TRANSFORMATION AND EXPRESSION OF GLUTAREDOXIN-2 GENE INTO TOMATO PLANTS

By

NEAMA HUSSEIN OSMAN ABDEL-ATI
B.Sc. Agric. Sci. (Biotechnology), Fac. Agric., Cairo Univ., 2001
M.Sc. Agric. Sci. (Genetics), Fac. Agric., Cairo Univ., 2007

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APPROVAL SHEET

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ABSTRACT

These studies were conducted during the period from 2008 to 2014 at Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt, to improve tomato tolerance to salinity using biotechnological techniques. Development of transgenic tomato plants harboring glutaredoxin-2 gene (*GRX-2*) was aimed. In the first experiment, a regeneration system has been established for five tomato cvs, i.e. Flora-Dade, Marmande, Summer Prolific, Castlerock and Super Strain B. Cotyledon and hypocotyl explants from the five tomato cvs were cultured on MS medium supplemented with different concentrations of plant growth regulators. Data showed that, cultivars and BA concentration significantly affected callus induction, shoot induction, and regeneration frequencies for each type of explants. The best cultivar was “Castlerock” at 6 mg/l BA. The second experiment was conducted to describe the possibility of producing transgenic tomato plants harbouring the *GRX-2* gene, conferring salinity tolerance. To achieve this goal, tomato cotyledon explants, and seeds were transformed using *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary plasmid pRI101 on DNA which contains *GRX-2* gene, and the selectable marker gene neomycin phosphotransferase II (*nptII)* under the control of a *CaMV35S* promoter and nopaline synthase (*nos*) terminator. Molecular analysis using PCR, DNA sequencing, and dot blot hybridization proved the presence and integration of the transgenes in the genome of the transgenic plants. RT-PCR detected successfully the expression of *GRX-2* gene. Field experiment was conducted to investigate the effect of the introduced *GRX-2* gene on tomato salt tolerance. The data showed that the transgenic lines expressed different levels of salt tolerance as expressed by the performance of plants dry weight, and Na^+ concentration. These results show that the *GRX-2* gene enhance salt tolerance. The results of the present study can be seen as a step towards development of salinity tolerant transgenic tomato.

Key words: Tomato, Glutaredoxin, Salinity, Transformation, Regeneration.

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INTRODUCTION

Tomato, *Solanum lycopersicum* L., is an economically important crop species and the focus of a large agricultural industry. Also, tomato is a model organism for genetic, developmental, and physiological research. Tomatoes are among the most widely consumed vegetables in the world, and many of the compounds found in tomatoes have received much interest in recent years for their potential health benefits. Tomato plant growth and development are adversely affected by salinity, which is a major environmental stress that limits agricultural production. From an agricultural point of view, salinity is the accumulation of dissolved salts in the soil water to an extent that inhibits plant growth (Gorham, 1992). Damage caused by high salinity to plants is observed as either loss of plant productivity or plant death.

Recent statistics suggested that 67% of world agricultural area has potential for transient salinity, a type of ground water associated salinity (Rengasamy, 2006). The total global area of salt-affected soils including saline and sodic is 831 million hectares (Martinez-Beltran and Manzur, 2005), extending over all the continents including Africa, Asia, Austrlia and the Americas (Rengasamy, 2006; Schoups *et al*., 2005). More importantly, 33% of the cultivated land (Ghassemi *et al*., 1995), which comprises only 3% of total land area in Egypt, is already salinized. The reduction in production of soils affected by salinity is about 30% (El-Lakany *et al*., 1986), threatening the livelihoods of the poor farming and having a significant negative impact on the food production in Egypt as whole. Moreover, the Egyptian Government has spent large sums on reclamation, mainly on drainage projects (more than US$ 30 million annually) to solve salinity problems in irrigated area, but the annual average net income from crops grown with drainage system is more limited than for those grown without drainage system (Amer *et al*., 1989). Therefore, genetic improvement for salt tolerance in major crops, particularly because this approach is perhaps the easiest, safest, most practical, less expensive, and best-environment-friendly than other, has become an urgent task dealing with salinity problems in Egyptian agriculture sector.

Most commercial cultivars of tomato are moderately sensitive to salinity at all stages of plant development and as a result, their economic yield is substantially reduced under salt stress (Bolarin *et al*., 1993). Genetic resources for salt tolerance have been identified within tomato related wild species, *viz*., *S. peruvianum*, *S. cheesmanii*, *S. habrochaites, S. pennellii* and *S. pimpinellifolium* (Sarg *et al*., 1993).

Although there are comparatively salt tolerant relatives of the cultivated tomato, it was proved difficult to enrich elite lines with genes from wild species that confer tolerance, due to the several reasons including the large number of genes involved in salt tolerance, most of these genes with small effect in comparison to the environment. Salt tolerance in tomato, as well as many other plant species, at each stage of plant development is often independent of tolerance at other stages. There are difficulties in interspecific crosses between cultivated tomato and wild species, the unavailability of a simple and reliable method for assessment of tolerance, and the high costs of recovering the genetic background of the receptor cultivar (Kalloo, 1991).

The paucity of success achieved by past attempts to generate salt-tolerant genotypes, both through conventional breeding programmes and through some biotechnological approaches (such as *in vitro* selection; Flowers, 2004), have fuelled hopes that the problems might be solved *via* transgenesis. The introduction of genes conveying salt tolerance to elite cultivars or elite parents of current hybrids, by transformation, is a very attractive idea because, hypothetically, susceptible but productive cultivars should be converted to tolerant cultivars, while maintaining all the very valuable characters current cultivars possess.

Recent developments in plant tissue culture and genetic engineering have made it possible to develop superior cultivars of several economically important crop plants including tomato. Till date, transformed tomato plants have been produced by *Agrobacterium*-mediated genetic transformation (Horsch *et al.*, 1985; McCormick *et al.*, 1986; Hamza and Chupeau, 1993; Roekel *et al.*, 1993; Frary and Earle, 1996; Moghaieb *et al.*, 2000; Vidya *et al.*, 2000; Hu and Phillips, 2001; Park *et al.*, 2003; Qiu *et al.*, 2007; Li, 2011; and Guo *et al.*, 2012), followed by *in vitro* regeneration either by direct or indirect somatic embryogenesis. Though these methods are widely used, they have some disadvantages such as requiring highly sterile conditions, genotype consumption, occurrence of somaclonal variations, genotype specificity and recalcitrance. Moreover during hardening, the valuable transgenic plantlets may fail to acclimatize, ultimately leading to less transformation efficiency. Thus, considerable refinements of current transformation systems are required to achieve commercial application of transgenics.

Recently, *in planta* transformation has been established for several commercially valuable crops. *In planta* transformation is an alternative method which does not involve *in vitro* culture of plant cell or tissues, thereby reducing time, labor cost and most importantly avoiding somaclonal variation encountered during *in vitro* culture-mediated genetic transformation and regeneration. The transformation frequency obtained in rice and wheat by using *in planta* transformation was much higher than that of previously reported transformation methods (Supartana *et al*., 2005 and 2006).

As a first step in a local tomato breeding program for developing salt tolerant tomato, the present study was conducted to:

1- Establishing a reliable regeneration system for tomato which can be used for introducing a foreign gene into tomato cells or tissues using *Agrobacterium*.

2- Developing transgenic tomato plants harboring *GRX-2* gene, which confer recipient plant tolerance to salinity.

3- Evaluating the transgenic tomato plants for salt tolerance using some analytical methods.

REVIEW OF LITERATURE

1. Salinity in agriculture

Salinity is a major constraint to food production because it limits crop yield and restricts use of land previously uncultivated. Salinity is a problem for agriculture because also only few crop species and genotypes are adapted to saline conditions. Regardless of the cause (ion toxicity, water deficit, and/or nutritional imbalance), high salinity in the root zone severely impedes normal plant growth and development, resulting in reduced crop productivity or crop failure. Although irrigation covers only about 15% of the cultivated land of the world, irrigated land has at least twice the productivity of rain-fed land, and may therefore produce one-third of the world's food. The reduced productivity of irrigated lands due to salinity is, therefore, a serious issue (Flowers, 2004 and Foolad, 2004).

With the projected increase in populations of 1.5 billion people over the next two decades coupled with increased urbanization in developing countries, the world’s agriculture is faced with an enormous challenge to maintain, let alone increase, our present level of food production (Owen, 2001). Ways must be found to achieve this without resorting to unsustainable farming practices and without major increases in the amount of new land under cultivation, which would further threaten forests and biodiversity. It is estimated that productivity should be increased by 20% in the developed countries and by 60% in the developing countries. In the light of these demographic, agricultural and ecological issues, the threat and effects of salinity become even more alarming. Reducing the spread of salinization and increasing the salt tolerance of crops and improving crop genotypes for salt tolerance, particularly the high yielding ones are, therefore, issues of global importance.

According to Epstein *et al.* (1980) two major approaches have been proposed and employed to minimize the deleterious effects of high soil/water salinity in agriculture. First, a technological approach of implementing large engineering schemes for reclamation, drainage and irrigation with high-quality water. Although this approach has been effective in some areas, the associated costs are high and it often provides only a temporary solution to the problem. The second approach is generating crops that can tolerate high levels of salinity. This approach has been found to be more promising for growing plants in saline soils and can be used in combination with the first method (Epstein *et al.*, 1980). Great efforts have been made to improve the salinity tolerance of many crops by means of traditional breeding programmes and more recently by genetic transformation (Cuartero *et al.*, 2006).

2. Effects of salinity on plants

Plants main requirements for their life cycle are mineral nutrients (elements) and energy from sunlight. There are certain elements called essential mineral nutrients and it has been determined that plants need them to grow and develop. These elements are of importance in numerous biological functions and each has its own function in the cell. Although essential mineral nutrients are imperative for plant survival, excessive soluble salts in the soil have deleterious effects on most plants. In addition, plant growth is more influenced by salt than other toxic substances (Xiong and Zhu, 2002). According to their response to high salt concentrations, plants can be divided into glycophytes which are sensitive to high salinity and halophytes which have tolerance to saline soils and they comprise a wide spectrum of families (Flowers *et al.*, 1977). Halophytes are experimentally of importance since they have the ability to cope with salt stress and studies of them have led to the discovery of salt tolerance mechanisms (Flowers, 2004).

In general, salinity can inhibit plant growth by the four following major ways (Greenway and Munns, 1980 and Xiong and Zhu, 2002): (i) osmotic stress, salt stress alters the water potential in the environment and this causes osmotic stress to plants, where, plants lose their turgor; (ii) specific ion toxicity, usually associated with either excessive chloride or sodium uptake; (iii) nutrient deficiency, nutrient ion imbalance when the excess of Na^+ or Cl^- leads to a diminished uptake of K^+, Ca^{2+}, NO^{3-} or P, or to impaired internal distribution of one or another of these ions; and (iv) oxidative stress that occurs as a secondary effect of salinity and it is caused by excessive reactive oxygen species (ROS) such as, hydrogen peroxide, hydroxyl radicals and superoxide anions. In fact generation of ROS usually involves normal cellular reactions. However, when plants are subjected to stress, the amount of ROS in the cells increases.

Salinity generally affects growth rate by the osmotic stress and it results in plants with smaller leaves, shorter stature and sometimes fewer leaves by reducing growth rate (Munns and Termaat, 1986).

Also, salinity by osmotic stress changes the roots structure by reducing their length and mass; therefore roots may become thinner or thicker (Shannon and Grieve, 1999). Flowering of tomato plants is delayed by salinity. Moreover, ionic effects of salinity are generally seen in leaf and meristem damage or typical nutritional disorder symptoms. Burning of leaves is an effect that is caused by both salinity and nutritional disorders (Shannon and Grieve, 1999). In spite of negative effects, salinity may have some good effects on yield, quality and disease resistance of tomato. For example, at early stage of fruit development, salt stress can increase soluble sugar content and sugar acid ratio in tomato mature fruit (Jiang *et al.*, 2007 and Balibrea *et al.*, 2003).

3. Tomato and salinity

Tomato production has been limited by a high level of salinity in the soil or irrigation water. Tomato is sensitive to moderate levels of salinity like most crop plants. All stages of plant development including seed germination, vegetative growth and reproduction show sensitivity to salt stress and economic yield is reduced under salt stress (Jones *et al.*, 1988; Maas, 1986 and Bolarin *et al.*, 1993). The potential source of useful genes for salt tolerance breeding are present in several wild species. On the other hand, the cultivated species contribute limited variation for salt tolerance (Rick, 1979). Salt tolerance in tomato appears to be a developmentally regulated, stage-specific phenomenon. There is often no correlation in salt tolerance at different plant developmental stages and tomato salt tolerance increases with plant age. Because of these reasons each stage such as germination and emergence, seedling survival and growth, and vegetative growth and reproduction should be studied separately (Foolad and Lin, 1997a).

4. Salinity tolerance of plants and their mechanisms

Salinity is one of the major abiotic stresses that severely affects the growth and productivity of crop plants (Lopez *et al.* 2002). It changes the morphology, physiology and metabolism of plants (Hila *et al.*1998), ultimately diminishing growth and yield (Ashraf and Harris 2004). Salinity affects plants in different ways such as osmotic effects, specific-ion toxicity and/or nutritional disorders (Läuchli and Epstein 1990). The extent by which one mechanism affects the plant over the others depends upon many factors including the species, genotype, plant age, ionic strength and composition of the salinizing solution, and the organ in question. Along with these effects, the secondary stresses resulting from high salt stress, such as reactive oxygen species (ROS) formation, may cause further damage to plant cells (Dat *et al.* 2000).

High salt stress disrupts homeostasis in water potential and ion distribution. This disruption of homeostasis occurs at both the cellular and the whole plant levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death. To achieve salt tolerance, three interconnected aspects of plant activities are important. First, damage must be prevented or alleviated. Second, homeostatic conditions must be re-established in the new, stressful environment. Third, growth must resume, albeit at a reduced rate (Zhu 2001a).

Salt tolerance is a complex trait involving the coordinated action of many gene families that perform a variety of functions such as control of water loss through stomata, ion sequestration, metabolic adjustment, osmotic adjustment and antioxidative defense (Abogadallah 2010). The mechanisms responsible for salt tolerance in crop plants include exclusion, inclusion, compartmentation and homeostasis (Saqib *et al.* 2005 and Tahir *et al.* 2006), enhanced plant water status, immobilization of toxic Na+ ion (Liang *et al.* 2003), reduced Na+ and enhanced K+ uptake (Tahir *et al.* 2006) and higher K+:Na+ selectivity (Hasegawa *et al.* 2000). Consequently, better crop growth, physiological efficiency, balanced nutrition, and increased nutrient uptake is maintained in salinity stressed plants (Murillo-Amador *et al.* 2007) by diluting salts accumulated in saline environment.

Responses to abiotic stress at the gene level fall into one of three types:

1. Genes coding proteins that play an important role in signaling cascades and in transcriptional control (Zhu 2001b).
2. Genes whose products immediately confer protection on membranes and proteins (Bray 1997).
3. Genes involved in water and ion uptake and transport, such ion transporters (Blumwald 2000).

a. Halotolerant genes engineered to tomato

Despite considerable efforts in the area of genetic transformation, limited attempts have been made to develop transgenic tomatoes with enhanced salt tolerance.

A notable progress has been achieved in the development of tomato plants overexpressing *AtNHX1*, a single gene controlling vacuolar Na^+/H^+ antiport protein, introduced from *Arabidopsis thaliana* (Apse and Blumwald, 2002; El-Awady *et* al., 2014; Yamaguchi and Blumwald, 2005 and Zhang and Blumwald, 2001). The overexpression of this gene was previously shown to improve salt tolerance in *Arabidopsis* (Apse *et al.*, 1999). Transgenic tomato plants overexpressing this gene were reported to have the ability to grow, set flower and produce fruit in the presence of 200 mM NaCl in greenhouse hydroponics, whereas the control plants did not survive in these salinity conditions. The transgenic plants were reported to have acquired a halophytic response to salt tolerance, accumulating salts in the vacuoles. This is unlike the normal response of the cultivated tomato to salt stress, which is exclusion of salts from cells at the root shoot level, a glycophytic response.

Accordingly, under high salinity conditions, transgenic tomato plants accumulated high concentrations of Na^+ and Cl^- in their leaves. The overproduction of the vacuolar Na^+/H^+ antiport protein enhanced the ability of the transgenic plants to sequester Na^+ in their vacuoles, averting its toxic effects in the cell cytosol. At the same time Na^+ was used to maintain an osmotic balance to drive water into the cell, and thus used salty water for cell expansion and growth. This was the first reported example of a single-gene transformation in any crop species that resulted in such a significant enhancement in plant salt tolerance (Apse *et al.*, 1999). Subsequently, transfer and overexpression of the same gene resulted in transgenic plants with enhanced salt tolerance under controlled saline conditions in canola, *Brassica napus* (Zhang *et al.*, 2001), corn (Yin *et al.*, 2004) and wheat (Xue *et al.*, 2004). However, the transgenic plants are yet to be evaluated for salinity tolerance under field conditions and examined for their commercial value. Obviously, much more research is needed to gain a better understanding of the genetics, biochemical, and physiological basis of plant salt tolerance using the transformation technology. However, knowledge of various tolerance components and identification, cloning and characterization of responsible genes may allow development of plants harboring multiple transgenes and production of highly salt-tolerant transgenic plants. With the recent advances in molecular biology of stress tolerance in tomato, this expectation may not be unlikely.

Wheat vacuolar sodium antiporter *TNHX1* and pyrophosphatase *TVP-1* genes were introduced to tomato plant to improve its salinity tolerance (Khoudi *et al.*, 2009). Transgenic tomato plants overexpressing these genes exhibited a better appearance than their non-transgenic counterparts at 200mM NaCl. Total chlorophyll determinations showed that *TNHX-1* and *TPV-1* transgenic plants retained 4-7 times more chlorophyll, respectively, than their non-transgenic plants. This phenotype is likely due to enhanced capacity of transgenic tomato plants to sequester sodium in their vacuoles which prevents its toxicity in the cytosol and the damage to the photosynthesis apparatus.

Glycine Betaine (GB - N, N, N-trimethyl glycine) is a quaternary ammonium compound found in bacteria, haemophilic archaebacteria, marine invertebrates, plants and mammals (Chen and Murata, 2002; Rhodes and Hanson, 1993; and Takabe *et al*., 2006). GB is synthesized; either by, the oxidation (or dehydrogenation) of choline *via* betaine aldehyde, but different enzymes are involved in the process, or by the N-methylation of glycine (Chen and Murata, 2002). GB stabilizes the structure and function of enzymes, protein complexes and maintains the integrity of membranes against excessive cold, heat and freezing (Gisbert *et al.*, 1999). Many important crops such as rice, potato, and tomato that do not accumulate GB are, therefore, potential targets for engineering betaine biosynthesis (Mohanty *et al.*, 2002).

Bansal *et al.* (2008) transformed the *pGAH/codA* gene, cloned from *A. globiformis*, into Indian tomato cv. Pusa Ruby using *A. tumefaciens* EHA101. The *codA* gene encodes enzyme choline oxidase, that converts choline to GB in a single oxidation step. A few 11-day-old cotyledonary explants with apical and basal portion removed were used for transformation experiments. The infected cotyledonary explants were cultured on MS-B5 medium supplemented with 2 mg/l BAP, 25 mg/l kanamycin and 250 mg/l cefotaxime. The regenerated transgenic shoots were then transferred on half strength MS medium supplemented with 25 mg/l kanamycin for root induction. Transformed shoots were multiplied by auxiliary bud formation. All transformed plants showed normal vegetative growth, flower induction, fruit set and seed production. The transgenic status and gene expression in primary T_0 plants and progeny was confirmed by molecular characterization. The number of active loci was determined by segregation analysis. *In* *vitro* salt tolerance assay was performed using T_2 plants by challenging them with an increasing concentration of 0-200 mM NaCl.

Recently, Goel *et al*. (2011) introduced the *codA* gene encoding choline oxidase, which revealed synthesize GB, from *A. globiformis* into tomato. The *codA*-transgenic plants showed higher tolerance to salt stress during seed germination, and subsequent growth of young seedlings than wild-type plants. The *codA* transgene enhanced the salt tolerance of whole plants and leaves. Mature leaves of *codA*-transgenic plants revealed higher levels of relative water, chlorophyll, and proline content than those of wild-type plants under salt and water stresses. Results from their study suggested that the expression of the *codA* gene in transgenic tomato plants induces the synthesis of GB and improves the tolerance of plants to salt and water stresses.

Jia *et al.* (2002) transformed the betaine aldehyde dehdrogenase (*BADH*) gene, cloned from *Atriplex hortensis* and controlled by two 35S promoters of the cauliflower mosaic virus, into a salt-sensitive tomato cv. Bailichun using *A. tumefaciens* strain LBA4404 carrying a binary vector pBin438, and using a leaf regeneration system. Polymerase chain reaction and Southern hybridization analyses demonstrated that the *BADH* gene had integrated into the genome of tomato. Transgenic tomato plants showed significantly higher levels of mRNA and BADH enzyme activity than wild-type plants. Observations on rooting development and relative electronic conductivity suggested that the transgenic plants exhibited tolerance to salt stress, with these plants growing normally at salt concentrations up to 120 mM.

An *Arthrobacter globiformis COX* gene isolated independently by Deshnium *et al.* (1995) has been transferred to plants, which are non-accumulator of GB such as *A. thaliana* (Hayashi *et al.*, 1997, 1998a and 1998b; and Sakamoto *et al.*, 2000), *B. napus* (Huang *et al.*, 2000), *B. juncea* (Sakamoto and Murata, 2001), *Diospyros kaki* Thunb. (Gao *et al.*, 2000), *N. tabacum* (Maas, 1986; and Lilius *et al.*, 1996), *O. sativa* (Mohanty *et al.*, 2002), *S. lycopersicum* (Park *et al.*, 2004). There is enough evidence to support that tomato is a non-accumulator of GB in natural condition (Zhang and Blumwald, 2001); when foliar spray of GB was applied, the plants were able to take it and provide stress tolerance response (Horsch *et al.*, 1985; McCormick *et al.*, 1986; Makela *et al.*, 1996; and Makela *et al.*, 1998) but, failed to provide stable expression or integration of the trait in the progeny.

Trehalose is a nonreducing disaccharide of glucose that has been correlated with tolerance to different stress conditions. Cortina and Culiáñez-Maciá (2005) produced transgenic tomato plants by introducing the yeast trehalose-6-phosphate synthase (*TPS1*) gene, under control of *CaMV35S* gene for expression in plants. Using *Agrobacterium*-mediated transfer, the gene was incorporated into the genomic DNA and constitutively expressed in tomato cv. UC82B plants. TPS1 transgenic tomato plants exhibited pleiotropic changes such as thick shoots, rigid dark-green leaves, erected branches and an aberrant root development. Additionally, leaves of transgenic TPS1 tomato plants showed a chlorophyll and starch content higher than wild-type plants. Under drought, salt and oxidative stress, TPS1 tomato plants improved tolerance than wild type, suggesting that carbohydrate alterations produced by trehalose biosynthesis be linked to the stress response. These results indicate the feasibility of engineering tomato for increased tolerance of abiotic stress, without decreasing productivity, under both stress and non-stress conditions through trehalose biosynthesis.

Several halotolerance (*HAL*) genes have been isolated from yeast (Serrano and Gaxiola, 1994). It has been demonstrated that the overexpression of the yeast genes *HAL1* and *HAL2* improves yeast growth under salt stress (Gaxiola *et al.*, 1992 and Serrano and Gaxiola, 1994). *HAL1* confers salt tolerance by modulating cation transport systems, while, maintaining a high internal K^+ concentration and decreasing intracellular Na^+ during salt stress (Gaxiola *et al.*, 1992). In turn, *HAL2* overexpression increases tolerance to high lithium and sodium concentrations, because it encodes a cation-sensitive nucleotidase required for sulfate assimilation (Murguía *et al*., 1995 and 1996) and RNA processing (Dichtt *et al.*, 1997).

Arrillaga *et al.* (1998) introduced *HAL2* gene into tomato cv. UC82B by *Agrobacterium*-mediated transformation method. Five to six percent of the explants produced transgenic plants. *HAL2* expressing transformants were allowed to self-pollinate and salt tolerance assays were performed *in vitro* on progenies from two independent transgenic plants with different levels of expression of the transgene. Under salt stress, callus formation from hypocotyl explants was higher on both transgenic-derived progenies than in the control. In addition, progenies from the plant with the highest expression of the transgene also showed a higher level of root production on NaCl-supplemented medium.

These results suggested a positive effect of the yeast *HAL2* gene on the level of salt tolerance in progenies derived from transgenic plants.

Gisbert *et al*. (1999) introduced the yeast gene *HAL1* into tomato by *A. tumefaciens*-mediated transformation. Results from different tests indicated a higher level of salt tolerance in the progeny of two different transgenic plants bearing four copies or one copy of the *HAL1* gene. In addition, measurement of the intracellular K^+ to Na^+ ratios showed that transgenic lines were able to retain more K^+ than the control under salt stress. Although plants and yeast cannot be compared in an absolute sense, these results indicate that the mechanism controlling the positive effect of the *HAL1* gene on salt tolerance may be similar in transgenic plants and yeast.

