 aagatcgaag aagacgagga cgagcttgcc aaggtcatga tgggcgctgga ccgccccgagg
12301 gcagagccat gactttttta gccgctaaaa cggcgggggg gtgcgcgtga ttgccaagca
12361 cgtccccatg cgcctcctca aagaagcgcg cttcgcgagcttgtaagat aacacccgca
12421 cgagcaaggg cagacggagc gcttttgccg cgcctca