REPUBLIC OF TURKEY
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF SCIENCES

CLONING OF OLIVE *OeSRC1* AND COMMON BEAN *DEHYDRIN LEA* GENES AND FUNCTIONAL EVALUATION IN TOBACCO PLANTS

A THESIS SUBMITTED TO THE DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (MSc)

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12210261

FEBRUARY 2016
SAMSUN

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MASTER OF SCIENCE (MSc) THESIS

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Date of Examination : 02.02.2016

SUPERVISED BY: Asst. Prof. Dr. Musa KAVAS

This thesis was supported by the Project management Office of Ondokuz Mayıs University (Project number:PYO.ZRT.1904.15.010)

Ondokuz Mayıs University Institute of Sciences

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ACKNOWLEDGEMENTS

For to us a child is born, to us a son is given, and the government will be on his shoulders. And he will be called Wonderful Counselor, Mighty God, Everlasting Father, Prince of Peace (Holy Bible). Indeed my life has been an embodiment of your abundant graces and mercies oh, LORD. Thank you for the ultimate sacrifice. I am highly grateful.

Secondly, I would like to thank Assistant Professor Doctor Musa KAVAS for his immense support and fatherly guidance during the course of this work. To you I say May the LORD ALMIGHTY bless you beyond human comprehension.

I am grateful to YTB for awarding me a scholarship and affording me the opportunity to study in the beautiful country of Turkey.I would also like to thank the faculty members and the entire student body of the department of Agricultural Biotechnology for the extraordinary brotherliness they showed towards me. I admit I enjoyed every moment I shared with you and I strongly believe I will cherish these memories for a very long time.

Special thanks also go to my work mates Elif Seda ATABAY and Gökhan GÖKDEMİR. I pray that the LORD will shine his light on your paths and cause you to excel. My heartfelt gratitude also goes to each and every one who in one way or another contributed to the success of this work. To all I say you are so precious without you I would not have been here. May my God bless you all in Jesus' name.

February, 2016 ADUSE POKU SAMUEL.

DEDICATION

This piece of work is dedicated to my best friend,THE COMFORTER.

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ABBREVIATIONS

PSII	Photosystem II
FAO	Food and Agricultural Organisation
LEA	Late Embryogenesis Abundance
ROS	Reactive Oxygen Species
Hsp	Heat Shock Proteins
FAD	Fatty Acid desaturase
BLT	Barley Low Temperature
PEG	Polyethylene glycol
NaCl	Sodium chloride
HptII	*Hygromycin phosphotransferase* gene
MS	Murashige-Skoog basal salt medium
NAA	Naphthalene acetic acid
BA	Benzylaminopurine
YEB	Yeast Extract Broth
PCR	Polymerase Chain Reaction
LB	Luria Broth

ZEYTİN *OESRC1* VE FASULYE *DEHİDRİN LEA* GENLERİNİN KLONLANMASI VE TÜTÜN BİTKİSİNDE FONKSİYONEL KARAKTERİZASYONU.