Late-embryogenesis-abundant (LEA) proteins constitute a superfamily of proteins that were detected for the first time during the maturation phase of cotton embryogenesis, which is the stage when acquisition of desiccation tolerance occurs in the embryo, when they accumulate in high concentrations, a characteristic that gave rise to their name (Dure and Chlan, 1981 and Dure and Galau, 1981). There have been several studies of specific members of group 2 of LEA proteins that confirm their accumulation during seed desiccation and in response to water deficit induced by drought, low temperature, or salinity (Ismail *et al*., 1999 and Nylander *et al*., 2001). Since the expression of dehydrins is significantly induced by abiotic stresses such as drought, cold and high salinity, it has been suggested that a positive correlation exists between dehydrin expression and abiotic stress tolerance in plants (Saavedra *et al*., 2006 and Brini *et al*., 2007).

The *tas14* dehydrin gene was isolated and characterized in tomato (Godoy *et al*., 1990). This gene was induced in tomato seedlings and adult plants under osmotic stress (NaCl and mannitol) and abscisic acid (ABA) (Godoy *et al*., 1994), but the physiological role played by this gene during drought and salt stress in tomato still remains unknown. In a study by Muñoz-Mayor *et al.* (2012), transgenic tomato plants overexpressing *tas14* gene under the control of the 35SCaMV promoter were generated to assess the function of *tas14* gene in drought and salinity tolerance. The plants overexpressing *tas14* gene achieved improved long-term drought and salinity tolerance without affecting plant growth under non-stress conditions. A mechanism of osmotic stress tolerance *via* osmotic potential reduction and solutes accumulation, such as sugars and K^+ is operating in *tas14* overexpressing plants in salinity conditions. Moreover, the overexpression of *tas14* gene increased Na^+ accumulation only in adult leaves, whereas in young leaves, the accumulated solutes were K^+ and sugars, suggesting that plants overexpressing *tas14* gene are able to distribute the Na^+ accumulation between young and adult leaves over a prolonged period in stressful conditions. Measurement of ABA showed that the action mechanism of *tas14* gene is associated with an earlier and greater accumulation of ABA in leaves during short-term periods.

b. Using glutaredoxin gene to improve tolerance to abiotic stress

Reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($HO^{\bullet}$) are redox signals essential to many physiological processes in both prokaryotes and eukaryotes (Apel and Hirt, 2004 and

Rouhier, 2010). In higher plants, chloroplast and mitochondria are two major organelles that contribute to production of ROS during photosynthesis and carbon metabolism. Although ROS are signals essential for plant development, high concentration of ROS can damage macromolecules and thus disrupt normal signaling in plant. Many environmental stresses such as drought, salinity, heavy metals, and abnormal temperature can induce excessive accumulation ROS in plants, which will damage macromolecules, thus change normal signal condition (Miller *et al.*, 2010), and leads to inhibition of plant growth and development (Gill and Tuteja, 2010; Jaspers and Kangasjarvi, 2010 and Suzuki *et al.*, 2011).

To manage oxidative damage and simultaneously regulate signaling event, plants have orchestrated an elaborate antioxidant network system (Foyer and Noctor, 2005 and Rouhier *et al.*, 2008). As part of this network, glutaredoxins (Grxs) are small ubiquitous proteins of the thioredoxin (Trx) family and mediate reversible reduction of disulfide bonds of their substrate proteins in the presence of glutathione (GSH) *via* a dithiol or monothiol mechanisms (Rouhier *et al.*, 2008). These enzymes have emerged as key regulators in diverse cellular processes such as controlling plant development, DNA synthesis, signaling, [Fe-S] assembly and oxidative stress responses by regulating cellular redox state and redox-dependent signaling pathway (Rouhier *et al.*, 2004 and 2006), and are conserved in both prokaryotes and eukaryotes (Shelton *et al.*, 2005; Lillig *et al.*, 2008; Rouhier *et al.*, 2008 and Cheng *et al.*, 2011).

Three major groups of Grxs have been classified (Vlamis-Gardikas and Holmgren, 2002 and Fernándes and Holmgren, 2004). Classical Grxs RE 10-kDa proteins with a CPYC active site (Grx1 and Grx3 in *E. coli* and Grx1 and Grx2 in yeast). A second group, with a CGFS active site, corresponds to yeast Grx3, Grx4, and Grx5 (Rodriguez-Manzaneque *et al*., 1999). The third type, represented by *E. coli*, Grx2 is structurally related to the glutathione S-transferase (Xia *et al*., 2001). Heterologous expression in yeast (*Saccharomyces cerevisiae*) mutant cells has been used to establish some conserved functions among Grx (Cheng *et al*., 2006 and Cheng, 2008). Grxs appears to be ubiquitous in plants (Rouhier *et al*., 2006 and Garg *et al*., 2010), but only a few have been characterized (Guo *et al*., 2010; Sundaram and Rathinasabapathi, 2010 and Cheng *et al*., 2011).

Cyanobacterium synechocystis strain PCC6803 contains two genes (*slr1562* and *ssr2061*) encoding two glutaredoxins (Grx1 and Grx2, respectively). The amino acid sequences deduced from both proteins share high identity with those of Grxs from other organisms.

Gaber *et al***.** (2007) found that the steady-state transcript levels of *ssr2061* were increased in the wild-type of *Cyanobacterium* cells under oxidative stress conditions imposed by high salinity (NaCl), chilling or application of H_2O_2, methylviologen or t-butyl hydroperoxide. Moreover, the protein Grx2 encoded by *ssr2061* was successfully overexpressed as a soluble fraction in *Escherichia coli* JM109. The transformed *Escherichia coli* cells showed high tolerance to NaCl (more than 700 mM) mediating growth inhibition compared to cells transformed with the vector alone.

Marteyn *et al*. (2009) found that *GRX1* and *GRX2* of *Synechocystis* PCC6803 strain are active, and that Grx2 but not Grx1 is crucial to tolerance to hydrogen peroxide and selenite; the predominant form of selenium in the environment.

Guo *et al*. (2010) isolated and characterized a novel cDNA fragment (SlGRX1) from tomato encoding a protein containing the consensus Grx family domain with a CGFS active site. Southern blot analysis indicated that *SlGRX1* gene had a single copy in tomato genome. Quantitative real-time RT-PCR analysis revealed that *SlGRX1* was expressed ubiquitously in tomato including leaf, root, stem and flower, and its expression could be induced by oxidative, drought, and salt stresses. Virus-induced gene silencing mediated silencing of *SlGRX1* in tomato led to increased sensitivity to oxidative and salt stresses with decreased relative chlorophyll content, and reduced tolerance to drought stress with decreased relative water content. In contrast, over-expression of *SlGRX1* in *Arabidopsis* plants significantly increased resistance of plants to oxidative, drought, and salt stresses. Furthermore, expression levels of oxidative, drought and salt stress related genes *Apx2, Apx6*, and *RD22* were up-regulated in *SlGRX1* overexpressed *Arabidopsis* plants when analyzed by quantitative real-time PCR.

Moreover, transgenic *Arabidopsis* lines expressing a GRX of the fern, *Pteris vittata,* PvGRX5, were more tolerant to high temperature stress than control lines (Sundaram and Rathinasabapathi, 2010).

Wu *et al*. (2012) demonstrated that *AtGRXS17* has conserved functions in anti-oxidative stress and thermotolerance in both yeast and plants. In yeast, *AtGRXS17* co-localized with yeast ScGrx3 in the nucleus and suppressed the sensitivity of yeast grx3grx4 double-mutant cells to oxidative stress and heat shock. In plants, GFP-AtGRXS17 fusion proteins initially localized in the cytoplasm and the nuclear envelope but migrated to the nucleus during heat stress. Ectopic expression of *AtGRXS17* in tomato plants minimized photo-oxidation of chlorophyll and reduced oxidative damage of cell membrane systems under heat stress. This enhanced thermotolerance correlated with increased catalase (CAT) enzyme activity and reduced H_2O_2 accumulation in *AtGRXS17*-expressing tomatoes. Furthermore, during heat stress, expression of the heat shock transcription factor (HSF) and heat shock protein (HSP) genes was up-regulated in AtGRXS17-expressing transgenic plants compared with wild-type controls. Thus, these findings suggest a specific protective role of a redox protein against temperature stress and provide a genetic engineering strategy to improve crop thermo-tolerance.

5. Tomato tissue culture and transformation

The development and improvement of stress tolerance of crops are primary targets for plant molecular and genetic breeding. Plant transformation has become a versatile tool for cultivar improvement as well as to study gene function in plants. A commonly accepted definition of plant transformation is: "the introduction of exogenous genes into plant cells, tissues or organs employing direct or indirect means developed by molecular and cellular biology" (Jenes *et al.*, 1993). Prerequisites for successful genetic transformation include an *in* *vitro* regeneration, DNA delivery system, functionally introduced DNA (integration of the introduced DNA into the chromosome for stable transformation), selection of transformed cells (promoters and markers) and their regeneration (Hansen and Wright, 1999). For *in vitro* regeneration, tomato has been shown to be a particularly amenable plant (Bhatia *et al.*, 2004). There were various reports about the tissue culture studies and transformation of tomato cultivars that are reviewed here after, with emphasis on *Agrobacterium*-mediated gene transfer method, which is the most widely used method to transfer genes into plants.

a. Tomato regeneration

Most techniques for genetic transformation and regeneration depend on the use of plant growth regulators in complex and nearly empirical combinations adapted to each particular situation. Development of protocols independent of exogenous plant growth regulators could standardize techniques for different species and cultivars; thereby, reducing problems of regeneration efficiency and elongation of regenerated roots. Although, tomato transformation *via Agrobacterium* through adventitious shoot regeneration is not considered a real problem, the interest to obtain more efficient, reliable, simple, rapid and universal methods for tomato transformation is well documented in the literature.

The success in tomato regeneration response has been found to depend largely on the genotype, explant and plant growth regulators used in the culture medium (El-Farash *et al.*, 1993).

1. Explant type

Researchers have used various types of explants *viz.* cotyledon, hypocotyl, pedicel, peduncle, leaf, stem sections and inflorescence for organogenesis. The type of explants used not only determines the proportion of explants, which show organogenesis, but also the number of shoots produced per explant. Duzyaman *et al.* (1994) found that the degree of shoot regeneration was in the order of leaves ≥ cotyledons ≥ hypocotyls, and all cultivars responded similarly. Plastira and Perdikaris (1997) reported differential regeneration frequency of various explants in the order of hypocotyl > cotyledon > leaf.

Preferential regeneration was also demonstrated from hypocotyl explants better than from cotyledon explants (Gunay and Rao, 1980 and Ajenifujah-Solebo *et al*., 2013). In contrast to these findings, Schutze and Wieczorrek (1987) reported that *in vitro* shoot production from cotyledon explants was better than that from hypocotyl explants. Most tissues of tomato seem to have high totipotency; however the choice of the right explant may vary with the genotype.

The influence of explant on the growth and development of organs depends on several factors, including the genotype, the age of explant, the size of explant and the method of inoculation. El-Farash *et al.* (1993) found an interaction between genotype, explant type, and the age of explant donor plant for shoot regeneration rate and the number of shoots produced per explant.

2. Plant growth regulators

Tomato regeneration, although plants have endogenous growth hormones, they are sometimes required to be supplemented under *in* *vitro* conditions to obtain optimal results. A wide variety of plant growth regulators have been used at varying concentrations. The concentration of growth regulators employed is dependent on the cultivar being cultured and the particular cytokinin or auxin being employed. Changing the exposure time results in variations in the time required for organogenesis and numbers of shoots formed on an explant (Chen *et al.*, 1999; Costa *et al.*, 2000 and Venkatachalam *et al.*, 2000). Usually plantlets are regenerated either directly (Dwivedi *et al.*, 1990), or from primary callus (Jawahar *et al.*, 1997). Subculture of unorganized callus to a medium in which the ratio of cytokinin to auxin is increased, or in which there is only cytokinin present, leads to shoot differentiation (Gresshoff and Doy, 1972).

Four major cytokinins *viz*. zeatin, 2-iP, BA, and kinetin, can be used either separately or in combination with auxins for organogenesis in tomato.

b. Tomato transformation

There were various reports on transformation of tomato cultivars. Tomato regeneration capacity and transformation frequencies show variation among cultivars. Transformation frequencies have ranged from 6 to 49.5% (Qiu *et al.*, 2007; Jabeen *et al.*, 2009 and Raj *et al.*, 2005). Many reports were related to only optimization of transformation protocol and other related to transform for production of tolerance to abiotic stress in tomato from various sources.

The first report of tomato transformation using *Agrobacterium* was by Horsch *et al.* in 1985. They produced transformed tomato plants by means of a novel leaf disk transformation-regeneration method.

Surface sterilized leaf disks were inoculated with an *A. tumefaciens* strain containing a modified tumor inducing plasmid and cultured for 2 days. The leaf disks were then transferred to selective medium containing kanamycin. Shoot regeneration occurred within 2 to 4 weeks, and transformants were confirmed by their ability to form roots in medium containing kanamycin.

Saker and Rady (1999) developed a reproducible transformation system for the tomato cv. Edkawy using *A. tumefaciens* and cotyledon explants. PBI 121 construct, in which the selectable marker gene *Gus* was replaced by nopaline synthase-phosphinothricin acetyl transfrase (*bar*) and introduced into tomato cotyledon explants using the *A. tumefaciens* strain LBA4404. Transformed explants were firstly selected on kanamycin and secondly on BASTA herbicide containing media. Regeneration of transformed shoots *via* organogenesis was achieved on MS medium containing 2 mg/l zeatin, 0.2 mg/l IAA and 50 mg/l kanamycin. Factors governing the efficiency of *Agrobacterium*-mediated transformation, including origin of explants, bacterial concentration, co-cultivation time, explants wounding and inclusion of acetosyringone were optimized. PCR analysis was used to confirm the insertion of *nptII* gene into tomato genome.

Moghaieb *et al.* (2000) showed that, the hypocotyl segments of tomato cvs UC-97 and Pontaroza were transformed by *BADH* gene using *Agrobacterium rhizogenes* DC-AR2 as a mediated gene transfer. The transformation frequency was 69.3% and 51.0% in the cultivars UC-97 and Pontaroza, respectively. Hairy root lines were established from both cvs. The resulted hairy root lines show mikimopine activity confirming the transformation status. The stable integration of *BADH* gene was confirmed by PCR and Southern analysis.

Park *et al.* (2003) developed an efficient method to transform tomato cvs Micro-Tom, Red Cherry, Rubion, Piedmont and E6203, using leaf, cotyledon and hypocotyls explants on 7 different regeneration media. Pre-culturing for one day on 1 mg/l BA and 0.1 mg/l NAA and 3 days co-cultivation with the *Agrobacterium* on the same medium followed by transfer to a medium with 2 mg/l zeatin and 0.1 mg/l IAA for 4-6 weeks resulted in a greater than 20% transformation frequency for all five cultivars tested. Transmission of the transgene in T_1 plants was confirmed by southern blot analysis.

Cortina and Culiáñez-Maciá, (2004) studied *Agrobacterium* mediated transformation of *S. lycopersicum* cv. UV82B using cotyledonary explants. Bacterial strain used was LBA4404 harboring *nptII* marker gene. Optimal shoot regeneration rate was obtained with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} zeatin. Acetosyringone at 200 µM, enhanced the transformation efficiency (12.5%) and neomycin resistant shoots (50%).

Park *et al.* (2004) reported that transformation of tomato cv. Moneymaker with a *codA* gene which encodes choline oxidase, while accumulating GB in their leaves. GB-accumulating plants are more tolerant of chilling stress than their wild-type counterparts, yield 10–30% more fruit following chilling stress. Exogenous application of either GB or H_2O_2 improves chilling and oxidative tolerance as well concomitant with enhanced catalase activity.

Shahriari *et al.* (2006) recorded rapid regeneration and transformation of three tomato cvs KalG, Kal-early and Su2270 with *A. tumefaciens* (pGV3850) using cotyledon and hypocotyls. Optimal regeneration was obtained *via* MS medium supplemented with 2 mg/l zeatin and 2 mg/l BAP. Transformation efficiency of 17% for Kal-early and 35% for KalG was observed, confirmed by GUS and PCR analyses.

Qiu *et al.* (2007) established a protocol for *Agrobacterium*-mediated transformation of tomato cv. Micro-Tom for incorporation of the carotenoid biosynthetic gene. Cotyledons used as explant source were cultured for 1 day on the medium containing 2 mg/l zeatin and 0.1 mg/l IAA, submerged in *Agrobacterium* (OD_{600} = 0.2) for 20 min and co-cultivated for 3 days on the same medium. Cotyledons were shifted to pre-selection medium with 500 mg/l cefotaxime for 3 days and shifted to selection medium with 100 mg/l kanamycin and 500 mg/l carabenicillin for 6-8 weeks. 20% transformation efficiency was observed.

Sharma *et al.* (2009) described a highly efficient and reproducible *Agrobacterium*-mediated transformation protocol applicable to several tomato cvs (Pusa Ruby, Arka Vikas and Sioux). Conditions such as co-cultivation period, bacterial concentration, concentration of BAP, zeatin and IAA were optimized. Co-cultivation of explants with a bacterial concentration of 108 cells/ml for three days on 2 mg/l BAP, followed by regeneration on a medium containing 1 mg/ml zeatin resulted in a transformation frequency of 41.4%. Transformation of tomato plants was confirmed by southern blot analysis and β-glucuronidase (GUS) assay. The optimized transformation procedure is simple, efficient and does not require tobacco, petunia, tomato suspension feeder layer or acetosyringone.

Islam *et al.* (2010) established an efficient transformation protocol for tomato cvs. Bina tomato-3, Bina tomato-5, Bahar and Pusa Ruby by reducing complexity and increasing transformation efficiency. Transformation of cotyledonary leaf explant was performed with *Agrobacterium tumefaciens* strain LBA4404, harboring binary vector pBI121 having *GUS* and *nptII* marker genes. Frequency of transient GUS expression showed that the transformation competence in tomato was highly influenced by several factors, like optical density of *Agrobacterium* suspension, incubation period, co-cultivation period etc. Cotyledonary leaf explants from all four cvs tested found to be efficiently transformed by bacterial suspension having optical density (OD600) of 0.79 with 15 min incubation and 3 days of co-cultivation period. All these conditions along with pre-culture of explants prior to transformation gave better regeneration response following *Agrobacterium* infection. Moreover, for successful regeneration of transformed shoot, 200 mg/l kanamycin was found to be most effective selection pressure. As all four varieties showed similar response, their protocol was considered as a simple and genotype-independent reproducible protocol.

Paramesh *et al.* (2010) established an efficient and reproducible transformation protocol for tomato cv. L15 using *Agrobacterium* strain GV 2260 carrying pCAMBIA 1301 plasmid with *β-GUS* and *hpt* genes. The use of pre-cultured cotyledon, leaf and hypocotyl, bacterial density

(OD600) and co-cultivation time of 48 hours and a cefotaxime concentration of 300 mg/l were found to be ideal to keep the *Agrobacterium* under control during the transformation experiments. 2.83 % of selection was observed and GUS assay of the explants were used to evaluate transformation efficiency in early steps.

Li (2011) carried out a study on the optimization of *Agrobacterium*- mediated genetic transformation system of cotyledon explants of tomato cv. Meifen No. 1. The highest transformation efficiency was obtained when the explants were cultivated for 2 d on MS medium supplemented with 2.0 mg/l 6-BA and 0.5 mg/l IAA and then infected with *Agrobacterium* EHA105 (OD = 0.4) for 5 min; it was proved by PCR analysis that the target *nptll* gene had been integrated into the genome of regenerated plants.

Guo *et al*. (2012) studied the effect of four parameters on transformation frequency of cotyledon explants of tomato cv. Micro-Tom: the concentration of bacterial suspension, time of dip in bacterial suspension, co-cultivation time, and concentration of carbenicillin. Also, they studied the effect of these parameters on contamination rate, necrosis rate, mortality, cut-surface browning rate, and undamaged explant rate. Both the bacterial and carbenicillin concentrations had a significant influence on the rate of infected explants. The time of co-cultivation also had a significant influence on the transformation parameters. The optimal transformation protocol consisted of an *Agrobacterium* suspension of 0.5×10^8 cells/ml ($OD_{600} = 0.5$) and an infection time of 5 min, one day of co-cultivation and 500 mg/l carbenicillin. Under these conditions, the transformation efficiency of the shoots reached 5.1%; the mean transformation frequency was 3.9%.

6. *In planta* transformation

Genetic transformation of plants occurs naturally (Hooykass and Schilperoort, 1992). Scientists have been able to carry out controlled plant transformation with specific genes since the mid-1970s although many different techniques have been tested for gene transformation to plant cells; *Agrobacterium* mediated transformation has been extensively employed. The first transgenic plant of *Nicotinana tobaccum* was produced *via Agrobacterium* mediated transformation (Horsch *et al.*, 1984). With this success, many crop plants were transformed *via Agrobacterium*. This is the simplest method now available for transferring genes into intact plant tissue.

Developments in plant tissue culture and genetic engineering have made it possible to develop superior cultivars of several economically important crop plants including tomato. Till date, transformed tomato plants have been produced either by *Agrobacterium*-mediated genetic transformation (Horsch *et al.*, 1985; McCormick *et al.*, 1986; Moghaieb *et al.*, 2000; Qiu *et al.*, 2007; Sharma *et al.*, 2009; Islam *et al.*, 2010; Li, 2011; Guo *et al.*, 2012), followed by *in vitro* regeneration either by direct or indirect somatic embryogenesis. Though these methods are widely used, they have some disadvantages such as requiring highly sterile conditions, genotype consumption, occurrence of somaclonal variations, genotype specificity and recalcitrance. Moreover during hardening, the valuable transgenic plantlets may fail to acclimatize, ultimately leading to less transformation efficiency. Thus, considerable refinements of current transformation systems are required to achieve commercial application of transgenics. *In planta* transformation is an alternative method which does not involve *in vitro* culture of plant cell or tissues, thereby reducing time, labor cost and most importantly avoiding somaclonal variation encountered during *in vitro* culture-mediated genetic transformation and regeneration. The transformation efficiency obtained through *in planta* transformation was much higher than conventional tissue culture-based transformation (Supartana *et al*., 2005 and 2006).

First *in planta* transformation was carried out in *Arabidopsis* (Feldmann and Marks, 1987). Since then, *in planta* transformation was successfully adapted in monocotyledonous plants such as rice (Supartana *et al*., 2005), wheat (Supartana *et al*., 2006) and maize (Chumakov *et al*., 2006 and Mamontova *et al*., 2010) and dicotyledonous plants such as radish (Park *et* al., 2005), *Medicago truncatula* (Trieu *et al*., 2000), peanut (Rohini and Rao, 2000), pigeon pea (Rao *et al*., 2008), strawberry (Spolaore *et al*., 2001), and tomato (Saker *et al*., 2008 and Yasmeen *et al*., 2009). *In planta* transformation was carried out by infecting germinating seeds of radish, wheat, rice, cotton and *Brassica napus* (Park *et al*., 2005; Supartana *et al*., 2005 and 2006; Keshamma *et al*., 2008 and Li *et al*., 2009), floral buds of *Arabidopsis* and shoot apical node of *Medicago* (Tague and Mantis, 2006), mature embryo of rice (Lin *et al*., 2009), through fruit injection of tomato (Yasmeen *et al*., 2009), floral dip of *Arabidopsis*, *Medicago* *truncatula*, wheat, radish and tomato (Clough and Bent, 1998; Trieu *et al*., 2000, Curtis and Nam, 2001; Saker *et al*., 2008 and Agarwal *et al*., 2009), pistal dip of maize and cotton (TianZi *et al*., 2010 and Chumakov *et al*., 2006) and *via* pollen tube pathway in soybean (Hu and Wang, 1999).

a. *In planta* seed transformation

To the best of our knowledge, a few reports on *in planta* seed transformation and there is no report on successful *in planta* seed transformation in tomato. The following review was screened the previous reports on in planta transformation of some crops.

The first report of *in planta* seed transformation was by Feldmann and Marks (1987) on *Arabidopsis thaliana*, who described a procedure with a disarmed *A. tumefaciens* strain C58Clrif that carried the pGV3850:pAKI003 plasmid containing the *npt-II* genes. Seeds of T_1 generation imbibed for 12 h before a 24 h exposure to *Agrobacterium* gave rise to the highest number of transformed progeny (T_2 generation). The low transformation efficiency and different yields of transformants in different replications was found. As comes out from the paper of Feldmann (1991), approximately one mutation is recovered from about ten seeds treated. Therefore, Pavingerová and Ondřej (1995) attempted improvement of *A. thaliana* seed transformation efficiency. *A. tumefaciens* induced transgenosis by treatment of germinating *A. thaliana* seed embryos has been achieved with different *Agrobacterium* strains including the strain LBA4404, which was ineffective in seed transformation experiments of the other authors. The frequency of transgenosis was increased several times by application of acetosyringone to the growing *A. tumefaciens* suspension cultures. The DNA demethylating agent 5-azacytidine partly restored the distorted Mendelian segregation ratios in the offspring of transgenic plants.