ÖZET

Kültüre alınan en eski bitkilerden biri olan *Olea europaea* L., Oleaceae familyasında yer alan ve 20-25 tür barındıran bir cinstir (Zohary *et al*., 2000). Herdem yeşil, yavaş gelişim gösteren ve kuraklık stresine dayanıklı bir tür olan zeytin ağacı, 500 yıla kadar sürebilen çok uzun bir ömre sahiptir (Zohary *et al*., 2000). Fasulye (*Phaseolus vulgaris* L.) dünyada yetiştiriciliği en yaygın düzeyde yapılan bitkilerden biridir ve insanlarca tüketilen en önemli baklagillerdendir (CIAT, 2001, Emam *et al*. 2010). Zeytin ve fasulye, dünya genelinde düşük gelirli kesimler için zengin besin kaynağı oluşturan ekonomik açıdan öneme sahip bitkilerdir. Zeytin ve fasulye üretimi düşük ve yüksek sıcaklık, tuzluluk, kuraklık, sel, oksidatif stres ve ağır metal toksisitesi gibi çeşitli abiyotik stres etmenlerince sınırlandırılabilmektedir. İklim değişimine bağlı olarak iklim koşullarının sürekli olarak kötüye gidişi sonucunda, yukarıda sözü edilen stres etmenlerinin zeytin ve fasulye bitkileri üzerindeki etkilerinin daha da kötü olacağı tahmin edilmektedir. Bu nedenle bu bitkilere abiyotik stres etmenlerine karşı dayanıklılık kazandırabilecek aday genlerin belirlenmesine yönelik çalışmalar önem arz etmektedir. Bu çalışmanın amacı da; sırasıyla fasulye (*Phaseolus vulgaris* L.) ve zeytin (*Olea europaea* L.) transkriptom belirleme çalışmaları sırasında bulunmuş olan "*LEA-dehydrin* (late embryogenesis abundant-dehydrin)" ve "*OeSRC1*" adlı iki yeni dehidrin benzeri genin başarılı bir şekilde klonlanması ve bu genlerin işlevlerinin tütün bitkisi üzerinde araştırılmasıdır. Hedef genlerin klonlanması "gateway klonlama sistemi" ile gerçekleştirilmiştir. Genler daha sonra tütün yaprak disklerine transforme edilmiştir. Kuraklık ve tuzluluk stresi testleri yalnızca *OeSRC1* hatları üzerinde gerçekleştirilebilmiştir. Çalışma sonucunda *OeSRC1* transgenik hatlarının yabani tip bitkilere kıyasla, kuraklık ve tuzluluk stresi koşulları altında daha iyi geliştiği ve fizyolojik performans sergilediği gözlemlenmiştir.

Anahtar Kelimeler: *OeSRC1*, *LEA-dehydrin,* fasulye, tütün transformasyonu

CLONING OF OLIVE *OeSRC1* AND COMMON BEAN *DEHYDRIN LEA* GENES AND FUNCTIONAL EVALUATION IN TOBACCO PLANTS

ABSTRACT

Olea europaea L. belongs to a genus of about 20-25 species in the family Oleaceae (Zohary *et al*., 2000) and it is one of the earliest cultivated plants. The olive tree is an evergreen, slow-growing species, tolerant to drought stress and extremely long-lived, with a life expectancy of about 500 years (Zohary *et al*., 2000). Common bean (*Phaseolus vulgaris L*.) is one of the widely cultivated plants in the world. It is considered as one of the most important legumes for human consumption (CIAT, 2001, Emam *et al*., 2010). Olive and common bean are economically important plants that serve as affordable rich sources of nutrients for most poor households around the World. Olive and common bean productions are often limited by a host of abiotic stress factors such as low temperatures, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. With the continuous deterioration of the climate due to the global phenomenon of climate change, the effects of the aforementioned stress factors on the olive and common bean plant are expected to worsen even further. There is therefore the urgent need for research aimed at identifying candidate genes that can confer resistance to abiotic stresses in these plants. The aims of this study was therefore to successfully clone two novel dehydrin-like genes; *LEA-dehydrin* (late embryogenesis abundant-dehydrin) and *OeSRC1* identified during the transriptome profiling of common bean (*Phaseolus vulgaris* L.) and olive (*Olea europaea* L.) respectively and the investigation of the functions of these genes in tobacco plants. The cloning of our genes of interest was achieved with the gateway cloning system. Tobacco leaf discs were afterwards successfully transformed with our genes of interest by means of the *Agrobacterium*-mediated transformation method. Drought and salt stress test were only carried out on *OeSRC1* transgenic lines. It was observed at the end of the study that *OeSRC1* transgenic lines displayed better growth and physiological performances compared to wild-type plants when grown under drought and salt stressed conditions.

Keywords: *OeSRC1*, *LEA-dehydrin*, common bean, tobacco plant transformation.

1 INTRODUCTION

1.1 Stresses

Plants are often exposed to a host of abiotic stress factors such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. In addition to abiotic stress factors, bacteria, fungi, and viruses as well as herbivores cause a lot of havoc to plants. These stress conditions are detrimental to plants and do not allow them them to attain their full genetic potential by limiting crop productivity worldwide (Bray *et al*., 2000).