Park *et al.* (2005) developed a protocol for producing transgenic radish (*Raphanum sativus*) by using both ultrasonic and vacuum infiltration assisted. Where, germinating seeds inoculated with 100 ml overnight liquid culture of *A. tumefaciens* LBA4404 contained the binary vector pBI121-LEA through sonication for 0-12.5 min with an interval of 2.5 min. After sonication treatment, inoculated germinating seeds placed in a vacuum system for further treatment by vacuum infiltration for 0-12.5 min. After vacuum infiltration, radish seeds blotted on sterile papers in Petri dishes for co-culture in the dark at 25 ºC for 3 days then rinsed three times in sterilized distilled water containing 500 mg/l of claforan (cefotaxime sodium) and blotted on dry sterile paper towel and transferred to the seedling soil in pots for full development. One month after transferring to soil pots, the number of surviving seedlings was recorded and the surviving plants were subjected to transgene confirmation by PCR, Southern and Western blot analysis. With the increase in treatment time by sonication and vacuum infiltration, the surviving rate of *Agrobacterium* inoculated germinating seeds decreased, implying that these physical treatments had negative effect on the growth of germinating seeds. Transformation was successful in 2.5-7.5-min sonication combined with 2.5-7.5-min vacuum treatments, in which the transgenic efficiency was 2–4%, suggesting that sonication combined with vacuum infiltration helped to improve transformation efficiency.

Supartana *et al*. (2005) developed a simple and efficient *in planta* transformation method for rice using *A. tumefaciens.* Seeds of rice soaked in water for 2 d. then, the embryo containing an apical meristem inoculated with *A. tumefaciens* by piercing a site of the husk overlying the embryonic apical meristem with a needle that had been dipped in an *A. tumefaciens* inoculum. The inoculated seeds were then grown to maturation (T_0 plants) and allowed to pollinate naturally to set seeds (T_1 plants) in pots under nonsterile conditions. To examine the transformation by various means, three different strains of *A. tumefaciens* were used for transformation: an M-21 mutant, which is an avirulent mutant with a Tn5 insertion in the iaaM gene, and two LBA4404 strains each with a different binary vector. Transformation efficiency of T_1 plants was estimated to be 40% and 43% by PCR and a histochemical assay of β-glucuronidase, respectively.

In another attempt, Supartana *et al*. (2006) developed a simple and efficient *in planta* transformation method for wheat using *A. tumefaciens*. Wheat seeds soaked in water at 22°C for 1 d. Thereafter, the embryo of the soaked seeds inoculated with *A. tumefaciens* by piercing a region of the embryonic apical meristem with a needle that had been dipped in an *A. tumefaciens* inoculum. The inoculated seeds incubated at 22°C for 2 d and sterilized by cefotaxime (Claforan) (1000 ppm water solution) treatment and then vernalized at 5°C for 25 d. Finally, the seedlings grown to maturation (T_0 plants) and allowed to pollinate naturally for seed setting (T_1 plants) in pots under nonsterile condition. The transformation efficiency of T_1 plants estimated to be 33% by PCR analysis, 75% by Southern hybridization and 40% by plasmid rescue.

Keshamma *et al*. (2008) produced transgenic cotton plants by a tissue-culture independent *A. tumefaciens*-mediated transformation procedure. *Agrobacterium* strain LBA4404 harboring the binary vector pKIWI105 that carries *GUS* and *npt-II* genes was used for transformation. Apical meristem of the differentiated embryo of the germinating seedling is infected with *Agrobacterium*. Since the transgene is integrated into the cells of already differentiated tissues, the T_0 plants will be chimeric and stable integration can be seen only in the T_1 generation. The first proof of transformability in the T_0 generation was indicated by the GUS histochemical analysis of the seedlings, five days after co-cultivation and subsequently in the pollen and lint. T_1 transformants were identified by PCR analysis and subsequently confirmed by Southern. Three plants (T_1) with single copy insertions were selected for continuing into the next generations.

Li *et al*. (2009) suggested that seed transformation has a potential use in genetic transformation of rape, where, a seed transformation of sonication-assisted, no-tissue culture to rapidly produce transgenic *Brassica napus* plants. This method comprises the steps of treating seeds by ultrasonic wave, inoculating *Agrobacterium tumefaciens* with a recombinant *ChIFN*-α gene and germinating directly of treatment seed on wet filter papers. The obtained transformants were verified by GUS histochemical assay and nested PCR amplification.

Mayavan *et al*. (2013) developed an efficient, reproducible and genotype independent in planta transformation method for sugarcane using seed as explant. Transgenic sugarcane production through *Agrobacterium* infection followed by *in vitro* regeneration is a time-consuming process and highly genotype dependent. To obtain more number of transformed sugarcane plants in a relatively short duration, sugarcane seeds were infected with *A. tumefaciens* EHA 105 harboring pCAMBIA 1304-bar and transformed plants were successfully established without undergoing *in vitro* regeneration. Various factors affecting sugarcane seed transformation were optimized, including pre-culture duration, acetosyringone concentration, surfactants, co-cultivation, sonication and vacuum infiltration duration. The transformed sugarcane plants were selected against BASTA and screened by GUS and GFP visual assay, PCR and Southern hybridization. Among the different combinations and concentrations tested, when 12-h pre-cultured seeds were sonicated for 10 min and 3 min vacuum infiltered in 100 µM acetosyringone and 0.1 % Silwett L-77 containing *Agrobacterium* suspension and cocultivated for 72-h showed highest transformation efficiency. The amenability of the standardized protocol was tested on five genotypes. It was found that all the tested genotypes responded favorably, though CoC671 proved to be the best responding cultivar with 45.4 % transformation efficiency. The developed protocol is cost-effective, efficient and genotype independent without involvement of any tissue culture procedure and can generate a relatively large number of transgenic plants in approximately 2 months.

MATERIALS AND METHODS

This study was carried out to establish a reliable regeneration system for tomato which can be used for introducing *GRX-2* gene into tomato for improving salt tolerance using *Agrobacterium* during the period from 2008 to 2014 at the Department of Genetics, Faculty of Agriculture, Cairo University, Giza.

1. *In vitro* tomato culture

a. Plant materials

Five tomato cvs. Flora-Dade (LA3242, C. M. Rick, University of California, Davis); Marmande (PI157850) and Summer Prolific (PI303791) (Northeast Regional PI Station, USDA, ARS); Castlerock and Super Strain B (Horticultural Research Institute, Agricultural Research Center), were used in this study.

b. Growth conditions

Basal Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 7g/l agar was used. Several plant growth regulators (PGR), *i.e.*, BA, IAA, and Kinetin, were used in different concentrations (Table 1) to find out suitable media combinations for the growth and differentiation of hypocotyl and cotyledon explants for the used tomato cvs. The pH for media was adjusted to 5.6 – 5.8 after adding PGR, then autoclaved at 121^{o} C and 1.5 lb for 15 min.

Seeds were surface sterilized by dipping in 70% ethanol for 1 min, followed by immersion in 50% sodium hypochlorite (commercial bleach is mainly hypochlorite) for 10 min, and three rinses in sterile

Table 1. Media used in tomato tissue culture.

Media	Composition
M1	MS + 30 g/l sucrose + 7 g/l agar + 2.5 mg/l BA + 1.0 mg/l IAA.
M2	MS + 30 g/l sucrose + 7 g/l agar + 1.0 mg/l BA + 0.2 mg/l IAA.
M3	MS + 30 g/l sucrose + 7 g/l agar + 4.5 mg/l BA.
M4	MS + 30 g/l sucrose + 7 g/l agar + 6.0 mg/l BA.
M5	MS + 30 g/l sucrose + 7 g/l agar + 3.0 mg/l Kin + 0.3 mg/l IAA.

distilled water. Seeds were dried on sterilized Whatman filter papers. The sterilized seeds were germinated in jars containing MS + 30 g/l sucrose + 7 g/l agar. The glass jars were incubated at 25±2° C under darkness for 48 h then transferred into a 16/8 h light/dark photoperiodic regime (1000 lux).

The *in vitro* grown 10 day-old seedlings were used as a source of explants, the cotyledonary leaves and the meristematic ends of the hypocotyls (3 mm in length) were isolated. Both types of explants were cultured in glass jars with different MS media (Table 1). Each jar contained 40 ml medium and 10 explant segments and all the treatments were performed with 3 replications. The jars were incubated at 25±2° C under a 16/8 h light/dark photoperiodic regime (1000 lux). The explants were sub-cultured every 2 weeks on corresponding medium freshly prepared. Data were collected on the number of explants inducing callus, number of callus inducing shoots, number of shoots per callus, and number of rooting shoots; and the following parameters were calculated:

$$\textbf{Callus induction frequency (\%)} = \frac{\textbf{No. of explants inducing calli}}{\textbf{the total number of explants in the culture}} \times \mathbf{100}$$

$$\textbf{Shoot induction frequency (\%)} = \frac{\textbf{No. of explants gaving shoots}}{\textbf{the total number of explants in the culture}} \times \mathbf{100}$$

c. Statistical analysis

A factorial experiment passed on a completely randomized design (CRD) with three factors (5 cvs, 5 media, and 2 explant types) and with 3 replications was used. The analysis of variance (ANOVA) and mean comparisons (the least significant differences (LSD) and Duncan's multiple range tests) analysis were performed using the software according to Maxwell and Delaney (1989).

2. Tomato genetic transformation

a. Plant materials

According to tomato regeneration results, tomato cv. Castlerock was selected and used in this study.

b. Construction of plant transformation vector

The plant expression vector pRI 101-ON DNA (Eurofins MWG Operon, USA) in *Agrobacterium tumefaciens* strain LBA4404 was used for transformation. The binary vector is containing the Glutaredoxin-2 target gene (*GRX-2*), which was cloned from cyanobacterium *Synechocystis* PCC 6803, under the control of cauliflower mosaic virus 35S (*CaMV 35S*) promoter and nopaline synthase (*nos*) terminator, and the selective kanamycin resistance gene *nptII* (Fig. 1).

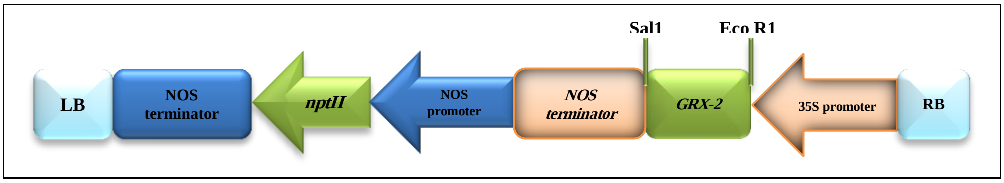


Fig. 1. Plasmid map of the transformation vector pRI 101-ON DNA GRX-2.

c. Transformation of the host *E. coli* DH5α cells with the plant expression vector

1. Preparation of competent cells

The competent cells of *E.coli* DH5α, were prepared following the protocol mentioned by Sambrook and Russell (2001) with minor modifications.

An isolated colony from fresh streak of the frozen stocks of the *E. coli* DH5α plate was inoculated into 5 ml Luria-Bertani (LB) broth liquid media (10 g/l Bacto-trypton + 5 g/l yeast extract + 10 g/l NaCl, pH 7.0-7.5) and incubated at 37°C overnight at 200 rpm. Next day, the culture was diluted to 1:100 by LB broth liquid media, *i.e.*, 0.5 ml of culture was added to 50 ml of LB liquid media, and incubated at 37^o C for 2-3 h till it attained an OD_{600} of 0.3 to 0.4. The culture was chilled on ice for 15 min and the cells were centrifuged for 10 min at 4000 rpm at 4^{o}C. The cells pellet was resuspended in 10 ml of ice-cold 0.1M $CaCl_2$. The cells were incubated on ice for 30 min, and then centrifuged as above and gently resuspended in 1ml ice-cold 0.1M $CaCl_2$ plus 15% glycerol. About 50-100 µl of DH5α competent was aliquots and chilled in -80 ^{o} C to be used in transformation.

2. Transformation of *E. coli* DH5α

To transform the competent cells, 5 µl of pRI 101-ON DNA vector was added to 50 µl of the competent cells and mixed gently. The mixture was chilled in ice for 30 min and heat shocked at 42^{o}C water bath for 40 sec, and then placed back directly into ice for 2 min. A 500 µl of LB broth medium was added to mixture and mixed gently and incubated at 37^{o}C at 200 rpm for 1h to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. LB agar plates supplemented with appropriates antibiotic (kanamycin) were prepared, and then the transformed competent cells were uniformly spread onto LB plates. The plates were left for about 10 min to dry then incubated at 37^{o}C for 16-24 h.

3. PCR confirmation of recombinant clones (pRI 101-ON with *GRX-2*)

a. Plasmid isolation

Colonies were inoculated to 5 ml LB broth with kanamycin (100 mg/ml) and incubated over night at 37°C with 175 rpm. Overnight grown culture was centrifuged at 14000 rpm for 5 minutes at 4°C in 1.5 ml micro centrifuge tubes. The supernatant was removed and pellet was resuspended in 100 µl of ice-cold solution I by vigorous vortexing. Later, 200 µl of freshly prepared alkaline lysis solution II was added to each tube and the contents were mixed by inverting the tubes for 4 to 5 times and kept on ice for about 5 minutes. To this suspension, 300 µl of solution III was added and again mixed thoroughly by gently inverting the tubes for 4-5 times. The tubes were stored on ice for 15 minutes and centrifuged at 14000 rpm for 10 minutes. The supernatant was transferred to fresh tubes and two volumes isopropanol was added. The contents were mixed and allowed to stand for 1h at -20 ºC. The solution was later centrifuged at 14000 rpm for 20 min. The supernatant was discarded and pellet was washed with 70 % ethanol and spun for 5 min at 14000 rpm to recover the plasmid. The supernatant was discarded; pellet was dried completely and dispensed into 30 µl of sterilized distilled water. Then the solution was stored at -20°C.

solution I Glucose: 50 mM
Tris-Cl (pH 8.0): 25 mM
EDTA-Ethylene Diamine Tetra Acetic Acid (pH 8.0): 10 mM
Autoclaved and stored at 4°C

solution II NaOH : 0.4 N
Sodium Dodecyl Sulphate (SDS): 2% (w/v)
(Prepared fresh and used at room temperature)

solution III 5 M potassium acetate : 60 ml
Glacial acetic acid : 11.5 ml
Double distilled water : 28.5 ml
Autoclaved and stored at 4°C

b. Polymerase chain reaction (PCR) analysis

Three pairs of specific primers were used in this study. The first pair was designed to amplify 279 bp of the *GRX-2* gene (namely F1 and R1), the second pair was designed to amplify 795 bp of the *nptII* gene (namely F2 and R2), and the third pair was designed to amplify 250 bp of the *35S promoter* (namely F3 and R3) as shown in Table 2. The primers were designed on the basis of the published sequence of the genes. The confirmation of the presence of cloned fragment was done by PCR amplification of clones with the specific primers for *GRX-2* gene. PCR amplification was performed in a 20 µl reaction mixture containing 40 ng DNA templates, 10 pmole of each forward and reverse primers, 10 µl of 2X power Taq PCR master mix (Biotecke Corporation) and volume was completed to 20 µl using sterilized distilled water. PCR temperature profile used for the amplification consisted of an initial denaturation cycle at 94 ºC for 5 min followed by 30 cycles of 94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 1 min, then a terminal extension cycle at 72 ºC for 5 min.

Table 2. The nucleotide sequence of the primers used for PCR analysis.

Genes	**Primers**[z]	**Sequence**	**Expected size (bp)**
GRX-2	F1	5$^{/}$ATGGCTGTCTCGGCAAAAATTG3$^{/}$	279
	R1	5$^{/}$CTAACTATGGAGCAGGGGGT3$^{/}$	
nptII	F2	5$^{/}$ATGATTGAACAAGATGGATTG3$^{/}$	795
	R2	5$^{/}$TCAGAAGAACTCGTCAAGAAG3$^{/}$	
CaMV	F3	5/AGGAAGGTCTTGGGAAGGAT3/	250
35S	R3	5/TGTGATAACATGGTGGAGCA3/	

[z]F: forward and R: reverse.

d. Transformation of *A. tumefaciens* with plant vector

The binary plasmid pRI 101-ON DNA-GRX2 was introduced into *A. tumefaciens* strain LBA4404, using an alternative, efficient, direct transformation procedure described by Tzfira *et al*. (1997) with some modifications.

1. Preparation of *Agrobacterium* competent cells

A single colony of *Agrobacterium* was inoculated in 2 ml LB liquid medium supplemented with 2μl streptomycin (100 mg/ml) and incubated overnight at 28°C with Shaker. Culture was transferred to 50 ml LB medium and kept at 28°C with shaking at 250 rpm until the optical density (OD_{600}nm) reached to 0.5-1(3-4 h). The culture was centrifuged at 5000 rpm for 5 min at 4°C. The pellet was gently resuspended in 1ml $CaCl_2$ (20mM) then incubated on ice for 20 min.

2. Transfer plasmid DNA to competent cells

Fifty ng of plasmid DNA was added to 50µl of competent cells and froze in liquid nitrogen. The cells were put in 37°C water bath for 5 min, then the cells were cooled on ice for 5 min. 500 µl of LB was added to the tube, then incubated for 3-4 h with gentle shaking at 28°C. After that the culture was transferred to LB plates containing kanamycin (100 mg/ml) as selection agent and streptomycin.

The presence of recombinant plasmid in the *Agrobacterium* was confirmed by PCR amplification as described with *E. coli* DH5α.

e. Tomato transformation

Agrobacterium cells containing pRI 101-ON DNA with *GRX-2* gene from *Synechocystis* PCC 6803 carrying *nptII* gene as selectable marker were used for transformation of tomato cv. Castlerock using two methods, *i.e.*, transformation of cotyledon explants and transformation of germinated seeds.

1. *Agrobacterium* culture

A single colony of *A. tumefaciens* strain LBA4404 grown on solid LB medium (10 g/l Bacto-trypton + 5 g/l Yeast extract + 10 g/l NaCl +15g/l agar) containing 100 mg/l kanamycin (kan) and 50 mg/l streptomycin (strep) was picked and grown in liquid LB medium containing 100 mg/l kanamycin + 50 mg/l streptomycin on a shaker at 28ºC and 200 rpm for 48 h. Bacterial concentration was diluted to optical density of 0.8 at 600 nm (5 ml of *Agrobacterium* culture was added to 15 ml fresh LB medium) and shaken at 28ºC for 2-3 h perior to co-cultivation with explants.

2. Transformation techniques

Two techniques were used to transform tomato cv. Castlerock with *Agrobacterium* cells containing pRI 101-ON DNA plasmid with *GRX*-2 gene. The first technique was infection of tomato cotyledon explants by dipping it into the *Agrobacterium* culture. The second technique was soaking of tomato seeds in suspension of *Agrobacterium* cells.

a. Transformation of tomato cotyledon explants

Cotyledon explants were placed in bacterial culture suspension for 10 min with gentle agitation, to increase the transformation rate; the explants were frequently dipped into the *Agrobacterium* culture during the cutting of the explants. Bacteria were poured off and the explants were blotted dry on sterile filter paper to get rid of the excess of inoculum. The explants were spread out on the best chosen regeneration medium from the regeneration experiment (MS containing 6 mg/l BA) and incubated in the growth room for 2-3 days in darkness.

After 2-3 days, explants were washed in sterile distilled water, then dried on sterile filter paper and placed on regeneration medium supplemented with 100 mg/l kanamycin and 500 mg/l cefotaxime then incubated in a growth room at 25ºC under 16 h/day photoperiod of 1000 lux and subsequently transferred to fresh medium every two weeks.

Shoot initiation was developed after 3-4 weeks on the regeneration medium. Shoots of 2-3 cm long were excised after 6-8 weeks and placed on elongation medium containing 100 mg/l kanamycin and 500 mg/l cefotaxime for 2-4 weeks (the first stage of transformation). Elongated shoots were transferred individually to rooting medium (MS + 30 g/l sucrose + 7 g/l agar + 1mg/l IBA) (the second stage). After 2-3 weeks, the rooted shoots (plantlets) were transferred to pots containing sterile mixture of peatmoss and vermaculite (1:1 v:v) and covered with plastic bags in the growth room at 25 ^{o}C under 16/18 h light /dark cycle. The plantlets were hardened by removing plastic bags gradually within 2 weeks. Uncovered plants were transferred after a few days to greenhouse.

b. Transformation of seeds

Prior to transformation, a primary culture of *Agrobacterium* was prepared by inoculating single colony from a freshly streaked LB agar plate, in 10 ml of autoclaved LB broth containing 10 mg/l of streptomycin and 50 mg/l of kanamycin. The culture was incubated for 24 h in a shaker at 180 rpm in dark at 28 ºC. Secondary culture was initiated by inoculating 0.5 ml of primary culture into a 250 ml flask containing 50 ml LB broth supplemented with the aforesaid antibiotics and grown under the same conditions. When the bacterial culture OD_{600} reached 1.0, the bacterial cells were harvested by centrifugation at 6000 rpm for 10 min at 4ºC. The bacterial cells were resuspended in liquid MS medium, and the OD_{600} of the bacterial suspension was adjusted to 0.8.

Tomato seeds cv. Castlrock were initially dipped in 70% ethanol for 1 min, and then disinfected with 50 % (v/v) sodium hypochlorite for 10 min and washed 3 times with sterile distilled water. The sterilized seeds were soaked in sterile distilled water overnight, and then infection consisted of adding 1 ml of an overnight culture of *A.*

tumefaciens to the seeds in 50 ml of liquid MS for 1 h with shaker 120 rpm at 25 ºC. After that the seeds were kept in MS liquid medium containing 500 mg/l cefotaxime overnight. The infected seeds were washed three times with sterile distilled water and dried then were sown in seedling trays (209 cells) filled with sterile mixture enriched with macro and micro elements of peatmoss and vermaculite (1:1 volume) in air conditioned at 25±2 ºC. Five week-old seedlings were transferred in greenhouse for full development.

To calculate the germination percentage, infected and non-infected seeds of tomato cv. Castlerock were cultured in gars containing sterile MS solid medium (10 seeds/gar with 5 replicates). The cultures were incubated at 25 ± 2 ºC for 3 days in dark and later under 16 h photoperiod for 20 days. Those seeds that developed prominent radicle and plumule were considered as germinated seedlings.

3. Calculation of transformation efficiency

The transformation efficiency was calculated using diagnosis DNA PCR result and sample size was based on No. of germinated seedlings from particular parameter tested and successfully transformed seedlings.

f. Molecular analysis of putative *GRX-2* transgenic tomato

Transgenic tomato plants of T_0 and T_1 were transferred in greenhouse for full development. Transformed and non-transformed tomato plants (based on polymerase chain reaction - PCR confirmation) were selfed to obtain the T_1 and T_2 seeds. Tomato fruits were collected after 4 months of transferring and seeds were extracted from collected fruits. The inheritance of transgene into the T_1 and T_2 resistant seedlings was confirmed by the PCR analysis, DNA sequencing, Dot blot hybridization and RT-PCR analysis.

1. PCR analysis

a. Isolation of plant genomic DNA

Total plant genomic DNA was isolated from putative transgenic tomato leaves using a CTAB (Cetyltrimethylammonium bromide) protocol described by Murray and Thompson (1980) with some modifications.

A 0.1 g of young leaf tissue (T_0, T_1, and T_2) was collected and quickly homogenized in liquid nitrogen, and then ground to fine powder using mortar and pestle. The powder was transferred to 1.5 ml microfuge tube. Then 700 µl of pre-heated (65ºC) DNA extraction buffer (0.1 M Tris-HCl, 20 mM Na_2EDTA, 1.4 M NaCl and 2% CTAB, pH 8.0) and 7µl 1% β-mercaptoethanol were added to each tube. The tubes were mixed well and incubated at 65ºC for 45 min with periodical inversion. A 700 µl of chloroform (24): Isoamyl (1) solution was added and the contents were mixed gently by inversion to form an emulsion. The tubes were centrifuged at 14000 rpm for 10 min at room temperature and the aqueous phase was gently transferred to new tubes with a wide bore tip pipette and the last step was repeated once again. The same volume of isopropanol and one tenth volume of 3 M NaOAc (sodium acetate) pH 5.0 were added and mixed gently then placed at -20ºC overnight. The samples were centrifuged at 14000 rpm for 20 min, and the supernatants were discarded. The pellets were washed with 70% ethanol and centrifuged at 14000 rpm for 10 min. The tubes were drained on clean absorbent paper and allowed to over dry for 10-15 min. Then, DNA was hydrated by 50 µl of sterilized distilled water and stored at -20ºC until use.

b. Purification of plant DNA

The dissolved DNA was treated with 50 □g/ml RNase at 37ºC for 1h and further purified by extracting with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), to remove any trace of protein and RNase. It was followed by chloroform: isoamyl alcohol (24:1) extraction. The DNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.0) and two volumes of chilled 97% ethanol. The mixture was kept at □20ºC for 30-45 min for precipitation. The pellet was recovered by centrifugation at 14000 rpm at 4ºC for 15 min. The DNA pellet was washed with 70 % ethanol for 5-10 min. DNA pellet was air-dried and dissolved in 50 l of sterilized distilled water.

c. Polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from young leaves of putative transgenic tomato plants and then analyzed by PCR. Three pairs of specific primers were used for the detection of the *GRX-2*, *nptII* genes and 35S *promoter* by PCR (Table2). PCR amplification was performed as described above. The PCR program for *35S promoter* was similar to *GRX-2* gene, while the annealing temperature of *nptII* gene was 54ºC.

Electrophoresis of PCR products was carried out in 1% agarose gel stained with ethidium bromide. A 1 kb ladder DNA was used as molecular weight size marker which covers a range of DNA fragment size between 10000 bp and 250 bp. The DNA bands were visualized on ultraviolet (UV) trans illuminator and photographed with gel documentation system.

d. Agarose gel preparation

One gram of agarose powder was added to a 100 ml of 1xTAE buffer (0.04 M Tris pH 8.0 with acetic acid and 0.001 M EDTA) the slurry was heated in a microwave oven until the agarose dissolved. The solution was cooled down to 50º C, and then 0.5µl of ethidium bromide (10 mg/ml) was added. The warm agarose solution was poured into its tray with well-forming comb and allowed to complete solidification (about 30 min at room temperature). Then, the comb was removed carefully and the gel was placed on submarine electrophoresis unit. Enough electrophoresis buffer (1x TAE) was added to cover the gel. DNA samples were mixed with loading buffer, which contained 5-10% glycerol, 7% sucrose, and 0.025% bromophenol blue and loaded into the sample well of the submerged gel. Electrophoresis was done under constant voltage of 100 V.

2. DNA sequencing

The automated DNA sequencing reactions were conducted by Macrogen Company, Germany for seven DNA samples of T_0, T_1 and T_2 transgenic plants. In order to obtain larger amounts of DNA for the sequencing reaction, new PCR reactions were carried out in a 50 µl volume, with the same components described above and using the same amplification profile of *GRX-2* and *nptII* genes. Amplification products were purified from the gel using QIAquick PCR purification kit (QIAGEN).

Computer analysis for sequenced fragments was done using BLAST program from National Center for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov/BLAST).

3. Dot blot hybridization analysis

Total genomic DNA was isolated from transformed and non-transformed plants according to a method described previously by Murray and Thompson (1980), then denatured at 100ºC for 5 min, then rapidly cooled in ice. Samples were spotted onto presoaked nitrocellulose membrane. Hybridization was performed overnight at 42ºC in a buffer containing 5X denhardt's solution, 6X SSC, 0.5% SDS and 50% (v/v) deionized formamide and followed by the addition of *GRX-2* as probe. Membrane was washed twice at room temperature in 2X SSC/ 0.1% SDS for 10 min followed by two washes in 0.1X SSC/ 0.1% SDS for 20 min at 65ºC. Labeling of the probe, hybridization and detection was carried out using the Biotin Chromogenic Detection kit #K0661, #K0662 (Thermo Scientific).

4. RT-PCR expression analysis

To confirm the transgenic nature of tomato plants, RT-PCR technique was used. Total RNA was isolated from leaf tissue of transgenic (T_1 and T_2 generation) and non-transformed control plants using Biozol-total RNA extraction reagent (Bioflux). Reverse transcription reaction were performed using first strand cDNA synthesis kit (Thermo Scientific RevertAid). First strand cDNA was used as template for PCR amplification using the same set of *GRX-2* specific primers and conditions described above for screening of putative transgenic plants. RT-PCR was performed with approximately 0.1 µg RNA as a template for cDNA synthesis. Following the linear phase of DNA amplification (30 cycles), the PCR products were examined by electrophoresis in 1% agarose gel.

3. Evaluating transgenic tomato plants to salt tolerance

Three lines of *GRX-2* transgenic tomato and non-transgenic plants of cv. Castlerock were used as plant materials to evaluate effect of the *GRX-2* on salinity tolerance in tomato. Seeds were sowed in seedling trays filled with mixture enriched with macro and micro elements of peatmoss and vermiculate (1:1). Five week-old seedlings were transferred into plastic pots (3 l) containing a mixture of peatmoss, vermiculate and sand (1:1:1, v:v:v) in a randomized complete block design (RCBD) with three replicates. The plants were subjected to salt stress by the addition of zero, 100, 200 and 300 mM NaCl.

Plant height was measured after two weeks from starting salt treatment and also, chlorophyll content in the fifth leaf from the top of plant was measured by chlorophyll meter SPAD-502 (Konica Minolta Sensing, Inc., Japan). The several parts of harvested plants (root, stem and leaves) were dried at 70 ºC in an air-forced draught oven for more than three days, and then weighed.

To determin Na^+ ion concentration in plant, portions of 0.2 g of dried plant were digested using 10 ml of a mixture of concentrated acids of HNO_3, H_2SO_4 and $HClO_4$ at ratio of 5:1:2 (Imamul Huq and Alam, 2005). The digestion tubes containing plant samples and mixture of acids were covered and left overnight. Then, the tubes were placed on a hot plate and digestion proceeds until the appearance of white fumes and the sample turned colorless. The concentration of Na^+ was measured in the digestion solution using Flame Photometer (Corning, 410).

Data obtained were statistically analyzed and mean comparisons were based on Duncan's multiple range tests by using the software according to Maxwell and Delaney (1989).

RESULTS AND DISCUSSION

1. *In vitro* tomato culture

Tomato is one of the most studied higher plants because of its importance as a crop species, and of several advantages for genetic, molecular and physiological studies (Mccormick *et al.*, 1986). The *in vitro* morphogenic responses of cultured plant tissues are affected by the different components of the culture media, especially by concentration of growth hormones, and it is therefore important to evaluate their effects on plant regeneration. Development of an efficient protocol for tomato transformation and its subsequent regeneration is a pre-request for the production of transgenic plants. Several studies have demonstrated that cotyledons and hypocotyls were superior to leaves for promoting shoot organogenesis in tomato (Hamza and Chupeau, 1993 and Ling *et al*., 1998). Based on these reports, cotyledon and hypocotyl explants were used as explants for tomato regeneration, in the present study.

Two explant types derived from the cotyledonary leaf and meristematic end of the hypocotyl were isolated from 10 days old seedlings of five tomato cvs. Fifty segments from each type of explant and from each cultivar were cultured on MS medium supplemented with different growth regulators. Most of the available references on tomato used cotyledon and hypocotyl as explants. On the other hand, Applewhite *et al.* (1994) used pedicels and peduncles of flowering plants for tomato regeneration. Also, Compton and Veilleux (1991) regenerated *de novo* shoots, roots and flowers using inflorescence explants. Previous studies demonstrated that 8 to 10 day old cotyledons of tomato were superior to other sources of explants, including hypocotyls, stem and leaves for promoting shoot organogenesis of tomato (Hamza and Chupeau, 1993).

In the present study a simple protocol was used for the regeneration of the five cvs of tomato. This protocol allows regeneration of tomato plants within 6-8 weeks. Young regenerated tomato plants can be transplanted to the soil after 11-15 weeks. The *in vitro* grown 10 day-old seedlings (Fig. 2B) used as a source of the cotyledonary leaves (Fig. 2C) and the meristematic ends of the hypocotyls (3 mm in length – Fig. 2D). Both types of explants were cultured in glass jars with five different MS media.

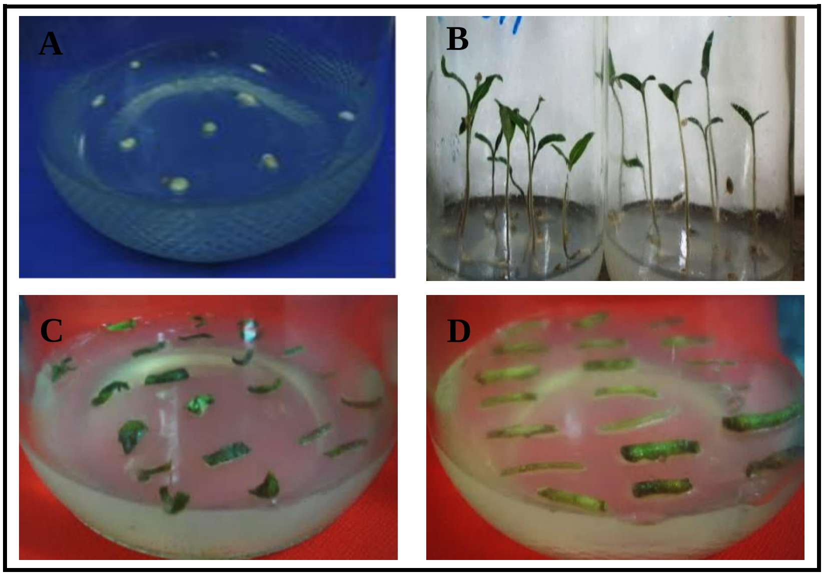


Fig. 2. Steps of tomato explants preparation. A, Seeds culture; B, Germinated seeds; and C and D, Preparation and culturing of cotyledon and hypocotyl explants, respectively.

a. Callus induction frequency

Data concerning effects of explant type, cultivar and media and their interactions on callus induction frequency are presented in Table 3 and Figs 3 and 4. Callus induction frequency was significantly high for cotyledon explant. "Castlerock" and "Super Strain B" cultivars were significantly higher in callus induction frequency (33%) than others (Fig. 3), this was true with cotyledon explant (26.27%) which indicates the importance of interaction between explant type and cultivar.

Table 3. Callus induction frequency for hypocotyl and cotyledon explants of 5 tomato cvs culturing on 5 different media.

Explant type	Cultivar	Media					
		M-1	**M-2**	**M-3**	**M-4**	**M-5**	**Mean**
Hypocotyl	**Castlerock**	33.33 g	0 u	33.33 g	60.00 d	33.33 g	**32.00**
	Flora-Dade	26.67 k	30.00 i	30.00 i	0 u	0 u	**32.00**
	Marmande	20.00 m	0 u	43.33 e	36.67 f	6.67 s	**17.33**
	Summer Prolific	33.33 g	23.33 l	33.33 g	33.33 g	20.00 m	**21.33**
	Super Strain B	33.33 g	0 u	0 u	93.33 a	33.33 g	**32.00**
Mean		**29.33**	**10.67**	**28.00**	**44.67**	**18.67**	**25.4**
cotyledon	**Castlerock**	33.33 g	16.67 o	18.33 n	71.67 c	30.00 i	**34.00**
	Flora-Dade	28.33 j	31.67 h	18.33 n	15.00 p	15.00 p	**21.67**
	Marmande	6.67 s	8.33 r	23.33 l	10.00 q	5.00 t	**10.67**
	Summer Prolific	31.67 h	36.67 f	26.67 k	20.00 m	18.33 n	**26.67**
	Super Strain B	10.00 q	30.00 i	18.33 n	83.33 b	28.33 j	**34.00**
Mean		**22.00**	**24.67**	**21.00**	**40.00**	**19.33**	**26.27****
	Castlerock	33.33 c	8.33 r	25.83 j	65.83 b	31.67 e	**33.00**
	Flora-Dade	27.50 h	30.83 f	24.17 k	7.50 s	7.50 s	**19.50**
	Marmande	13.33 p	4.17 u	33.33 c	23.33 l	5.83 t	**16.00**
	Summer Prolific	32. 50 d	30.00 g	30.00 g	26.67 i	19.17 n	**27.67**
	Super Strain B	21.67 m	15.00 o	9.17 q	88.33 a	30.83 f	**33.00**
		25.67	**17.67**	**24.50**	**42.33**	**19.00**	

$LSD_{0.05}$ Cultivar = 0.121 Media = 0.12

Explant type × Cultivar = 0.17 Explant type × Media = 0.17

In the interactions between cultivar × media and explant type × cultivar × media, values followed by a letter in common are not significantly different at the 0.05 level according to Duncan's multiple range test.

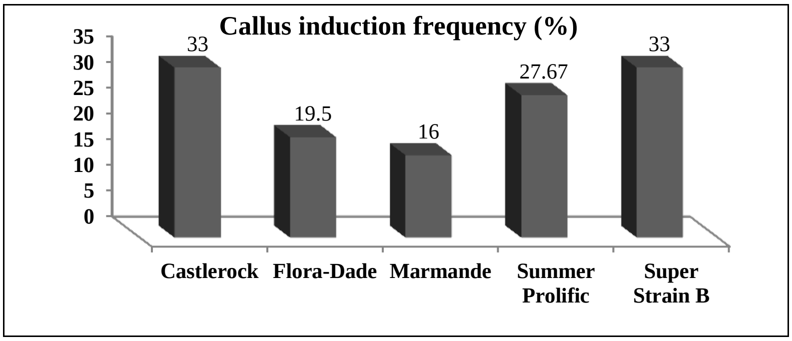


Fig. 3. Means of callus induction frequency of 5 tomato cvs culturing on 5 different media by using 2 explants.

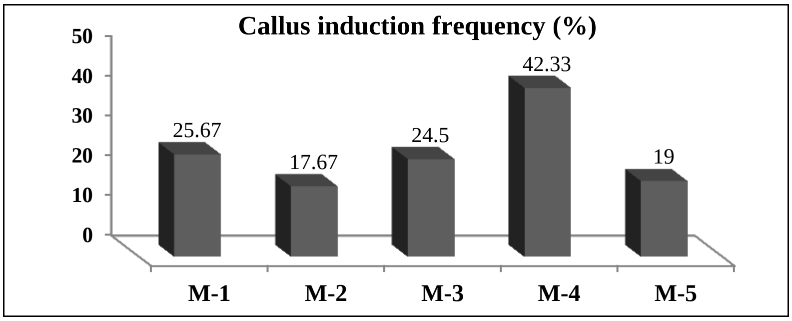


Fig. 4. Means of callus induction frequency on 5 different media for 2 explants of 5 tomato cvs.

With regard to the effect of media on calli induction frequency, five different media were employed (Table 3) the data confirmed that using M-4 (6 mg/l BA - 42% - Fig. 4) and cotyledon explant produced the highest callus induction frequency followed by hypocotyl explant. Also, using of this medium with "Super Strain B" and "Castlerock" was produced highly callus induction frequency.

The interaction among explant type, cultivar and medium revealed that using hypocotyl of cv. Super Strain B with M-4 medium resulted in the highest percentage of callus induction frequency followed by using cotyledon explant of the same cv on the same medium.

b. Shoot induction frequency

Data concerning effects of explant type, cultivar and media and their interactions on shoot induction frequency are presented in Table 4 and Figs. 5 and 6.

As shown in Table (4), there are significant differences between hypocotyl and cotyledon explants in shoot induction frequency, where cotyledon explants showed the highest percentage of shoot induction frequency (12.27%).

Table 4. Shoot induction frequency for hypocotyl and cotyledon explants of 5 tomato cvs on 5 different media.

Explant type	Cultivar	Media					
		M-1	**M-2**	**M-3**	**M-4**	**M-5**	**Mean**
Hypocotyl	**Castlerock**	0 r	0 r	10.00 k	40.00 c	0 r	**10.00**
	Flora-Dade	0 r	1.10 q	3.33 o	13.33 i	3.33 o	**4.22**
	Marmande	6.67 m	0 r	3.33 o	10.00 k	0 r	**4.00**
	Summer Prolific	16.67 g	6.67 m	16.67 g	16.67 g	3.33 o	**12.00**
	Super Strain B	6.67 m	0 r	0 r	53.33 b	0 r	**12.00**
Mean		**6.00**	**1.55**	**6.67**	**26.67**	**1.33**	**8.44**
cotyledon	**Castlerock**	13.33 i	15.00 h	10.00 k	55.00 a	21.67 e	**23.00**
	Flora-Dade	13.33 i	21.67 e	5.00 n	5.00 n	10.00 k	**11.00**
	Marmande	3.33 o	3.33 o	8.23 l	1.67 p	3.33 o	**4.00**
	Summer Prolific	20.00 f	26.67 d	8.33 l	6.67 m	11.67 j	**14.67**
	Super Strain B	3.33 o	16.67 g	8.33 l	0 r	15.00 h	**8.67**
Mean		**10.67**	**16.67**	**8.00**	**13.67**	**12.33**	**12.27****
	Castlerock	6.67 l	7.50 k	10.00 h	47.50 a	10.83 g	**16.50**
	Flora-Dade	6.67 l	11.38 f	4.17 o	9.17 i	6.67 l	**7.61**
	Marmande	5.00 n	1.67 p	5.83 m	5.83 m	1.67 p	**4.00**
	Summer Prolific	18.33 c	16.67 d	12.50 e	11.67 f	7.50 k	**13.33**
	Super Strain B	5.00 n	8.33 j	4.17 o	26.67 b	7.50 k	**10.33**
		8.33	**9.11**	**7.33**	**20.17**	**6.83**	

$LSD_{0.05}$ Cultivar = 0.177 Media = 0.18

Explant type × Cultivar = 0.25 Explant type × Media = 0.25

In the interactions between cultivar × media and explant type × cultivar × media, values followed by a letter in common are not significantly different at the 0.05 level according to Duncan's multiple range test.

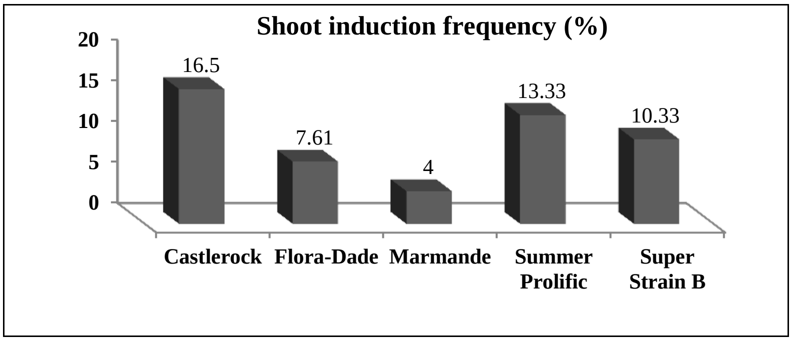


Fig. 5. Means of shoot induction frequency of 5 tomato cvs culturing on 5 different media by using 2 explants.

Regarding cultivar effect, "Castlerock" had the highest percentage of shoot induction frequency (16.50%) followed by "Summer Prolific" (13.33% - Fig. 5). With regard to the effect of medium, using M-4 significantly increased shoot induction frequency (Fig 6).

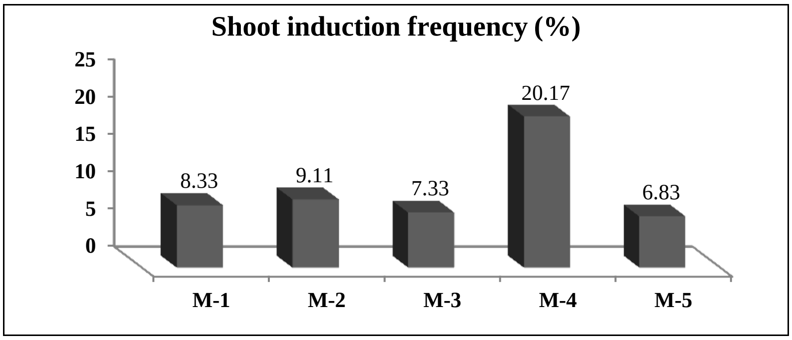


Fig. 6. Means of shoot induction frequency on 5 different media for 2 explants of 5 tomato cvs.

The interaction between explant type and cultivar revealed that using cotyledon explant of "Castlerock" had the highest percentage of shoot induction frequency (23%) and followed by "Summer Prolific" (14.67%).

Regarding the interaction between explant type and medium, culturing hypocotyl explant on M-4 led to the highest shoot induction frequency (26.67%).

The interaction between tomato cultivars and media, data showed that shoot induction frequency was significantly higher in "Castlerock" with M-4 (47.5%) and followed by "Super Strain B" with the same medium (26.67%).

With regard to the interactions among explant type, cultivar and medium, there was a significant effect on shoot induction frequency. Using cotyledon of "Castlerock" as explant and culturing it on M-4 gave the highest percentage of shoot induction frequency (55%) and followed by culturing hypocotyl explant of "Super Strain B" on the same medium (53%).

c. Number of shoots per explant

Data concerning effects of explant type, cultivar and media and their interactions on shoot induction frequency are presented in Table 5 and Figs 7 and 8.

Table 5. Number of shoots per explant for hypocotyl and cotyledon explants of 5 tomato cvs on 5 different media.

Explant type	Cultivar	Media					
		M-1	**M-2**	**M-3**	**M-4**	**M-5**	**Mean**
Hypocotyl	**Castlerock**	0 m	0 m	1.00 l	2.20 c	0 m	**0.64**
	Flora-Dade	0 m	0 m	1 .00 l	1.00 l	3.00 a	**1.00**
	Marmande	1.00 l	0 m	1.00 l	2.30 c	0 m	**0.86**
	Summer Prolific	1.20 k	2.00 d	1.20 k	1.00 l	2.00 d	**1.48**
	Super Strain B	1.00 l	0 m	0 m	1.90 de	0 m	**0.58**
Mean		**0.64**	**0.40**	**0.84**	**1.68**	**1.00**	**0.91**
cotyledon	**Castlerock**	1.40 ij	1.80 ef	1.70 fg	1.50 hi	1.80 ef	**1.64**
	Flora-Dade	1.50 hi	1.50 hi	1.00 l	1.00 l	2.30 c	**1.46**
	Marmande	1.00 l	1.00 l	1.00 l	1.00 l	1.50 hi	**1.10**
	Summer Prolific	1.30 jk	1.60 gh	1.20 k	1.00 l	1.60 gh	**1.34**
	Super Strain B	2.50 b	2.60 b	1.00 l	0 m	2.00 d	**1.62**
Mean		**1.54**	**1.70**	**1.18**	**0.90**	**1.84**	**1.43****
	Castlerock	0.70	0.90	1.35	1.85	0.90	**1.14**
	Flora-Dade	0.75	0.75	1.00	1.00	2.65	**1.23**
	Marmande	1.00	0.50	1.00	1.65	0.75	**0.98**
	Summer Prolific	1.25	1.80	1.20	1.00	1.80	**1.41**
	Super Strain B	1.75	1.30	0.50	0.95	1.00	**1.10**
		1.09	**1.05**	**1.01**	**1.29**	**1.42**	

LSD$_{0.05}$ Cultivar = 0.046 Media = 0.05

Explant type × Cultivar = 0.06 Explant type × Media = 0.06

In the interactions between cultivar × media and explant type × cultivar × media, values followed by a letter in common are not significantly different at the 0.05 level according to Duncan's multiple range test.

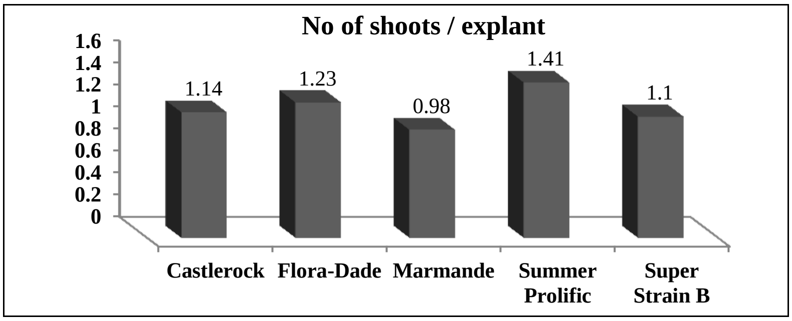


Fig. 7. No of shoots per explant of 5 tomato cvs culturing on 5 different media by using 2 explants.

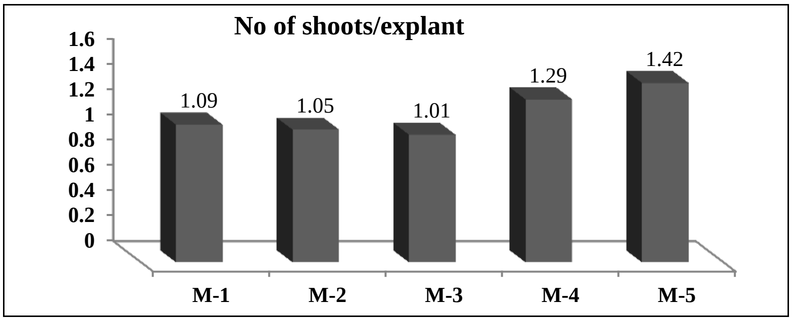


Fig. 8. No of shoots per explant on 5 different media for 2 explants of 5 tomato cvs.

Explant type had a significant effect on No. of shoots per explant. The highest No. of shoots per explant was achieved by using cotyledon explants (1.43 shoot/explant). Also, all tomato cvs had a significant influence on No. of shoots per explant. "Summer Prolific" showed significantly the highest number (1.41 shoot/explant) and followed by "Flora-Dade" (1.23%), as compared with "Castlerock" and "Super Strain B" (1.14 and 1.1 shoot/explant, respectively, Fig. 7).

Regarding the effect of the media on No. of shoots per explant, all used media had a significant effect on No. of shoots per explant, and the highest number was associated with M-5 (3.0 mg/l Kin + 0.3 mg/l IAA – 1.42 shoot/explant) and followed by M-4 (1.29 shoot/explant- Fig. 8).

The interaction between explant type and tomato cvs was significant. The highest number of shoots per explant was observed with cotyledon explants of "Castlerock" and "super Strain B" (1.64 and 1.62 shoot/explant, respectively). Also, the interaction between the explant types and the media as well as between the cultivars and the media significantly affected No. of shoots per explant. Moreover, culturing cotyledon explant on the medium M-5 (1.84 shoot/explant) and culturing of "Flora-Dade" on the medium M-5 (2.65 shoot/explant) increased No. of shoots per explant.

The interactions among explant type, tomato cultivars and the media revealed that culturing hypocotyl explant of "Flora-Dade" on the medium M-5 increased the No. of shoots per explant, where reached to 3 shoot/explant. Also, culturing cotyledon of "Super Strain B" on M-1 (2.5 mg/l BA + 1.0 mg/l IAA) and M-2 (1.0 mg/l BA + 0.2 mg/l IAA) increased the No. of shoots per explant, where recorded 2.50 and 2.6 shoot/explant, respectively.

d. Number of explants gave roots

Data concerning effects of explant type, cultivar and media and their interactions on shoot induction frequency are presented in Table 6.

As shown in Table 6, there are significant differences between hypocotyl explants and cotyledon explants in shoot induction frequency, where cotyledon explants gave the highest No. of explant that gave roots (0.96 explants).

Also, all tomato cvs had a significant influence on No. of shoots per explant. "Flora-Dade" showed significantly the highest number of explant gave root, where recorded 1.4 explants were gave root. Regarding the effect of the media on No. of explant gave root, all used media had a significant effect and the highest number was associated with M-1 (2.5 mg/l BA + 1.0 mg/l IAA) and M-2 (0.9 shoot gave root).

The interaction between explant type and cultivar revealed that using cotyledon explant of "Flora-Dade" had the highest No. of explants that gave roots (2.8 explants).

Table 6. Number of explant gave roots for hypocotyl and cotyledon explants of 5 tomato cvs on 5 different media.

Explant type	**Cultivar**	**Media**					
		M-1	**M-2**	**M-3**	**M-4**	**M-5**	**Mean**
Hypocotyl	**Castlerock**	0 f	0 f	0 f	0 f	1.0 e	**0.2**
	Flora-Dade	0 f	0 f	0 f	0	0 f	**0**
	Marmande	0 f	0 f	0 f	0 f	0 f	**0**
	Summer Prolific	0 f	0 f	0 f	0 f	0 f	**0**
	Super Strain B	0 f	0 f	0 f	0 f	0 f	**0**
Mean		**0**	**0**	**0**	**0**	**0.2**	**0.04**
Cotyledon	**Castlerock**	0 f	0 f	0 f	0 f	0 f	**0**
	Flora-Dade	5.00 b	7.00 a	0 f	0 f	2.00 d	**2.8**
	Marmande	3.00 c	1.00 e	0 f	0 f	1.00 e	**1.0**
	Summer Prolific	1.00 e	1.00 e	0 f	0 f	1.00 e	**0.6**
	Super Strain B	0 f	0 f	0 f	0 f	2.0 d	**0.4**
Mean		**1.8**	**1.8**	**0**	**0**	**1.2**	**0.96****
	Castlerock	0	0	0	0	0.5	**0.1**
	Flora-Dade	2.5	3.5	0	0	1.0	**1.4**
	Marmande	1.5	0.5	0	0	0.50	**0.5**
	Summer Prolific	0.5	0.5	0	0	0.5	**0.3**
	Super Strain B	0	0	0	0	1.0	**0.2**
		0.9	**0.9**	**0**	**0**	**0.7**	

LSD$_{0.05}$ Cultivar = 0.023 Media = 0.023
Explant type × Cultivar = 0.03 Explant type × Media = 0.03

In the interactions between cultivar × media and explant type × cultivar × media, values followed by a letter in common are not significantly different at the 0.05 level according to Duncan's multiple range test.

The interaction between tomato cultivars and media, data showed that No. of explants that gave roots was significantly the highest in "Flora-Dade" by using M-2 (3.5 explants).

With regard to the interactions among explant type, cultivar and medium, there was a significant effect on No. of explants that gave roots. Using cotyledon of "Flora-Dade" as explant and culturing it on M-2 gave the highest No. of explants that gave roots (7 explants).

Results of these experiments show the influence and importance of growth regulators on the number of shoots regenerated from tomato explants (cotyledons and hypocotyls). Although plants have endogenous growth hormones, they are sometimes required to be supplemented under *in vitro* conditions to obtain optimal results. *In vitro* morphogenic responses of cultured plant tissues are affected by the different components of the culture media, especially by concentration of plant growth regulators. These responses are also dependent on cultivar and explants type. The addition of plant growth hormones to the shoot regeneration medium could therefore enhance shoot regeneration in these cultivars and explants.

Cotyledon explants developed shoot buds better than those from hypocotyl explants. This result is in agreement with that of Duzyaman *et al.* (1994) and Schutze and Wieczorrek (1987) who reported that preferential regeneration is demonstrated from cotyledon explants better than from hypocotyl explants. In contrast to these findings, Gunay and Rao (1980), Plastira and Perdikaris (1997) and Ajenifujah-Solebo *et al.* (2013) reported *in vitro* shoot production from hypocotyl explants was better than that from cotyledon explants. Most tissues of tomato seem to have high totipotency; however the choice of the right explant may vary with the genotype.

The observed differences in shoot induction between tomato cultivars in different concentration of growth regulators were anticipated due to the genetic differences between them. These results are in agreement with those of Moghaieb *et al.* (1999) who reported that plant regeneration and somatic embryogenesis are genotype dependent. Also, El-Farash *et al.* (1993) reported that the success in tomato regeneration response has been found to depend largely on the genotype, explant and plant PGRs used in the culture medium.

According to the presented data of regeneration experiment, which showed that the cotyledon explants of "Castlerock" gave the best results of regeneration system on the M-4 medium (Fig. 9), therefore, this interaction was selected as a regeneration system in the following transformation experiment.

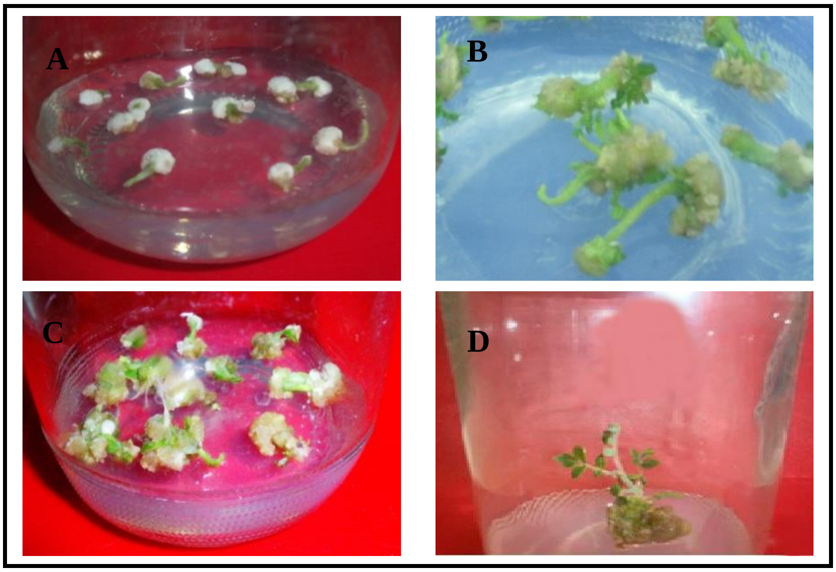


Fig. 9. Callus and organogenesis of cv. Castlerock. A and B: Hypocotyl callus and organogenesis; C and D Cotyledons callus and organogenesis.

2. Tomato genetic transformation

To improvement salinity tolerance in tomato cv. Castlerock, the *GRX-2* gene which has a role in defense against oxidative stress (Stroher and Mullar, 2012), was synthesized and cloned in the plant expression vector pRI 101-ON DNA by Eurofins MWG Operon, USA and this plasmid was used for transformation in the present study. We report for the first time the development of transgenic tomato plants harboring *GRX-2* gene.

a. Transformation of *E. coli* DH5α with vector pRI 101-ON DNA carrying *GRX-2*

To confirm the successful transformation of the pRI 101-ON DNA-GRX-2 vector into *E. coli* DH5α, the transformants were picked on LB containing kanamycin (100 mg/ml). PCR was utilized in this study for the screening of the presence of plasmid which contain *GRX-2* gene in *E.coli*. For PCR analysis a specific primers for the amplification of the full length of gene (279 bp) were used. For screening the transformed *E. coli* colonies by PCR, plasmid DNA was isolated from bacterial cells of several bacterium transformants and used as a template in PCR amplification (Fig. 10). The results showed that nine colonies were transgenic and contained the *GRX-2* gene (Fig.11).

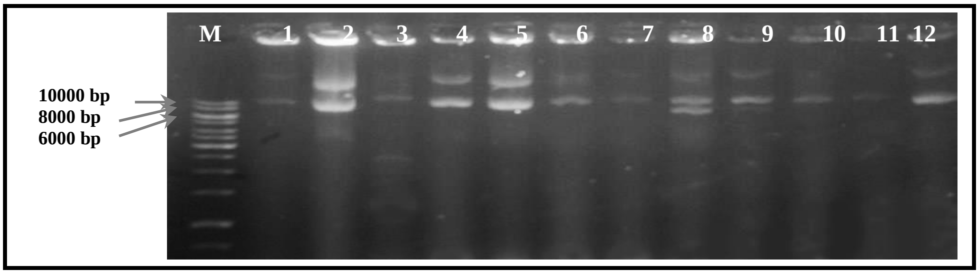


Fig. 10. The isolated plasmid of several transgenic *E. coli* colonies.

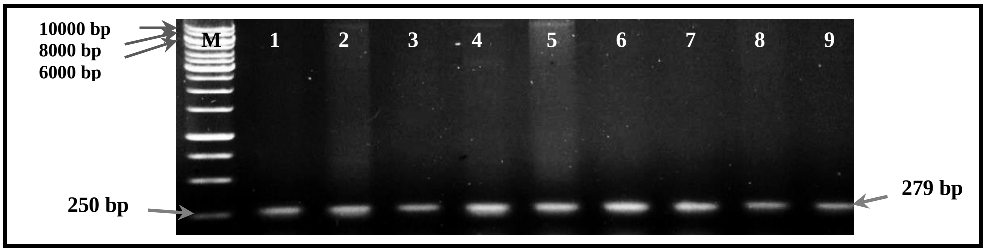


Fig. 11. PCR result of *E. coli* plasmid.

b. Transformation of *A. tumefaciens* strain LBA 4404

The bnary plasmid pRI 101-ON DNA-GRX-2 was introduced into *A. tumefaciens* strain LBA4404 using direct transformation procedure described by Tzfira *et al*. (1997) and produced 7 colonies. Those colonies were screened for the presence of the construct *GRX-2* gene by PCR amplification using specific *GRX-2* primers. As shown in Fig. 12, all *Agrobacterium* colonies produced were recombinant. This is to confirm that *A. tumefaciens* colonies were transgenic and contained the *GRX-2* gene.

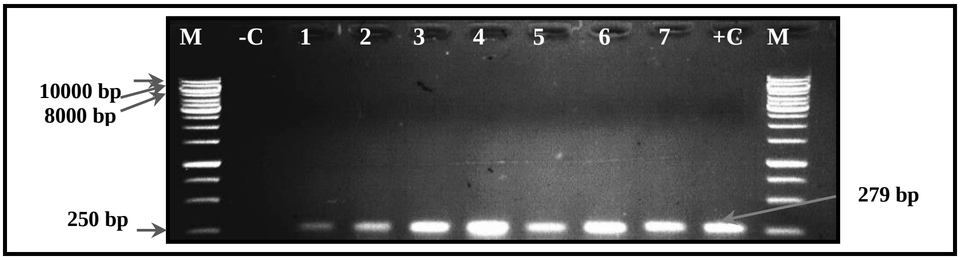


Fig. 12. PCR result of several transgenic *Agrobacterium* plasmids. M: Molecular DNA marker (1kb); -C, negative control (non-transgenic); +C, positive control (plasmid); and 1-7: different transgenic *Agrobacterium* colonies.

c. Tomato transformation

Many methods of tomato transformation are tissue culture based, requiring regeneration of whole plants from transformed cells. The utility of the techniques greatly depends on the establishment of tissue culture protocols in the species. Tissue culture is labor intensive and can be difficult. In addition, even under optimal transformation and regeneration conditions, tissue culture can result in somaclonal variation, morphological abnormalities, changes in chromosome number, and loss of fertility. The development tissue culture-independent transformation system is of great interest because such a system would avoid constraints imposed by genotype specificity in transformation and regeneration and avoid tissue culture induced genetic variation. In addition, transgenic plants would be produced inexpensively and rapidly. For non-tissue culture approaches, either *Agrobacterium* or tungsten particles have been used in a number of species to transform tissues or apical meristem cells that are subsequently allowed to grow. Recently, *in planta* transformation is an alternative method which does not involve *in vitro* culture of plant cell or tissues, thereby reducing time, labor cost and most importantly avoiding somaclonal variation encountered during *in vitro* culture-mediated genetic transformation and regeneration (Supartana *et al*., 2005 and 2006).

Two methods for introduction and expression of pRI101-ON-GRX-2 were employed in this work. The first method depended on the establishment of tissue culture protocol and the second was named seed transformation and was developed by this work.

1. Transformation of tomato cotyledon explants

Cotyledon explants of tomato cultivar Castlerock were wounded and infected with a single cell culture of *A. tumeficiens* strain LBA4404 carrying the *GRX-2* and *nptII* genes in the pRI 101-ON DNA plasmid vector. Organogenic calli derived from cotyledon explants were selected on M4 medium containing Kanamycin as a selective agent (Fig. 13). T_0 plants that regenerated independently from these calli were selected as putative transgenic plants.

In order to test that T_0 plants carrying *GRX-2* and *npt-II* genes in their tissue, total genomic DNA was purified from T_0 plant tissues and subjected for PCR analysis using *GRX-2* and *npt-II* genes primers.

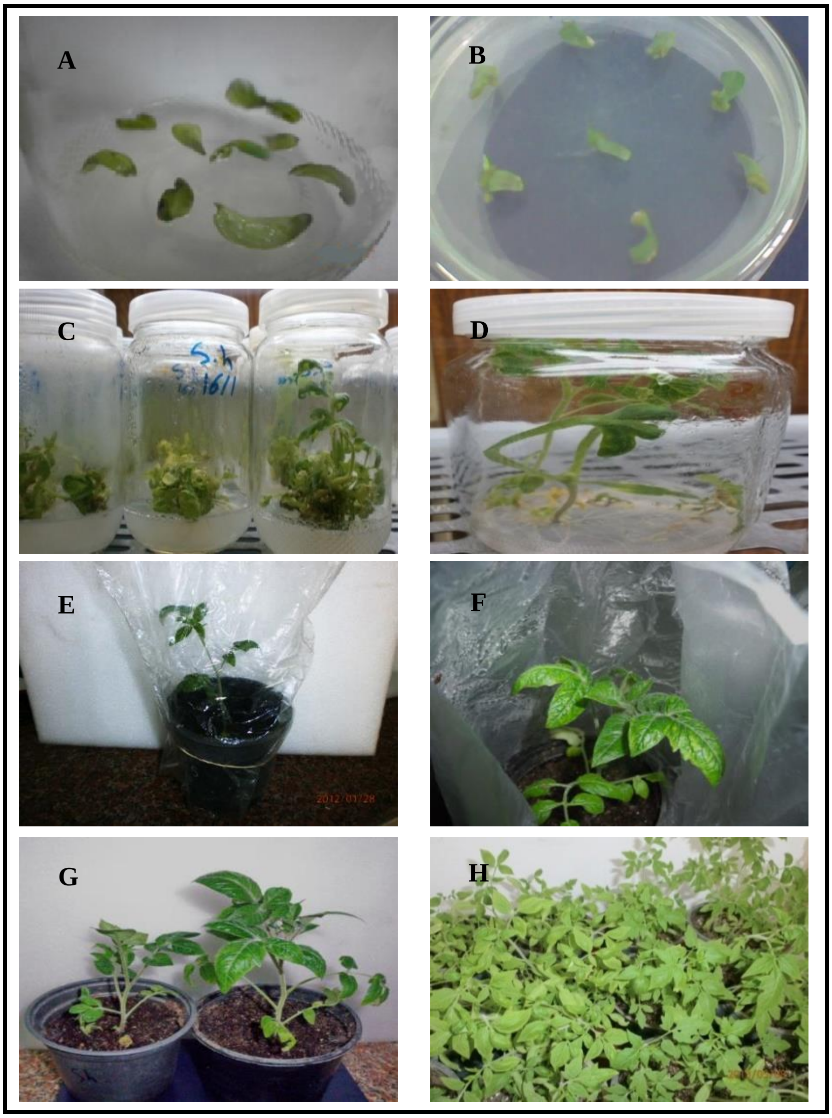


Fig. 13. ***GRX-2*** **transformed plantlets adaptation and transplanted in greenhouse. A: Culturing of infected cotyledon; B and C: Explants Differentiation; D: Transformed plantlet; E: Plantlets culturing in pots containing mixture of peatmoss and vermiculite and covered with plastic cover; F: Removing gradually plastic cover from on plantlets; G: Adapted plantlets; H: Culturing adapted plantlets in greenhouse.**

The PCR results confirmed the presence of a DNA band of 279 bp when *GRX-2* primers were used and a DNA band 795 bp (Fig. 14) when *npt-II* primers were used. This in to show that DNA purified from

T_0 regenerated plants carrying both genes that were transformed from the vector and it's transgenic plants, however, no amplification product was observed in untransformed plants. In order to calculate the transformation frequency in T_0 plants, it was found that only 44 out of 150 putatively transformed plants were confirmed to be transgenic, representing a percentage of 29.3%.

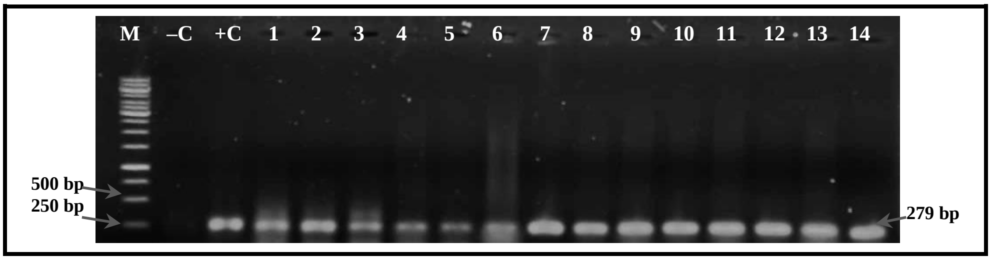


Fig. 14. PCR results of T_0 *GRX-2* transgenic tomato. M: Molecular DNA marker (1kb); -C, negative control (non-transgenic); +C, positive control (plasmid); and 1-14: different transgenic tomato plants.

2. Transformation of tomato seeds

The sterilized tomato seeds were soaked in sterile distilled water overnight and were inoculated with avirulent *Agrobacterium* LBA4404 which contained binary plasmid pRI 101-ON DNA (Fig. 15). It was noticed that the germination percentage of infected seeds was 70% (35 germinated seeds of 50 infected seeds) compared with 86% (43 germinated seeds of 50 seeds) on non-infected seeds.

The transformation efficiency obtained through *in planta* transformation was much higher than conventional tissue culture-based transformation (Supartana *et al.*, 2005 and 2006). According to number of positive plants determined by PCR analysis (Figs. 16 and 17), the estimated transformation frequency for T_0 generation was 38 % (21 confirmed transgenic T_0 plants out of 55 tested plants).

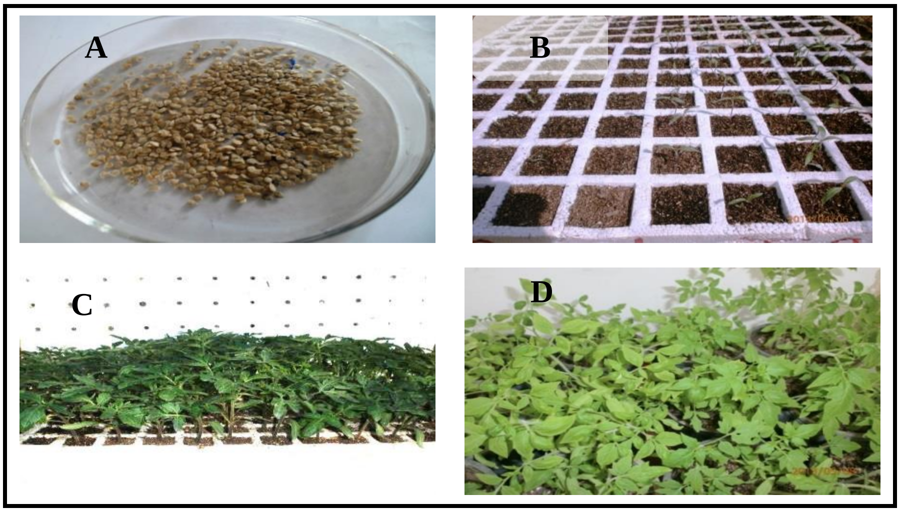


Fig. 15. ***Agrobacterium*-infected tomato cv. Castlerock seeds. A: Tomato seeds, B: Sowing of infected seeds in seedling tray, C: Five week-old seedlings, D: Transferring of infected-plants in pots.**

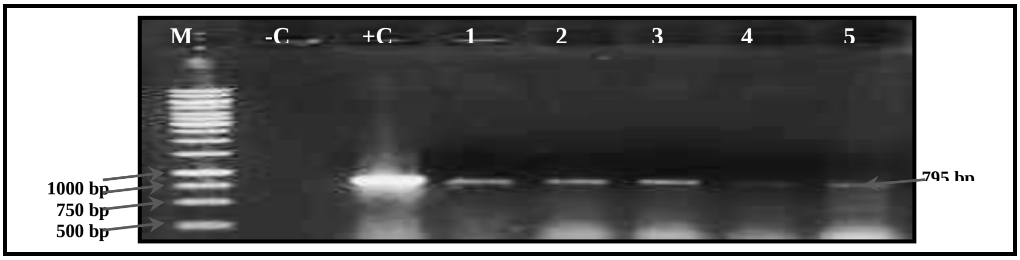


Fig. 16. PCR results of T_0 transgenic plants amplified with *nptII* primer. M: 1kb Molecular DNA marker; -C: Negative control (non-transgenic); +C: Positive control (plasmid); and 1-5: Different transgenic tomato plants.

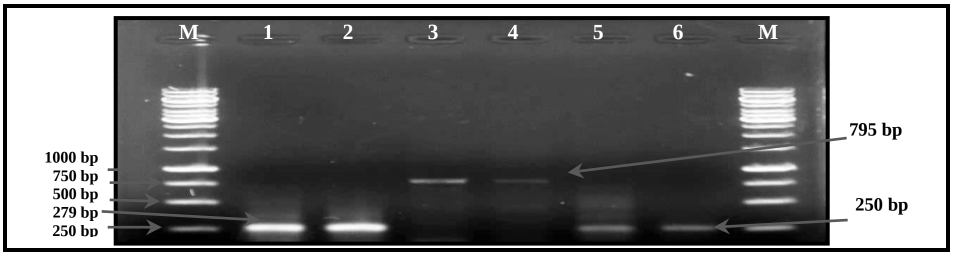


Fig. 17. PCR results of T_0 transgenic plants amplified with different primers (*GRX-2*, *nptII*, and CaMV 35S promoter). M: 1kb Molecular DNA marker; 1 to 6: T_0 transgenic tomato plants.

Tomato transformation efficiency values of conventional tissue culture-based transformation reported in the literature were 8% (Vidya *et al.*, 2000), 7–37% (Ling *et al.*, 1998), 9% (Roekel *et al.*, 1993), 11% (Frary and Earle, 1996), 14% (Hamza and Chupeau, 1993), 20% (Park *et al.*, 2003), 20% (Qiu *et al.*, 2007), 25% (Hu and Phillips, 2001) and 28-48% (Sun *et al.*, 2006). Also, in the present study, the transformation efficiency of conventional tissue culture was 29.3% compared with 38% of seed transformation. Results showed that the direct seed transformation is considered a suitable target tissue for genetic transformation of tomato without tissue culture steps and generate large numbers of transgenic plants rapidly.

The direct seed transformation method using *Agrobacterium*-mediated method is an alternative procedure that transgene can without tissue culture steps and generate large numbers of transgenic plants rapidly. The successful production of transgenic plants *via* seed *Agrobacterium*-mediated transformation has been reported in different plant species such as radish, wheat, rice, cotton, *Brassica napus* and *Morinda citrifolia* (Park *et al*., 2005; Supartana *et al*., 2005 and 2006; Keshamma *et al*., 2008 and Li *et al*., 2009). But the seed *Agrobacterium* transformation method using seed explant has not been reported in tomato transformation. This is the first successful report describing production of transgenic tomato plants from tomato cv. Castlerock using *in planta* seed transformation. The described system enabled us to produce putative transformants without the need of tissue culture system. Different successful protocol on tomato transformation was reported by Saker *et al*. (2008) who could develop transgenic tomato plants expressing defensing gene by using *in planta* floral dipping method. To increase seed transformation efficiency in tomato, transformation conditions such as, bacterial density, incubation periods and variable temperature degree, will be investigating for identifying the optimal conditions of direct seed transformation using *Agrobacterium*-mediated in tomato.

d. Integration and expression of the *GRX-2* gene in transgenic tomato

T_0 and T_1 transgenic plants were maintained in the greenhouse until maturity, selfed and produced fruits which contained viable seeds of transgenic generation T_1 and T_2, respectively were harvested (Fig. 18). Moreover, the inheritance of the transgenes into the T_1 and T_2 plants were confirmed and the detection of the expression of *GRX-2* gene was performed by PCR analysis, DNA sequencing, Dot blot and RT-PCR analysis.

1. Polymerase chain reaction (PCR) analysis

The inheritance of the transgenes into the T_1 and T_2 plants was confirmed by detection of *GRX-2* gene by PCR analysis employing *GRX-2* primers as shown in Figs. 19 and 20.

Fig. 18. Fruiting of *GRX-2* transgenic tomato plants and extracted transgenic seeds.

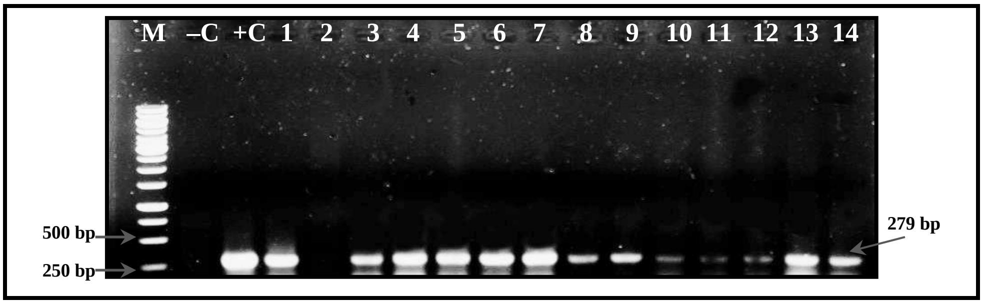


Fig. 19. PCR results of T_1 transgenic tomato. M: 1kb Molecular DNA marker; -C: Negative control (non-transgenic); +C: Positive control (plasmid); and 1-14: Different transgenic tomato plants.

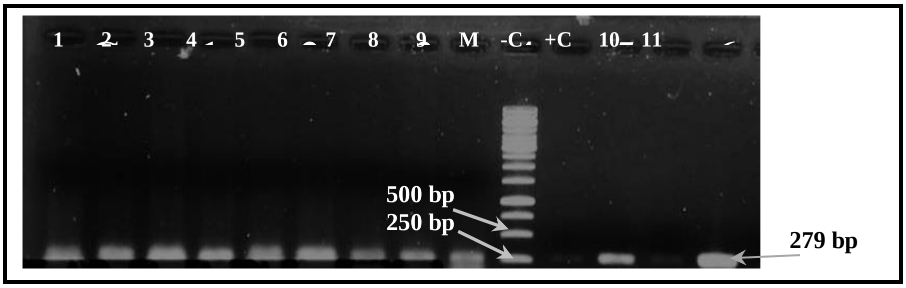


Fig. 20. PCR results of T_2 transgenic tomato. M: 1kb Molecular DNA marker; -C: Negative control (non-transgenic); +C: Positive control (plasmid); and 1-11: Different transgenic tomato plants.

The presence of amplified 279 bp DNA bands from the genome of T_1 and T_2 plants confirmed the integration of *GRX-2* gene and its inheritance to T_1 and T_2 generations.

2. DNA Sequencing

A BLAST sequence is a rapid sequence comparison tool that uses a heuristic approach to construct alignments by optimizing a measure of local similarity. Since BLAST compares protein and nucleotide sequences much faster than dynamic programming methods, it's widely used for database searches. A World Wide Web version of the program can be used interactively at the NCBI WWW site (http://www.ncbi.nlm.gov/BLAST/). The resulting alignments are presented in both graphical and text form (Tatusova and Madden, 1999).

In order to assure that the DNA bands from selective transformants at 795 bp and 279 bp are equivalent to DNA of the proposed *nptII* and *GRX-2* genes, respectively, sequencing was performed by Macrogen Company, Germany. Computer analysis using BLAST programs from National Center for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.gov/BLAST/). Fragment BLAST analysis results are shown in Table (7). DNA sequencing alignment the forward and reverse frame for 2 plants of T_0 with database of the *nptII* gene in Genebank was carried out. The results showed 99% similarity for kanamycin resistance gene (Figs. 21 to 23), while, the results of *GRX-2* showed 96% and 98% (forward and reverse, respectively) similarity for complete genome of *Synechocystis* sp. PCC6803 and 94% and 93% (forward and reverse, respectively) similarity for *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene. Also, the results of *GRX-2* T_1 plant showed 94% and 96% similarity for *Synechocystis* sp. PCC 6803 (forward and reverse, respectively) and 92% are similarity for *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene for reverse only. While, the results of T_2 plants were highly similarity (up to 95%) to *Synechocystis* sp. PCC 6803 and *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene.

Sequence analysis shows that the target fragment DNA of *GRX-2* contains 279 nucleotides (nt, accession no. 000911) (Fig. 24). In order to determine similarity between *GRX-2* and the inserted fragments in different generations of transgenic plants, multiple

Table 7. Transgenic plant with its percentage of similarity compared with our proposed kanamycin resistance and glutradeoxin genes sequence by alignment with database in Genbank.

Gene	Name	Sequence ID	Gene	Match	Total	Pct. (%)	E value
nptII	K4-F	gb_EU215433.1	Kanamycin resistance FRT vector pFKM2, complete sequence	758	759	99	0
		gb_EU215434.1	Kanamycin resistance loxP vector pLKM1, complete sequence	758	759	99	0
	K4-R	gb_EU215433.1	Kanamycin resistance FRT vector pFKM2, complete sequence	763	767	99	0
		gb_EU215434.1	Kanamycin resistance loxP vector pLKM1, complete sequence	763	767	99	0
	K9-F	gb_EU215433.1	Kanamycin resistance FRT vector pFKM2, complete sequence	762	766	99	0
		gb_EU215434.1	Kanamycin resistance loxP vector pLKM1, complete sequence	762	766	99	0
	K9-R	gb_EU215433.1	Kanamycin resistance FRT vector pFKM2, complete sequence	761	765	99	0
		gb_EU215434.1	Kanamycin resistance loxP vector pLKM1, complete sequence	761	765	99	0
GRX-2 (T_0)	G5-F	gb_CP003265.1	*Synechocystis* sp. PCC 6803, complete genome	236	240	96	5e-93
		dbj_BA000022.2	*Synechocystis* sp. PCC 6803 DNA, complete genome	236	240	96	5e-93
		gb_DQ398587.1	*Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds	137	147	94	1e-53
	G5-R	gb_CP003265.1	*Synechocystis* sp. PCC 6803, complete genome	236	240	98	2e-113
		dbj_BA000022.2	*Synechocystis* sp. PCC 6803 DNA, complete genome	236	240	98	2e-113
		gb_DQ398587.1	*Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds	137	147	93	9e-52
	G7-F	gb_CP003265.1	*Synechocystis* sp. PCC 6803, complete genome	209	222	94	4e-88
		dbj_BA000022.2	*Synechocystis* sp. PCC 6803 DNA, complete genome	209	222	94	4e-88

Continued

Table 7. Continued.

Gene	Name	Sequence ID	Gene	Match	Total	Pct. (%)	E Value
GRX-2 (T_0) (Contd.)	G7-F	gb_DQ398587.1	*Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds	143	152	94	6e-57
	G7-R	gb_CP003265.1	*Synechocystis* sp. PCC 6803, complete genome	168	173	97	3e-75
		dbj_BA000022.2	*Synechocystis* sp.PCC 6803 DNA, complete genome	168	173	97	3e-75
		gb_DQ398587.1	*Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds	144	152	95	1e-58
GRX-2 (T_1)	G2-F	gb_CP003265.1	*Synechocystis* sp. PCC 6803, complete genome	44	47	94	8e-08
		dbj_BA000022.2	*Synechocystis* sp. PCC 6803, complete genome	44	47	94	8e-08
	G2-R	gb_CP003265.1	*Synechocystis* sp. PCC 6803, complete genome	133	138	96	2e-55
		dbj_BA000022.2	*Synechocystis* sp. PCC 6803 DNA, complete genome	133	138	96	2e-55
		gb_DQ398587.1	*Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, parial cds	126	137	92	9e-45
GRX-2 (T_2)	A02-F	gb_CP003265.1	*Synechocystis sp*. PCC 6803, complete genome	210	210	100	1e-104
		dbj_BA000022.2	*Synechocystis sp*. PCC 6803 DNA, complete genome	210	210	100	1e-104
		gb_DQ398587.1	*Synechocystis sp*. AHZ-HB-MK glutaredoxin gene, partial cds	146	152	96	2e-62
	A02-R	gb-CP003265.1	*Synechocystis sp*. PCC 6803, complete genome	208	209	99	5e-103
		Dbj-BA000022.2	*Synechocystis sp*. PCC 6803 DNA, complete genome	208	209	99	5e-103
		gb-DQ398587.1	*Synechocystis sp*. AHZ-HB-MK glutaredoxin gene, partial cds	111	117	95	5e-43
	C02-F	gb_CP003265.1	*Synechocystis sp*. PCC 6803, complete genome	212	216	98	9e-101
		dbj_BA000022.2	*Synechocystis sp*. PCC 6803 DNA, complete genome	212	216	98	9e-101
		gb_DQ398587.1	*Synechocystis sp*. AHZ-HB-MK glutaredoxin gene, partial cds	142	149	95	2e-58
	C02-R	gb-CP003265.1	*Synechocystis sp*. PCC 6803, complete genome	209	209	100	4e-104
		Dbj-BA000022.2	*Synechocystis sp*. PCC 6803 DNA, complete genome	209	209	100	4e-104
		gb-DQ398587.1	*Synechocystis sp*. AHZ-HB-MK glutaredoxin gene, partial cds	111	117	95	5e-43

sequence alignment was performed. Sequence alignment suggested that, the similarity ratio of consensus sequance for T_0 sambles (G5 and G7) were 94% and 100% respectively (Table 7 and Figs. 25 to 27). While the similarity ratio for T_1 samble (G2) was 94% for forward primer only with *Synechocystis* sp. PCC 6803, complete genome (Table 7 and Figs. 28 and 29).

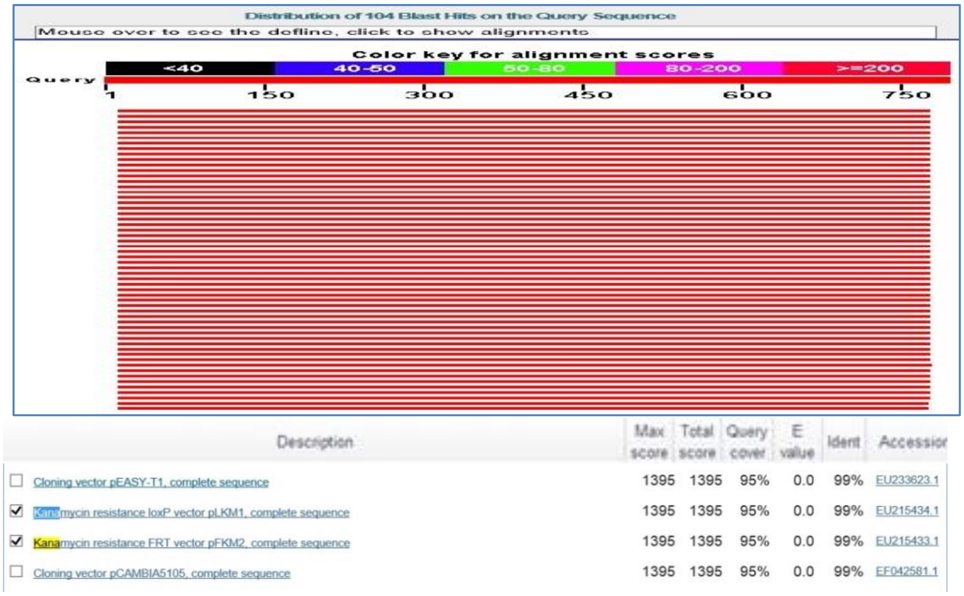
K4: 782bp

```
ACGAAGAGGCACCAGAGAGTGAAGGCACAAGACAAGAAAGAACTGAACCGAATC
GGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTT
TTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCG
CGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTT
GTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGAT
CTCCTGTCATCCCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCA
ATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCG
AAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAG
GATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGG
CTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCC
TGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGT
GGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGAT
ATTGCTAAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGT
ATCGCCGCTCCCGATGCGCAGCGCATCCCCTTCCACCGCCTTCTTGACGAGTTT
CTTCTGAAAACTTTTTTTCTTTTTTT
```

K9: 761bp

```
GCCGGGGGGGCTTTTTTATGAATTAAACCAGATGGAGCACCCAGGGTGGACCGA
CCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCT
GGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGG
AAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCA
CCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCA
TACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGA
GCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGA
AGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCAT
GCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATAT
CATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGT
GGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCT
TGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGA
TTCGCAGCGCTCGCTTATCGCCTTCTTGACGATTTCTTCTGAAAAAACCCCCCC
TTTCTTTCCCCCCCCTCTACCCCAGTTGTAAACATTTTATCCTTTAATTAAAAT
CCCTT
```

Fig. 21. Consensus sequence of *nptII* gene for T_0 plants.

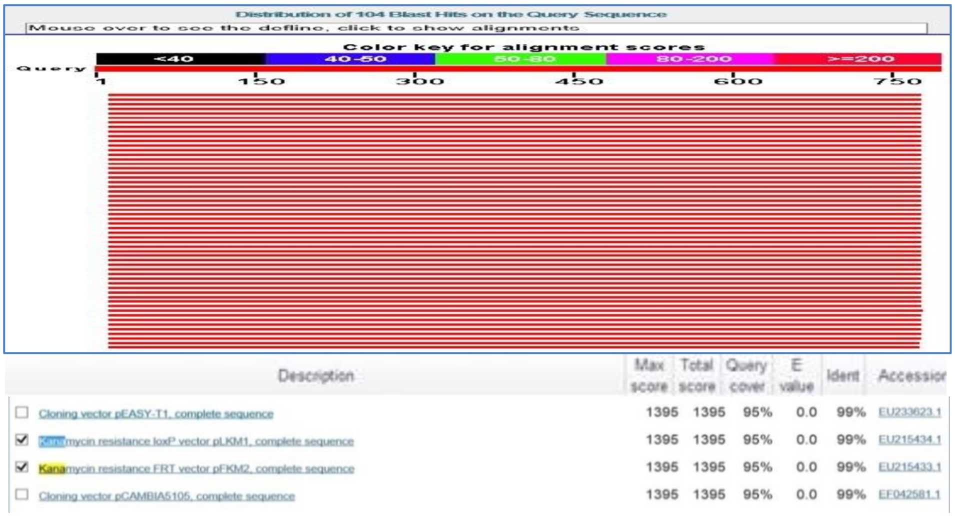


	Description	Max score	Total score	Query cover	E value	Ident	Accessior
☐	Cloning vector pEASY-T1, complete sequence	1395	1395	95%	0.0	99%	EU233623.1
☑	Kanamycin resistance loxP vector pLKM1, complete sequence	1395	1395	95%	0.0	99%	EU215434.1
☑	Kanamycin resistance FRT vector pFKM2, complete sequence	1395	1395	95%	0.0	99%	EU215433.1
☐	Cloning vector pCAMBIA5105, complete sequence	1395	1395	95%	0.0	99%	EF042581.1

Kanamycin resistance loxP vector pLKM1, complete sequence

```
Query  15    CGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGA  74
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2929  CGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGA  2988
Query  75    TGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCT  134
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2989  TGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCT  3048
Query  135   GTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGAC  194
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3049  GTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGAC  3108
Query  195   GGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCT  254
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3109  GGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCT  3168
Query  255   ATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCTCCTGCCGAGAAAGT  314
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3169  ATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCTCCTGCCGAGAAAGT  3228
Query  315   ATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATT  374
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3229  ATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATT  3288
Query  375   CGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGT  434
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3289  CGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGT  3348
Query  435   CGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAG  494
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3349  CGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAG  3408
Query  495   GCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTT  554
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3409  GCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTT  3468
Query  555   GCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGG  614
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3469  GCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGG  3528
Query  615   TGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGG  674
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3529  TGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGG  3588
Query  675   CGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCG  734
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3589  CGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCG  3648
Query  735   CATCGCCTTCTATCGCCTTCTTGACGAGTTTCTTCTGAA  773
|||||||||||||||||||||||||||||| ||||||||
Sbjct  3649  CATCGCCTTCTATCGCCTTCTTGACGAGTT-CTTCTGAA  3686
```

Fig. 22. Sequence alignment and identities of fragment *nptII* for T_0-K4 showing hits with kanamycin resistance loxP vector pLKM1, complete sequence.



	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Cloning vector pEASY-T1, complete sequence	1395	1395	95%	0.0	99%	EU233623.1
☑	Kanamycin resistance loxP vector pLKM1, complete sequence	1395	1395	95%	0.0	99%	EU215434.1
☑	Kanamycin resistance FRT vector pFKM2, complete sequence	1395	1395	95%	0.0	99%	EU215433.1
☐	Cloning vector pCAMBIA5105, complete sequence	1395	1395	95%	0.0	99%	EF042581.1

Kanamycin resistance FRT vector pFKM2, complete sequence

```
Query  8     TCT-CGGGCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGC-CAACAGACAATCGGC  65
             ||| ||| |||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  1384  TCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGC  1325
Query  66    TGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAG  125
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1324  TGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAG  1265
Query  126   ACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTG  185
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1264  ACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTG  1205
Query  186   GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGAC  245
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1204  GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGAC  1145
Query  246   TGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCTCCTGCC  305
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  TGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCCCACCTTGCTCCTGCC  1085
Query  306   GAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACC  365
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084  GAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACC  1025
Query  366   TGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCC  425
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1024  TGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCC  965
Query  426   GGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTG  485
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  964   GGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTG  905
Query  486   TTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGAT  545
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904   TTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGAT  845
Query  546   GCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC  605
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  844   GCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC  785
Query  606   CGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAA  665
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   CGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAA  725
Query  666   GAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGAT  725
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   GAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGAT  665
Query  726   TCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGATTTCTTCTGAA  771
             ||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  664   TCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAA  619
```

Fig. 23. **Sequence alignment and identities of fragment *nptII* for T_0-K9 showing hits with kanamycin resistance FRT vector pFKM2, complete sequence.**

gi|16329170:1050812-1051078 Glutaredoxin (GRX) *Synechocystis* sp. PCC 6803 chromosome, complete genome (NC_000911)

```
ATGGCTGTCTCGGCAAAAATTGAAATTTATACATGGAGCACTTGCCCTTTTTGC
ATGAGAGCCCTGGCTTTATTGAAACGTAAAGGAGTAGAGTTCCAAGAATATTGC
ATTGACGGCGACAACGAAGCAAGGGAAGCCATGGCGGCAAGGGCCAACGGCAAA
AGGAGCTTGCCCCAAATTTTTATTGACGACCAACACATTGGTGGCTGTGATGAC
```

Fig. 24. Sequence of Glutaredoxin-2 (*GRX-2*) gene.

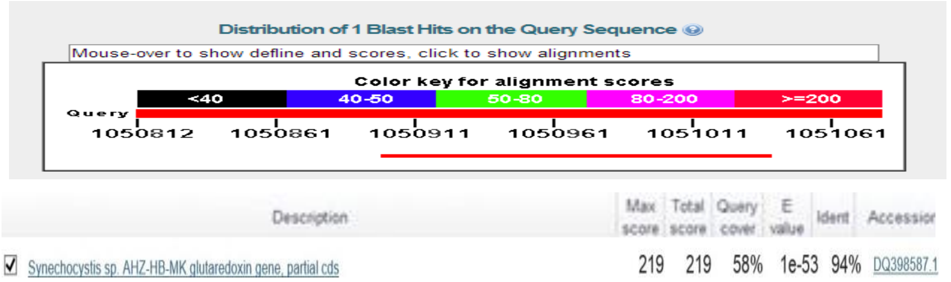
G5: 242 bp

```
GAATTGCAGATTTTCGTGAATCATAGAATATTAGAACGCACAAAGCGCCGATTA
TATTATAGTTCCAAGAATATTGCATTGACGGCGACAACGAAGCAAGGGAAGCCA
TGGCGGCAAGGGCCAACGGCAAAAGGAGCTTGCCCCAAATTTTTATTGACGACC
AACACATTGGTGGCTGTGATGACATCTATGCCCTAAGAGCGCAGAGGAAATATG
ACCCCTGCGACACCCAAACAGAAATG
```

G7: 145 bp

```
CTCAACGCATCGAGAACTAGACGCCATCAAAGAAACAATGGCTTAGTCAGATTA
GTAAACAACTTTATCACATTGGTGGCTGTGATGACATCTATGCCCTAGATGGTG
CAGGCAAGTTGGACCCCCTGCTCCATAGGCAGAAAAG
```

Fig. 25. Consensus sequence of *GRX-2* gene for T_0 plants.



	Description	Max score	Total score	Query cover	E value	Ident	Accession
☑	Synechocystis sp. AHZ-HB-MK glutaredoxin gene, partial cds	219	219	58%	1e-53	94%	DQ398587.1

***Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds**

```
Query  9     CTGC-CC-TCTTAGGGCATAGTATGTCATCACAGCCACCAATGTGTTGGTCGTCAATAAA  66
|||| || || |||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  145   CTGCACCATC-TAGGGCATAG-ATGTCATCACAGCCACCAATGTGTTGGTCGTCAATAAA  88
Query  67    AATTTGGGGCAAGCTCCTTTTGCCGTTGGCCCTTGCCGCCATGGCTTCCCTTGCTTCGTT  126
||||||||||||||||||| ||||||||||||||||||||||||| |||||||||||||
Sbjct  87    AATTTGGGGCAAGCTCCTTCTGCCGTTGGCCCTTGCCGCCATGGCCTCCCTTGCTTCGTA  28
Query  127   GTCGCCGTCAATGCAATATTCTTGGAA  153
||||| ||||||| ||||||||||||

Sbjct  27    ATCGCCATCAATGCTATATTCTTGGAA  1
```

Fig. 26. Sequence alignment and identities of fragment *GRX-2* gene of T_0-G5-showing hits with *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds.

Description	Max score	Total score	Query cover	E value	Ident	Accession
☑ Synechocystis sp. PCC 6803 DNA, complete genome	126	126	46%	5e-26	100%	BA000022.2
☑ Synechocystis sp. AHZ-HB-MK glutaredoxin gene, partial cds	93.5	93.5	34%	5e-16	100%	DQ398587.1

***Synechocystis* sp. PCC 6803 DNA, complete genome**

```
Query  9        CTGC-CC-TCTTAGGGCATAGTATGTCATCACAGCCACCAATGTGTTGGTCGTCAATAAA  66
                |||| || || |||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1051049  CTGCACCATC-TAGGGCATAG-ATGTCATCACAGCCACCAATGTGTTGGTCGTCAATAAA  1050992
Query  67       AATTTGGGGCAAGCTCCTTTTGCCGTTGGCCCTTGCCGCCATGGCTTCCCTTGCTTCGTT  126
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1050991  AATTTGGGGCAAGCTCCTTTTGCCGTTGGCCCTTGCCGCCATGGCTTCCCTTGCTTCGTT  1050932
Query  127      GTCGCCGTCAATGCAATATTCTTGGAACTCTACTCCTTTACGTTTCAATAAAGCCAGGGC  186
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1050931  GTCGCCGTCAATGCAATATTCTTGGAACTCTACTCCTTTACGTTTCAATAAAGCCAGGGC  1050872
Query  187      TCTCATGCAAAAAGGGCAAGTGCTCCATGTATAAATTTCAATTTTTGCCGAGACAGCCAT  246
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1050871  TCTCATGCAAAAAGGGCAAGTGCTCCATGTATAAATTTCAATTTTTGCCGAGACAGCCAT  1050812
```

***Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds**

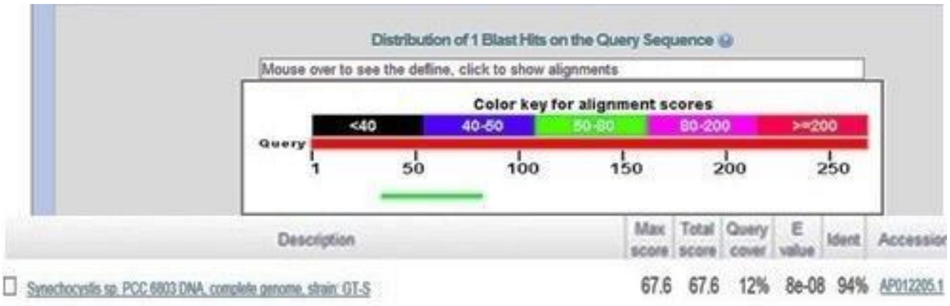
```
Query  9    CTGC-CC-TCTTAGGGCATAGTATGTCATCACAGCCACCAATGTGTTGGTCGTCAATAAA  66
            |||| || || |||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  145  CTGCACCATC-TAGGGCATAG-ATGTCATCACAGCCACCAATGTGTTGGTCGTCAATAAA  88
Query  67   AATTTGGGGCAAGCTCCTTTTGCCGTTGGCCCTTGCCGCCATGGCTTCCCTTGCTTCGTT  126
            ||||||||||||||||||| ||||||||||||||||||||||||| |||||||||||||
Sbjct  87   AATTTGGGGCAAGCTCCTTCTGCCGTTGGCCCTTGCCGCCATGGCCTCCCTTGCTTCGTA  28
Query  127  GTCGCCGTCAATGCAATATTCTTGGAA  153
             ||||| ||||||| ||||||||||||
Sbjct  27   ATCGCCATCAATGCTATATTCTTGGAA  1
```

Fig. 27. Sequence alignment and identities of fragment *GRX-2* gene of T_0-G7 showing hits with *Synechocystis* sp. PCC 6803, complete genome; and *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds.

G2-F: 341bp

```
GGAGCCTTGCCTTTTTGCTGAGAGCCCTGGCTTTATTGAAACGTAAGAACCATA
TACCCGAATTTGAATTGTTAGTAATCCGAAAAACACTCCCCATATGAGAGCAAG
AGAAAAATAAACTATTACCGTTTTTATATCTTTTTGTCATACAAAATTTAAACT
AAAAAACACCCCCCCATTTACTCTAACTGTGTTTCATATATATTTAATTTTTAT
ACCTAACACTTGCTTAAAAAAATCCCCCCGAAATACCCCACCCCCTTTTTTTTT
AATGCCGGTGTGCCTAAATTATTTGAGCTGAGAGGGTTCTTCTCGATGCCTAGA
GCGCCAAATAC
```

Fig. 28. DNA sequence of forward primer of *GRX-2* gene for T_1 plant.



Distribution of 1 Blast Hits on the Query Sequence

Mouse over to see the define, click to show alignments

☐	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Synechocystis sp. PCC 6803 DNA, complete genome, strain: GT-S	67.6	67.6	12%	8e-08	94%	AP012205.1

***Synechocystis* sp. PCC 6803 DNA, complete genome (Sequence ID: dbj|BA000022.2|)**

```
Query  9        AGC-CTTG-CCTTTTTGC-TGAGAGCCCTGGCTTTATTGAAACGTAA  52
                ||| |||| ||||||||| ||||||||||||||||||||||||||||
Sbjct  1050848  AGCACTTGCCCTTTTTGCATGAGAGCCCTGGCTTTATTGAAACGTAA  1050894
```

Fig. 29. Sequence alignment and identities of fragment *GRX-2* gene of T_1-G2-F showing hits with *Synechocystis* sp. PCC 6803 DNA, complete genome.

While, the results of T_2 plants as shown in Table 7 and Figs. 30 to 32 were highly similarity (98% and 100%) to *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene. In summary, the sequence similarity suggested the highest nucleotide sequence identity between inserted fragment and *GRX-2* gene.

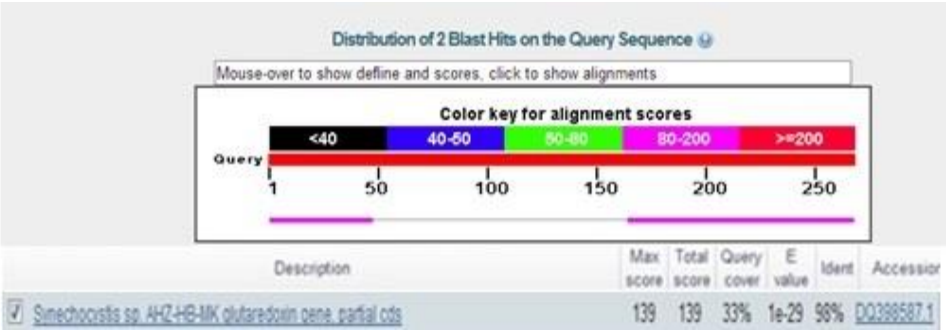
A02: 245bp

```
TTTGGCTGTCTCGGCAAAAATTGAAATTTATACATGGAGCACTTGCCCTGATTA
GTAAATTTATGAGCTTGCCCCAAATTTTTATTGACGACCAACACATTGGTGGCT
GTGAACACATCTATGCCCTAGATGGTGCAGGCAAGTTGGACCCCCTGCTCCATA
GTTAGAAGGAGATGTCTCACAGCCACAGGGAGGATGTCCTTAAATTGGGCAGTT
CTTTTGCCCTGCTTGGGTTTATTTTCTTT
```

C02: 268bp

```
TTATTGACGACCAACACATTGGTGGCTGTGATGACATCTATGCCCTAGATGGTG
CAGGCAATTGGACCCCCTGCTCCATATTAGAGAGACAGCCATATGGCTGTCTCG
GCAAAATTTGCCGAGACAGCCATATGGCTGTCTCGGCAATAATTGCCGAGACAG
CCATATGGCTGTCTCGGCAAATTTTGCCGAGACAGCCATATGGCTGTCTCGGCA
```

Fig. 30. Consensus sequence of *GRX-2* gene for T_2 plants.


Distribution of 2 Blast Hits on the Query Sequence

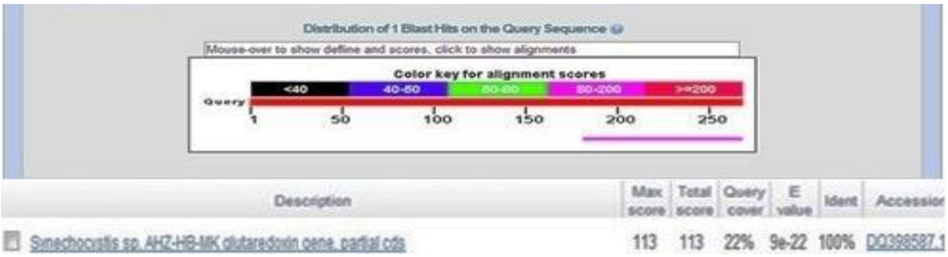
Mouse-over to show define and scores, click to show alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
☑ Synechocystis sp. AHZ-HB-MK glutaredoxin gene, partial cds	139	139	33%	1e-29	98%	DQ398587.1

***Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds**

```
Query  58   TTCCAAGAATATTGCATTGACGGCGACAACGAAGCAAGGGAAGCCATGGCGGCAAGGGCC  117
            |||||||||||| ||||||| |||||  ||||||||||||| ||||||||||||||||||
Sbjct  1    TTCCAAGAATATAGCATTGATGGCGATTACGAAGCAAGGGAGGCCATGGCGGCAAGGGCC  60
Query  118  AACGGCAAAAGGAGCTTGCCCCAAATTTTTATTGACGACCAACACATTGGTGGCTGTGAT  177
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AACGGCAGAAGGAGCTTGCCCCAAATTTTTATTGACGACCAACACATTGGTGGCTGTGAT  120
Query  178  GACATCTATGCCCTAGATGGTGCAGGCAAGTT  209
            ||||||||||||||||||||||||||||||||
Sbjct  121  GACATCTATGCCCTAGATGGTGCAGGCAAGTT  152
```

Fig. 31. Sequence alignment and identities of fragment *GRX-2* gene of T_2-A02-showing hits with *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds.


Distribution of 1 Blast Hits on the Query Sequence

Mouse-over to show define and scores, click to show alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
☐ Synechocystis sp. AHZ-HB-MK glutaredoxin gene, partial cds	113	113	22%	9e-22	100%	DQ398587.1

***Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds**

```
Query  26   ACAGCCACCAATGTGTTGGTCGTCAATAAAAATTTGGGGCAAGCTCCTTTTGCCGTTGGC  85
            ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  117  ACAGCCACCAATGTGTTGGTCGTCAATAAAAATTTGGGGCAAGCTCCTTCTGCCGTTGGC  58
Query  86   CCTTGCCGCCATGGCTTCCCTTGCTTCGTTGTCGCCGTCAATGCAATATTCTTGGAA  142
            ||||||||||||||| |||||||||||||  ||||| ||||||| ||||||||||||
Sbjct  57   CCTTGCCGCCATGGCCTCCCTTGCTTCGTAATCGCCATCAATGCTATATTCTTGGAA  1
```

Fig. 32. Sequence alignment and identities of fragment *GRX-2* gene of T_2-C02 showing hits with *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene, partial cds.

3. Dot blot hybridization

To examine the insertion and integration of *GRX-2* gene in tomato transgenic lines, genomic DNA from 7 T_2 transgenic lines and 2 non-transgenic plants were isolated, blotted, and hybridized with the

GRX-2 probe. The presence of transgenes in the T_2 plants was confirmed using dot blotting hybridization analysis. Dot blotting analysis indicated that *GRX-2* gene was inserted in 7 line genomes (Fig. 33).

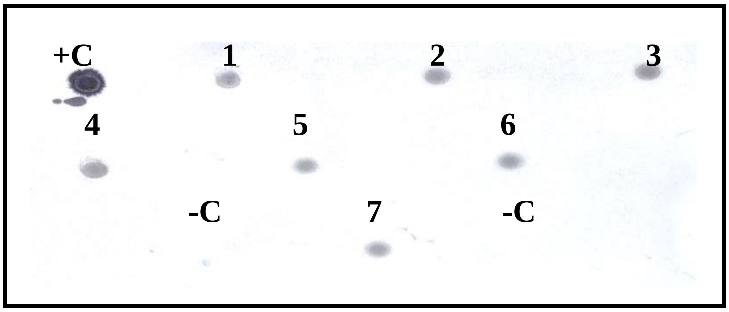


Fig. 33. Dot blot hybridization with *GRX-2* gene specific probe. Dot +C is positive control, dots -C are negative control (non-transgenic) and dots 1-7 are T_2 tomato transgenic plants.

4. RT-PCR

Total RNA was purified from T_1 and T_2 and negative control (Fig. 34A) using Biozal-total RNA reagents. Reverse transcription reaction was performed using first stand cDNA synthesis kit (Thermo Scientific Reverttid). First strand cDNA was used as a template for PCR amplification using the same set of *GRX-2* specific primers.

RT-PCR was used to test the expression of *GRX-2* mRNA messages in transgenic (T_1 and T_2) and a non-transformed plant was used as a negative control. The specific *GRX-2* primers successfully amplified fragment of 279 bp in transgenic plants. No signal was detected in the negative control (Fig. 34B). RT-PCR results indicating that the expression of *GRX-2* in both T_1 and T_2 plants.

These results are agreement with Guo *et al.* (2010) who found that RT-PCR analysis revealed that *SlGRX1* was expressed ubiquitously in tomato including leaf, root, stem and flower, and its expression could be induced by oxidative, drought, and salt stresses.

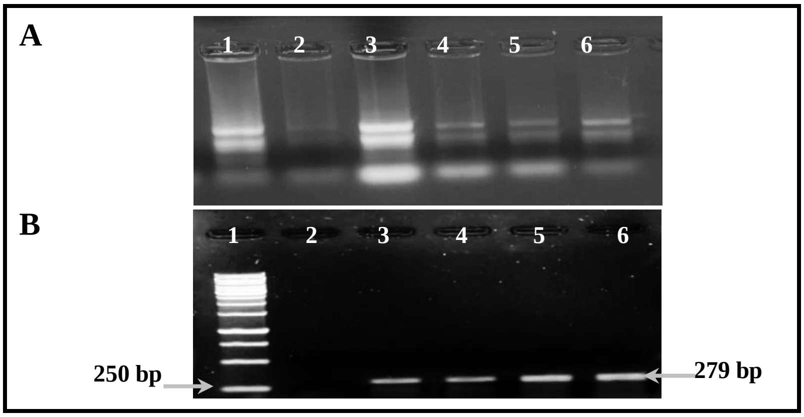


Fig. 34. RT-PCR confirmation of *GRX-2* expressing lines. A: RNA of non-transgenic plants (1-2), T_1 lines (3-4), and T_2 lines (5-6); B: RT-PCR product of *GRX-2* transgenic plants (1: 1kb DNA marker; 2: negative control; 3 and 4: cDNA from T_1 and 5 and 6: cDNA from T_2 plants).

3. Evaluating *GRX-2* transgenic tomato plants to salinity tolerance

Salt stress affects many aspects of plant metabolism and as a result, growth and yields are reduced. Excess salt in the soil solution may adversely affect plant growth either through osmotic inhibition of water uptake by roots or specific ion effects. Salinity impacts plants in two main ways: osmotic stress and ion toxicity (Munns and Termaat, 1986). Osmotic stress is caused by ions (mainly Na^+ and Cl^-) in the soil solution decreasing the availability of water to roots. Ion toxicity occurs when plant roots take up Na^+ and/or Cl^- ions and these ions are accumulated to detrimental levels in leaves.

Salinity stress results in a clear stunting of plant growth, which results in a considerable decrease in fresh and dry weights of leaves, stems and roots. Increasing salinity is also accompanied by significant reductions in shoot weight, plant height and root length (Greenway and Munns, 1980 and Munns and Termaat, 1986). Exposure of plants to salt stress usually begins in the roots. This leads to changes in growth, morphology and physiology of the root that will in turn change water and ion uptake and the production of signals that sends information to shoot. The whole plant is then affected when roots are growing in a salty medium. Tomato cultivars varied significantly in their response to different salinity levels. Increasing NaCl concentrations in nutrient solution adversely affect tomato shoots and roots, plant height (Foolad, 2004).

To assess the performance of *GRX-2* gene in transgenic lines under salinity stress, T_2 plants of three *GRX-2* transgenic lines and non-transgenic of cv. Castlerock (control) were subjected to different degrees of NaCl stress, *i.e.* 0, 100, 200, and 300 mM. Effects of salinity were studied on dry weight of the roots and leaves, plant height, and leaves contents of chlorophyll (SPAD value) and Na^+ percentage.

The visual symptoms of salt injury appeared after 2 weeks on the evaluated transgenic and non-transgenic plants. The symptoms on plants included chlorotic yellow and small sized leaves. Moreover, the plants that stopped growth became completely wilted. However, the moisture stress as judged by dropped leaves and then after few days the plants defoliated and died. With increasing salt concentration, the phenotypic differences between non-transgenic and transgenic plants became apparent (Fig. 35). Non-transgenic plants showed growth retardation at 200 mM and (Fig. 35A) and under 300 mM salt treatment the non-transgenic plants almost died. The transgenic plants on the other hand showed normal growth and phenotype with increasing level of salt stress (Figs. 35B and C).

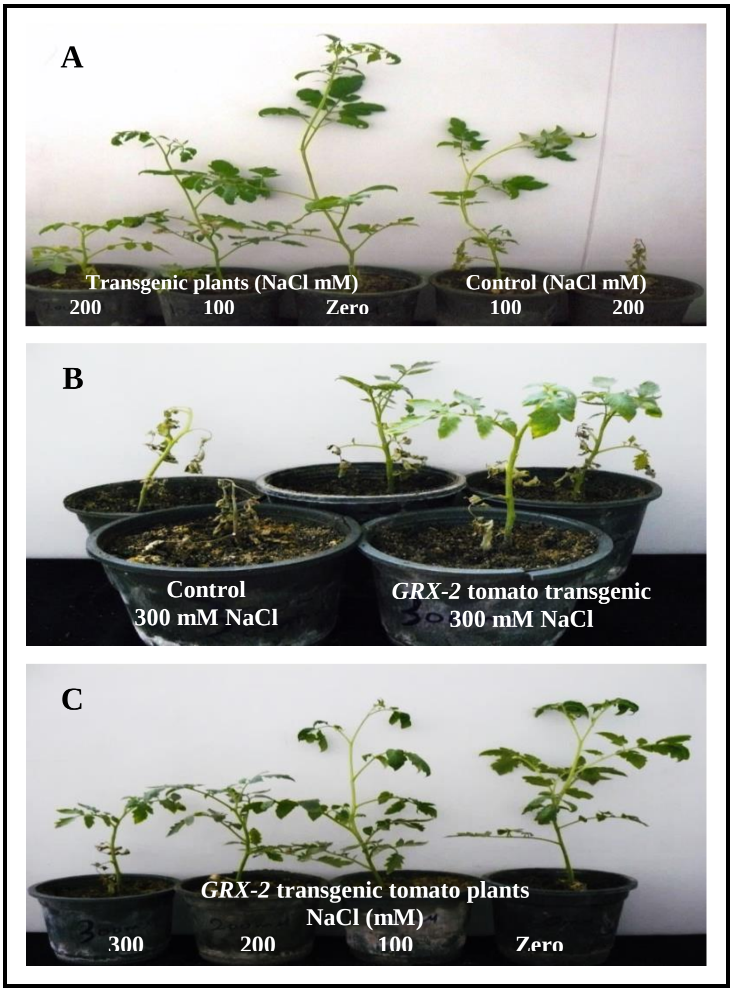


Fig. 35. Effects of NaCl stress on T_2 *GRX-2* transgenic lines and non-transgenic cv. Castlerock. A: Phenotype of transgenic and non-transgenic plants subjected to NaCl stress; B: Phenotype of transgenic plants at 300 mM NaCl as comparing with non-transgenic plants and B: Phenotype of transgenic plants with 0, 100, 200, and 300 mM NaCl.

The differences in plant height are presented in Table 8. The plant height of transgenic lines at different NaCl treatments was highly significant than non-transgenic plants and with increased NaCl level, the plant height of transgenic and non-transgenic plants was decreased (Fig. 35B). The data indicated that the expression of *GRX-2* gene in tomato leads to a vigorous change in plant height.

Table 8. Plant height; dry weight of root and leaves and leaves contents of chlorophyll and Na^+ for *GRX-2* transgenic tomato three lines and non-transgenic line, control, under different NaCl treatments.

NaCl treatment (mM)	Plant line	Plant height (cm)	Dry weight (g)		Chlorophyll (SPAD value)	Na^+ (%)
			Root	Leaves		
Zero	**T2-1**	42.47 a	0.127 e	0.48 f	44.53 ab	0.68 k
	T2-2	39.77 b	0.127 e	0.46 f	43.56 bc	0.91 j
	T2-3	38.80 c	0.133 de	0.44 f	45.03 a	0.74 k
	Control	39.40 b	0.133 de	0.47 f	44.78 a	0.96 j
100	**T2-1**	34.60 e	0.143 c-e	0.64 de	42.18 de	1.42 h
	T2-2	33.90 f	0.150 cd	0.68 cd	41.48 e	1.29 i
	T2-3	37.57 d	0.173 ab	0.62 de	43.01 cd	2.09 g
	Control	29.83 h	0.090 f	0.29 g	42.33 de	1.23 i
200	**T2-1**	32.43 g	0.160 bc	0.75 b	43.07 cd	2.59 e
	T2-2	27.57 j	0.183 a	0.67 cd	42.68 cd	2.56 e
	T2-3	28.43 i	0.180 ab	0.60 e	42.96 cd	2.21 f
	Control	24.60 k	0.090 f	0.31 g	40.11 f	2.02 g
300	**T2-1**	19.53 m	0.150 cd	0.67 cd	44.19 ab	3.09 b
	T2-2	21.37 l	0.170 ab	0.72 bc	43.69 bc	3.72 a
	T2-3	17.07 n	0.183 a	0.82 a	44.28 ab	2.98 c
	Control	13.37 o	0.057 g	0.30 g	38.86 g	2.70 d

[z] Values followed by a letter in common are not significantly different at the 0.05 level according to Duncan's multiple range test.

As a result of NaCl stress, leaves of non-transgenic plant became chlorotic yellow and small sized. Leaves content of chlorophyll was decreased from 44.78 (zero NaCl) to 38.86 (300 mM NaCl) with increase NaCl concentration. The data in Table 8 on chlorophyll content in leaves (SPAD value) shown that transgenic lines have high significant chlorophyll content in leaves compared to non-transgenic plants. These results indicate to the *GRX-2* gene enhanced plant growth of tomato under salt stress and also are agreement with those of Wu *et al*. (2012), who reported that ectopic expression of *AtGRX17* gene in tomato plants minimized photo-oxidation of chlorophyll and reduced oxidative damage of cell membrane system under heat stress. Also, Guo *et al.* (2010) found that silencing of *SlGRX1* in tomato led to increased sensitivity to oxidative and salt stresses with decreased relative chlorophyll content, and reduced tolerance to drought stress with decreased relative water content, while, over-expression of *SlGRX1* in *Arabidopsis* plants significantly increased resistance of plants to oxidative, drought, and salt stresses.

Effect of salt stress on the dry weight of roots and leaves of the evaluated plants appeared in Table 8 and Figs. 36 and 37. At the different levels of NaCl, dry weight of roots and leaves were high significant with *GRX-2* transgenic lines with comparing to non-transgenic cv. Castlerock. Also, dry weights of root and leaves of *GRX-2* transgenic plants were increased with increase NaCl level. Accordingly, the growth of *GRX-2*-expressing tomato plants was more tolerant to the same concentration of NaCl. After 2 weeks under different NaCl treatments, *GRX-2*-expressing tomato plants were significantly vigorous than non-transgenic plants (Fig. 35A).

Transgenic and non-transgenic plants of tomato were treated with different concentrations of NaCl and Na^+ concentration was measured in the plants. The results showed that, the Na^+ concentration was increased in *GRX-2* transgenic plants with comparing to non-transgenic plants. The Na^+ accumulated was increased in the transgenic plants with increased NaCl level, where, Na^+ content was ranged from

1.29-2.09% with 100mM NaCl and increased to 2.98-3.72% with 300mM NaCl.

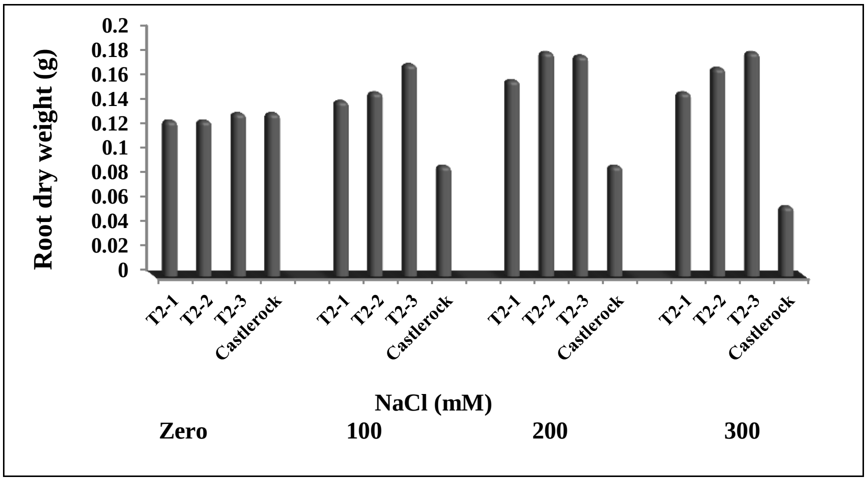


Fig. 36. Means of root dry weight of *GRX-2* transgenic lines and non-transgenic of cv. Castlerock under different NaCl levels.

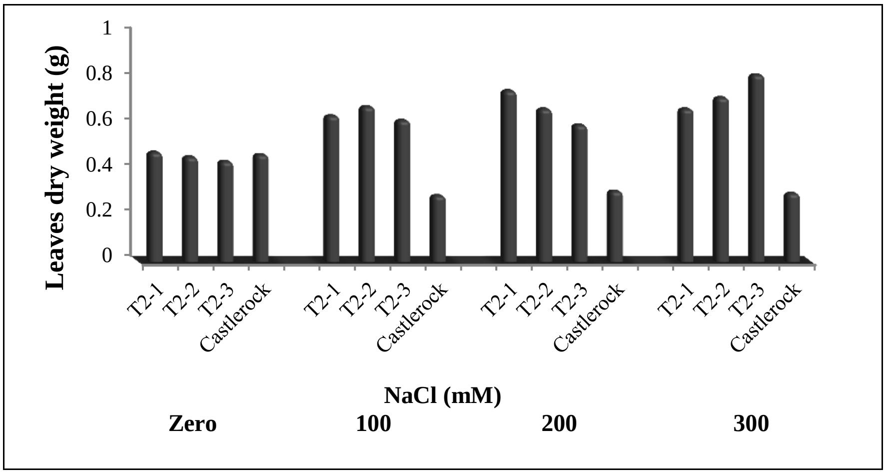


Fig. 37. Means of leaves dry weight of *GRX-2* transgenic lines and non-transgenic of cv. Castlerock under different NaCl levels.

The results of plant height, dry weight of roots and leaves, and leaves content of chlorophyll and Na^+ showed that overexpression of *GRX-2* gene significantly improved salt tolerance in the transgenic lines with comparing to non-transgenic cv. Castlerock. These results are agreement with Guo *et al.* (2010) who reported that the Grx gene *SIGRX1* from tomato plays an important role in regulating abiotic tolerance against oxidative, drought, and salt stress.

GRX, widely found in bacteria, plants, and mammalian cells, is an electron carrier for ribonucleotide reeducates and a general glutathione-disulfide reeducates of important for redox regulation. GRXs were previously described to have functions in controlling plant development, DNA synthesis, signaling and stress response, and [Fe-S] assembly (Cheng *et al.*, 2006; Cheng, 2008 and Rouhier *et al.*, 2004). Cyanobacterium *Synechocystis* strain PCC 6803 contains two genes *GRX-1* and *GRX-2* (Gaber *et al.*, 2006 and Li *et al*., 2005). Gaber *et al.* (2006) studied of transcript level of *GRX-2* in *Synechocystis* sp. PCC 6803 cell under different stress conditions, and cloned of the *GRX-2* gene of *Synechocystis* sp. PCC 6803 in the cytoplasm of *E. coli* and found that the transformed *E. coli* cells showed high tolerance to NaCl (over than 700mM). In this study, a *GRX-2* gene from *Synechocystis* sp. PCC 6803 was synthesized and cloning in plant vector to introducing it to tomato plant. This is the first report for the development of transgenic tomato plants harboring salt tolerance gene, *GRX-2*. Results of this study indicated that *GRX-2* plays a crucial role in salt stress and will provide useful information for studying the molecular mechanism of plant responses to environmental stresses and for genetically engineering of crops to improve their stress tolerance.

SUMMARY

This investigation was conducted during the period from 2008 to 2014 at Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt to improve tomato cultivars by using biotechnological techniques. Salt stress is one of the major abiotic stresses in the world, which causes significant alterations in both yield and quality in many crop species. The objectives of the study include:

1. Establishing a reliable regeneration system for tomato which can be used for introducing a foreign gene into tomato cells or tissues using *Agrobacterium*.
2. Developing transgenic tomato plants harboring *GRX-2* gene, which confer recipient plant tolerance to salinity.
3. Evaluating the transgenic tomato plants for salt tolerance using some analytical methods.

1. Tomato regeneration

Regeneration system has been established for five tomato cvs, *i.e.* Flora-Dade, Marmande, Summer Prolific, Castlerock and Super Strain B. In this study, two explant types, derived from cotyledons and hypocotyls, were isolated from seedlings of the 5 tomato cvs. One hundred and fifty hypocotyl segments and 180 cotyledon segments from each cv. were cultured on MS medium supplemented with different concentrations of plant growth regulators**.** Data were collected on the following criteria: callus induction frequency, shoot induction

frequency, No. of shoots per explant and No. of explants gave roots. Results obtained are summarized as follows:

a. Callus induction frequency

Callus induction frequency was significantly high of cotyledon explant. "Castlerock" and "Super Strain B" were significantly the highest of callus induction frequency.

With regard to the effect of the medium on callus induction frequency, using M-4 especially with hypocotyl explant produced the highest callus induction frequency followed by with cotyledon explant. This medium produced the highest callus induction frequency with "Super Strain B" followed with "Castlerock".

The interaction among explant type, cultivar and medium revealed that using hypocotyl of cv. Super Strain B with M-4 medium (6 mg/l BA) resulted in the highest percentage of callus induction frequency followed by using cotyledon explant of the same cv on the same medium.

b. Shoot induction frequency

There are significant differences between hypocotyl explants and cotyledon explants in shoot induction frequency, where cotyledon explants gave the highest percentage of shoot induction frequency.

Regarding cultivar effect, "Castlerock" had the highest percentage of shoot induction frequency followed by "Summer Prolific". With regard to the effect of medium, using M-4 significantly increased shoot induction frequency.

The interaction between explant type and cultivar revealed that using cotyledon explant of "Castlerock" followed of "Summer Prolific" had high percentage of shoot induction frequency.

Regarding the interaction between explant type and medium, culturing hypocotyl explant on M-4 led to increment in shoot induction frequency.

The interaction between tomato cultivars and media, data showed that shoot induction frequency was significantly the highest in "Castlerock" with M-4 followed by "Super Strain B" with the same medium.

With regard to the interactions among explant type, cultivar and medium, there were significant effect on shoot induction frequency. Using cotyledon of "Castelrock" as explant and culturing it on M-4 gave the highest percentage of shoot induction frequency followed by culturing hypocotyl explant of "Super Strain B" on the same medium.

c. Number of shoots per explant

Explant type had a significant effect on No. of shoots per explant. The highest No. of shoots per explant was achieved by using cotyledon explants. Also, all tomato cvs had a significant influence on No. of shoots per explant. "Summer Prolific" showed significantly highest number followed by "Flora-Dade", as compared with "Castelrock" and "Super Strain B".

Regarding the effect of the media on No. of shoots per explant, all used media had a significant effect on No. of shoots per explant, and the highest number was associated with M-5 (3.0 mg/l Kin + 0.3 mg/l IAA) followed with M-4.

The interaction between explant type and tomato cv was significant. The highest number of shoots per explant was shown with cotyledon explants of "Castelrock" and "super Strain B". Also, the interaction between the explant types and the media as well as between the cultivars and the media significantly affected No. of shoots per explant. Moreover, culturing cotyledon explant on the medium M-5 and culturing of "Flora-Dade" and on the medium M-5 increased No. of shoots per explant.

The interactions among explant types, tomato cultivars and the media revealed that culturing hypocotyl explant of "Flora-Dade" on the medium M-5 increased the No. of shoots per explant. Also, culturing cotyledon of "Super Strain B" on M-1 (2.5 mg/l BA + 1.0 mg/l IAA) and M-2 (1.0 mg/l BA + 0.2 mg/l IAA) increased the No. of shoots per explant.

d. Number of shoots giving roots

There are significant differences between hypocotyl and cotyledon explants in shoot induction frequency, where cotyledon explants gave the highest No. of explants that gave roots.

Regarding cultivar effect, "Flora-Dade" had the highest No. of explants that gave roots, followed by "Marmande". With regard to the effect of medium, using M-1 and M-2 significantly increased No. of explants that gave roots.

The interaction between explant type and cultivar revealed that using cotyledon explant of "Flora-Dade" had the highest No. of explants that gave roots.

The interaction between tomato cultivars and media, data showed that No. of explants that gave roots was significantly the highest in "Flora-Dade" by using M-2.

With regard to the interactions among explant type, cultivar and medium, there were significant effects on No. of explants that gave roots. Using cotyledon of "Flora-Dade" as explant and culturing it on M-2 gave the highest No. of explants that gave roots.

According to the presented data of regeneration experiment, it is observed that the cotyledon explants of "Castlerock" gave the best results of regeneration system on the M-4 medium, therefore, these conditions were selected for regeneration system in the following transformation experiment.

2. Tomato genetic transformation

Tomato cv. Castlerock was used in this experiment to improve its salinity tolerance by introducing cyanobacterium *Synechocystis sp*. PCC 6803 GRX (*GRX-2*) gene *via Agrobacterium* using *in planta* seed transformation technique and tissue culture transformation method.

a. Transformation of tomato cotyledon explants

Cotyledon explants of tomato cultivar Castlerock were wounded and infected with *A. tumeficiens* strain LBA4404 carrying the *GRX-2* and *nptII* genes in the pRI 101-ON DNA plasmid vector. Organogenic calli derived from cotyledon explants were selected on M-4 medium containing Kanamycin (100 mg/l) as a selective agent. T_0 plants that regenerated independently from these calli were selected as putative transgenic plants.

The presence of *GRX-2* and *nptII* genes in the putative transgenic plants of T_0 generation was screened by PCR using specific primers of transgenes. Although these plants gave an expected PCR amplicons (279 and 795 bp) for *GRX-2* and *nptII* genes, respectively no such amplicons was observed in untransformed (negative control) plants. It was recorded that only 44 out of 150 putatively transformed plants were confirmed to be transgenic, representing a percentage of 29.3%.

b. Transformation of tomato seeds

Tomato seeds were inoculated with avirulent *Agrobacterium* LBA4404 which contained binary plasmid pRI 101-ON DNA. Because of infection by the *Agrobacterium*, the germination percentage of infected seeds was 70% (35 germinated seeds of 50 infected seeds) compared with 86% (43 germinated seeds of 50 seeds) of non-infected seeds.

The transformation efficiency obtained through *in planta* transformation was much higher than conventional tissue culture-based transformation. According to number of positive plants determined by PCR analysis, the estimated transformation frequency for T_0 generation was 38 % (21 confirmed transgenic T_0 plants out of 55 tested plants).

c. Integration and expression of the *GRX-2* gene in transgenic tomato

Transgenic T_0 plants were maintained in greenhouse until maturity, selfed and produced fruits which contained viable seeds of transgenic generations $T_{1,}$ then T_2 were harvested.

1. PCR analysis

The inheritance of the transgenes into the T_0, T_1 and T_2 plants was confirmed by detection of *GRX-2* gene by PCR analysis. The results showed the integration of *GRX-2* gene into tomato genome.

2. DNA sequencing

In order to assure that the DNA bands from selective transformants at 795 bp and 279 bp are equivalent to DNA of the proposed *nptII* and *GRX-2* genes, respectively, sequencing was performed by Macrogen Company, Germany. DNA sequencing alignment the forward and reverase frame for T_0 plant with database of the *nptII* gene in Genebank was carried out. The results showed 99% similarity for kanamycin resistance, while, showed 96% and 98% (forward and reverse, respectively) similarity for complete genome of *Synechocystis* sp. PCC6803 and 94% and 93% (forward and reverse, respectively) similarity for *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene.

Also, the results of *GRX-2* T_1 plant showed 94% and 96% similarity for *Synechocystis* sp. PCC 6803 (forward and reverse, respectively) and 92% are similarity for *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene for reverse only.

While, the results of T_2 plants were highly similarity (up to 95%) to *Synechocystis* sp. PCC 6803 and *Synechocystis* sp. AHZ-HB-MK glutaredoxin gene. In summary, the comparison between consensus sequence of inserted fragment from different generations (T_0, T_1, and T_2) and synthesized *GRX-2* target gene sequence suggested the highest nucleotide sequence identity (94 - 100%) between inserted fragment and *GRX-2* gene.

3. Dot blot hybridization

The stable integration of the *GRX-2* gene into genome of the (T_2) plants was confirmed by dot blot hybridization analysis. Dot blotting hybridization analysis indicated that *GRX-2* gene was inserted in 7 line genomes.

4. RT-PCR analysis

To confirm the transgenic nature of tomato plants, RT-PCR technique was used. RT-PCR was performed with approximately 0.1 µg RNA as a template for cDNA synthesis. Following the linear phase of DNA amplification (30 cycles), the PCR products were examined by electrophoresis in 1% agarose gel. The results showed that, T_1 and T_2 plants expressed the gene of interest (*GRX-2*).

3. Evaluating *GRX-2* transgenic tomato plants to salinity tolerance

To assess the performance of *GRX-2* gene in transgenic lines under salinity stress, T_2 plants of three *GRX-2* transgenic lines and wild type of cv. Castlerock were subjected to different degrees of NaCl stress, *i.e.* 0, 100, 200, and 300 mM. The results showed that, with increasing salt concentration, the phenotypic differences between wild type and transgenic plants became apparent. Wild type of cv. Castlerock (non-transgenic, control) plants showed growth retardation at 200 mM and under 300 mM salt treatment the wild type plants almost died. The transgenic plants on the other hand showed normal growth and phenotype with increasing level of salt stress. The plant height of transgenic and non-transgenic plants was decreased with increased NaCl level, while, roots and leaves dry weight, chlorophyll and Na^+ content were increased compairing with non-transgenic plants.

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الملخص العربى

نقل و تعبير جين الجلوتاريدوكسين-2 إلى نباتات الطماطم

اجريت هذه الدراسة خلال الفترة من ٢٠٠٨-٢٠١٤ فى قسم الوراثة – كلية الزراعة – جامعة القاهرة، و ذلك بهدف تحسين أصناف الطماطم من خلال استخدام تقنيات التكنولوجيا الحيوية. و قد أشتملت هذه الدراسة على الأتى:

١- تطوير نظام استيلاد فى الطماطم يمكن استخدامه لنقل الجينات لنباتات الطماطم من خلال بكتريا الأجروباكتريم.

٢- تطوير نباتات طماطم محوره وراثياً تحمل جين الجلوتاريدوكسين-٢(*GRX-2*) لأكساب النباتات التحمل للملوحة.

٣- تقييم نباتات الطماطم المحوره لتحمل الملوحة باستخدام طرق تحليليه مختلفة.

١- إستيلاد الطماطم

تم اختبار خمسة بيئات على الاستيلاد من أجزاء نباتية من الأوراق الفلقية و السويقة الجنينية العليا لخمسة أصناف من الطماطم (كاسل روك، و فلوراديد، و مارموند، و سمربروليفك و سوبر سترين بى) المنزرعة على بيئة MS المحتويه علي منظمات نمو مختلفة و كانت النتائج كالتالى:

أ- نسبة تكوين الكالوس

نسبة تكوين الكالوس كانت عالية المعنويه مع الأجزاء النباتية من الأوراق الفلقية، و كان الصنفين "كاسل روك" و "سوبر استرين بى" أحسن الأصناف من حيث نسبة تكوين الكالوس.

بالنسبة لتأثير البيئات، كانت البيئة ٤ (٦ مجم BA /لتر) و خاصة مع السويقة الجنينية العليا أعلى من حيث نسبة تكوين الكالوس، و أعطت تلك البيئة أعلى نسبة مع الصنف " سوبر استرين بى " يليه الصنف "كاسل روك".

بخصوص التفاعل بين الجزء النباتى، و الصنف، و البيئة، ظهرت أعلى نسبة من تكون الكالس عند زراعة السويقة الجنينية العليا للصنف "سوبر استرين بى" على البيئة ٤، تلاها زراعة الأوراق الفلقية لنفس الصنف على نفس البيئة.

ب- نسبة تكوين الأفرع الخضرية

وجدت إختلافات معنوية بين كلا من الأجزاء النباتية المستخدمة فى نسبة تكوين الأفرع الخضرية، و أعطت الأوراق الفلقية أعلى نسبة. أما بالنسبة للصنف، فقد أعطى الصنف "كاسل روك" أعلى نسبة من تكون الأفرع الخضرية تلاه الصنف " سمربروليفك "، أما بخصوص البيئات فظهرت أعلى نسبة من تكون الأفرع مع البيئة ٤.

كان التفاعل الثنائى بين الثلاث عوامل محل الدراسة ذو تأثير معنوى على نسبة تكون الأفرع الخضرية ، و ظهرت أعلى نسبة عند زراعة السويقة الجنينية العليا على البيئة ٤، وزراعة الصنفين "كاسل روك" و "سوبر استرين بى" على نفس البيئة.

بالنسبة للتفاعل الثلاثى بين الجزء النباتى، و الصنف، والبيئة فكان ذو تأثير معنوى على نسبة تكون الأفرع الخضرية، و ظهرت أعلى نسبة عند زراعة الأوراق الفلقية للصنف "كاسل روك" على البيئة ٤، تلاها زراعة السويقة الجنينية العليا للصنف "سوبر استرين بى" على نفس البيئة.

جـ- عدد الأفرع الخضرية المتكونة لكل جزء نباتى

كان هناك تأثير معنوي للجزء النباتي المستخدم على عدد الأفرع الخضرية المتكونة لكل جزء نباتى، و ظهر أكبر عدد مع الأوراق الفلقية. أيضاً، كان هناك تأثير معنوى للصنف على العدد المتكون من الأفرع الخضرية لكل جزء نباتى، و أعطى الصنف "سمر بروليفيك" أكبر عدد تلاه الصنف "فلوراديد". أما بالنسبة للبيئة فكان لها ايضاً تأثير معنوى و أعطت البيئة ٥ (٣ مجم Kin/لتر + ٠,٣ مجم IAA/لتر) أكبر عدد من الأفرع الخضرية تلاها البيئة ٤.

كان التفاعل الثنائى بين الثلاث عوامل محل الدراسة ذو تأثير معنوى على عدد الأفرع الخضرية المتكونة لكل جزء نباتى، و ظهر أكبر عدد عند استخدام الأوراق الفلقية

للصنفين "كاسل روك" و "سوبر استرين بى"، و أيضاً ظهر عند زراعة الأوراق الفلقية على البيئة ٥، و استخدام الصنف "فلوراديد" مع البيئة ٥.

بالنسبة للتفاعل الثلاثى بين الجزء النباتى، و الصنف، والبيئة فكان ذو تأثير معنوى على العدد المتكون من الأفرع الخضرية لكل جزء نباتى، و أعطت السويقة الجنينية العليا للصنف "فلوراديد" المنزرعة على البيئة ٥ أكبر عدد، أيضاً زراعة الأوراق الفلقية للصنف "سوبر استرين بى" على البيئة ١ (٢,٥ مجم BA/لتر + ١ مجم IAA/لتر) و على البيئة ٢ (١ مجم BA/لتر + ٠,٢ مجم IAA/لتر) اعطى عدد كبير من الأفرع الخضرية لكل جزء نباتى.

د- عدد الأفرع الخضرية المعطية للجذور

تأثر عدد الأفرع الخضرية المعطية للجذور بالجزء النباتى المنزرع و أعطت الأوراق الفلقية اكبر عدد، و أيضاً كان للصنف تأثير معنوى على عدد الأفرع الخضرية المعطية للجذور و أعطى الصنف "فلوراديد" أكبر عدد تلاه الصنف "مارمند". أما البيئات فكان لها أيضاً تأير معنوى و أعطت البيئات ١ و ٢ أكبر عدد.

كان التفاعل الثنائى بين الثلاث عوامل محل الدراسة ذو تأثير معنوى على عدد الأفرع الخضرية المعطية للجذور، وظهر أكبر عدد مع استخدام الأوراق الفلقية للصنف "فلوراديد"، و عند زراعة نفس الصنف على البيئة ٢.

بالنسبة للتفاعل الثلاثى بين الجزء النباتى، و الصنف، والبيئة فكان ذو تأثير معنوى على عدد الأفرع الخضرية المعطية للجذور، و ظهر اكبر عدد عند زراعة الأوراق الفلقية للصنف "فلوراديد" على البيئة ٢.

وفقاً للنتائج السابقة، أختير الصنف كاسل روك على ان تستخدم الأوراق الفلقية منه و تزرع على البيئة ٤ نظراً لأعطائهم أحسن النتائج مع ذلك الصنف، و ذلك فى تجربة النقل الوراثى المعتمدة على زراعة الأنسجة النباتية.

٢. التحول الوراثى للطماطم

تم وضع نظام للتحول الوراثى لجين الجلوتاريدوكسين-٢ المعزول من بكتريا السيانوباكتريم *Synechocystis* sp. PCC 6803 فى الطماطم وقد استخدم صنف الطماطم

"كاسل روك"، و سلالة البكتريا *Agrobacterium tumefaciens* LBA4404 الحاملة للبلازميد pRI 101 المحتوى على جينات الـ *nptII* و الـ *GRX-2*. حيث تم اجراء العدوى بسلالة البكتريا لكل من الأوراق الفلقية النامية على البيئة المغذية و البذور، و تم تقدير كفاءة التحول الوراثى لكلاً منهما أعتماداً على تقدير عدد النباتات المحوره وراثياً و المكتشفة من خلال اختبار الـ PCR باستخدام المعلمات الجزيئية المتخصصة للجينات *nptII*، و *GRX-2*.

كانت كفاءة التحول الوراثى فى طريقة التحول التقليدية ٢٩,٣%، بينما كفاءة التحول الوراثى فى طريقة عدوى البذور فكانت أعلى و قدرت بـ ٣٨%.

أ- اكتشاف جين *GRX-2* و تحديد تعبيره فى نباتات الطماطم المحوره وراثياً

نباتات الجيل T_0 زرعت فى الصوبة، مع الإعتناء بها و تركها للتلقيح الذاتى للحصول منها على بذور كلا من الجيل T_1، الجيل T_2 التى استخلصت من الثمار و زرعت لأكتشاف الجين *GRX-2* بها و تقدير تعبيره فى النباتات النامية لـ T_1، T_2.

١- تفاعل البلمرة المتسلسل (PCR)

تم اثبات دخول واندماج جين الـ *GRX-2* فى جينوم نباتات الطماطم المحوره وراثيا للاجيال المختلفة (T_0،T_1،T_2) بواسطة استخدام اختبار ا لـ PCR.

٢- تتابع الدنا

تم إجراء تحليل لبندات الدنا الناتجة فى اختبار الـ PCR مع المعلمات الجزيئية المتخصصة للجينات *GRX-2* و *nptII*، لمعرفة تتابع القواعد النيتروجينية لهذه البندات و بمطابقة النتائج المتحصل عليها مع نتائج القواعد الجينية الموجودة ببنك الجينات تبين ان هناك تطابق بنسبه ٩٩% للجين *nptII* مع جين المقاومة للكاناميسين المعرف ببنك الجينات، و بنسبة تراوحت بين ٩٤-١٠٠% لجين *GRX-2* مع الجينوم الكامل للبكتريا *Synechocystis* sp. PCC 6803، و تراوحت بين ٩٢-٩٦% لجين الجلوتاريدوكسين لسلالة البكتريا *Synechocystis* sp. AHZ-HB-MK الموجود ببنك الجينات. و بمقارنة التتابع الاصلى لجين الجلوتاريدوكسين-٢ (*GRX-2*) الذى تم تخليقه بتتابع المقطع من الـ DNA الذى تم ادخالة فى الطماطم ظهرت نسبه تشابه مرتفعة تراوحت بين ٩٤-١٠٠% فى الاجيال المختلفة مع جين الجلوتاريدونسين-٢.

٣- تحليل Dot blot

تم التأكد من وجود جين الـ *GRX-2* فى النباتات المحوره وراثيا باستخدام تقنية الـ Dot blot.

٤- تحليل RT-PCR

تأكد نقل جين الـ *GRX-2* لنباتات الطماطم و تعبيره بها من خلال إجراء اختبار RT-PCR.

٣- تقييم مدى تحمل النباتات المحوره وراثيا للاجهادات الملحية

لتقييم مدى تحمل نباتات الطماطم المحوره وراثيا للاجهادات الملحية تم تعريض النباتات لتركيزات مختلفة من كلوريد الصوديوم. وقد اظهرت النتائج ما يلى:

تأثر النباتات الغير محوره وراثيا بالتعرض للتركيز ٢٠٠ ملمولرمن حيث الذبول واصفرار الاوراق بينما التعرض للتركيز ٣٠٠ ملمولرأدى الى موت النباتات وذلك بعكس النباتات المحوره وراثيا والتى اظهرت نموا عاديا. وجد ان طول النبات يتناقص بزيادة التركيز الملحى فى حين ان الوزن الجاف لكلا من الاوراق و الجذور و كذلك تركيز الصوديوم ومحتوى الكلوروفيل فى النباتات المحوره وراثيا يتزايد بزيادة التركيز الملحى بالمقارنة مع النباتات الغير محوره وراثيا.

نقل و تعبير جين الجلوتاريدوكسين-2 إلى نباتات الطماطم

رسالة مقدمة من

نعمه حسين عثمان عبدالعاطى
بكالوريوس في العلوم الزراعية (التكنولوجيا الحيوية)- كلية الزراعة - جامعة القاهرة، ٢٠٠١
ماجستير فى العلوم الزراعية (وراثة) – كلية الزراعة – جامعة القاهرة، ٢٠٠٧

للحصول على درجة

دكتوراه الفلسفة

في

العلوم الزراعية
(وراثة)

قسم الوراثة
كلية الزراعة
جامعة القاهرة
مصر

٢٠١٤

نقل و تعبير جين الجلوتاريدوكسين-2 إلى نباتات الطماطم

رسالة دكتوراه الفلسفة
فى العلوم الزراعية
(وراثة)

مقدمة من

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التاريخ ٣٠ / ٨ / ٢٠١٤

نقل و تعبير جين الجلوتاريدوكسين-2 إلى نباتات الطماطم

رسالة دكتوراه الفلسفة
فى العلوم الزراعية
(وراثة)

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اسم الطالب: نعمه حسين عثمان عبدالعاطى **الدرجة:** دكتوراه الفلسفة
عنوان الرسالة: نقل و تعبير جين الجلوتاريدوكسين-2 إلى نباتات الطماطم
المشرفون : دكتور: منى هاشم أحمد حسين
دكتور: محمد حسنين سليمان
دكتور: بسيطة عباس حسين
قسم: الوراثة **تاريخ منح الدرجة:** ٣٠ / ٨ / ٢٠١٤

المستخلص العربي

أجريت هذه الدراسة فى قسم الوراثة – كلية الزراعة – جامعة القاهرة خلال الفترة من ٢٠٠٨ إلى ٢٠١٤ و ذلك بهدف تحسين تحمل الطماطم للملوحه من خلال تقنيات التكنولوجيا الحيوية، و كان ذلك من خلال الحصول على نباتات طماطم محورة وراثياً تشفر لجين الجلوتاريدوكسين (*GRX-2*) لأكساب النباتات التحمل للملوحه. فى التجربة الأولى، تم اختبار تأثير خمس تركيزات مختلفه لمنظمات النمو على الاستيلاد من الأوراق الفلقية و السويقة الجنينية العليا لخمسة أصناف من الطماطم (كاسل روك، و فلوراديد، و مارموند، و سمربروليفك، و سوبر سترين بى) المنزرعة على بيئة MS. فأظهرت النتائج أن هناك تأثيرا معنويا لتركيز منظم النمو BA و الأصناف على نسب تكوين الكالوس، و تكوين الأفرع، و الاستيلاد فى كلا النوعين من الأجزاء النباتية المنزرعة. و كان أحسن الأصناف، الصنف كاسل روك النامى على تركيز ٦ مجم/لتر. فى التجربه الثانية تم وضع نظام للتحول الوراثى لجين الجلوتاريدوكسين فى الطماطم و ذلك باستخدم صنف الطماطم كاسل روك و سلالة البكتريا *Agrobacterium tumefaciens* LBA4404 الحاملة للبلازميد pRI 101 المحتوى على جينات الـ *nptII* و الـ *GRX-2*. تم عدوى كلا من الأوراق الفلقية مع استخدام نظام الإستيلاد المختار من التجربة الأولى و البذور بسلالة الأجروباكتريم الحاملة للبلازميد المحتوى على الجين، تم تأكيد إندماج الجينين المنقولين في جينوم نباتات الطماطم المحوره وراثيا باستخدام تفاعل البلمره المتسلسل PCR بواسطة بادئات خاصة لكل جين على حدى، وتم التأكد من وجود جين *GRX-2* فى النباتات المحورة وراثيا باستخدام تقنية الـ Dot blot وايضا تم التأكد للتعبير الجينى لجين *GRX-2* باستخدام تفاعل RT- PCR، وقد امكن تقييم مدى تحمل نباتات الطماطم المحوره وراثيا للملوحة حيث تم تعريض نباتات الجيل الثانى لتركيزات مختلفة من كلوريد الصوديوم (صفر، و ١٠٠، و ٢٠٠، و ٣٠٠ ملمولر). وتم تقدير كلا من الوزن الجاف وطول النبات وقياس تركيز عنصر الصوديوم وملاحظة التغير فى محتوى الاوراق من الكلوروفيل. وقد اظهرت النتائج المتحصل عليها من تحمل النباتات المحوره وراثيا للاجهاد الملحى. و تعتبرهذه خطوة هامه في انتاج نباتات طماطم متحمله للملوحه.

الكلمات الدالة: الطماطم، النقل الوراثى، الإستيلاد، الجلوتاريدوكسين، الملوحة.