Olea europaea L. belongs to a genus of about 20-25 species in the family Oleaceae and it is one of the earliest cultivated plants. The olive tree is an evergreen, slow-growing species, tolerant to drought stress and extremely long-lived, with a life expectancy of about 500 years (Zohary *et al*., 2000).

According to a 2013 FAO statistic, olive production in the world has reached 10.3 million ha and production quantity 20.4 million tones. Turkey's average share of the world's olive production is 10.9% and it's the fourth largest producer in the world, producing approximately 1.7 million tonnes of olive (FAO, 2013).

There are about 85 million olive trees in Turkey. Olive trees and olive production rates in the country are distributed as follows;

Marmara Region: Olives produced in this region is mostly used for corned olive production and production rate is 9.5% of Turkey's total olive production (http://hknbtrade.maximice.com.tr/turkish-aegean-olives/).

Aegean Region: This region has the best growing conditions for olive trees and it is the largest olive producing area in Turkey playing host to approximately 76% of the country's olive plants. The kind of olives produced here are most suitable for oil production (http://hknbtrade.maximice.com.tr/turkish-aegean-olives/).

Mediterranean Region: The mediterranean, the homeland of Turkey's olives lags behind other regions in the country in terms of olive production. 15% of olives produced in this area is consumed astable olive. The olive production rate in this region is 14% of all Turkey's production (http://hknbtrade.maximice.com.tr/turkish-aegean-olives/).

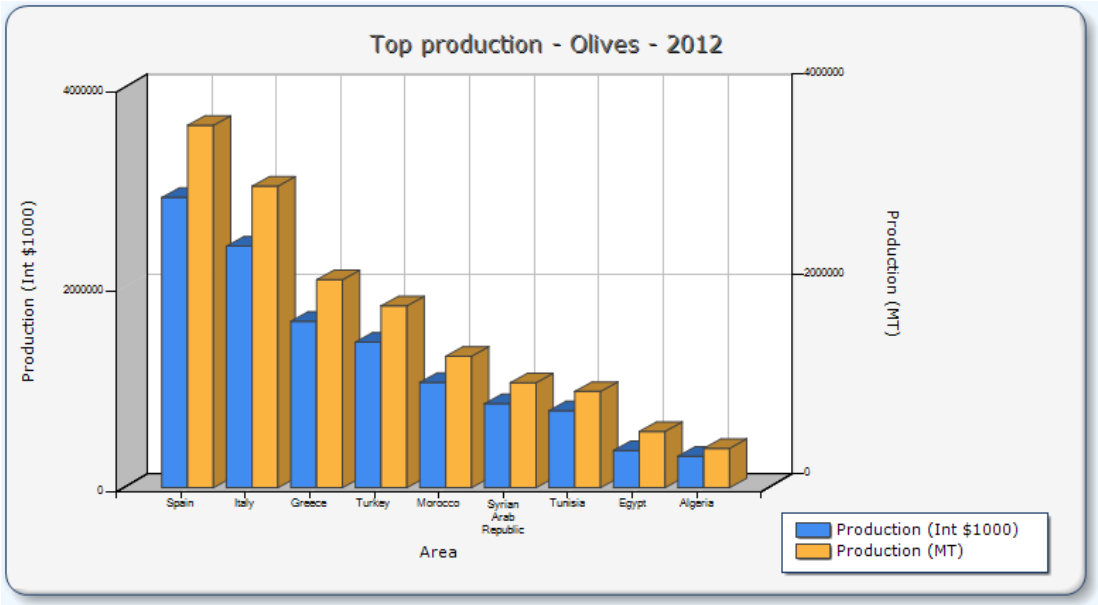

Figure 1.1. Top production – Olives -2012. FAO.

Although olive is regarded as an intermediate salinity and drought tolerant plant, water deficit and salinity stresses still remain the major cause of olive production losses in the Mediterranean region. Olive leaves are able to withstand extremely low water potential (up to -6 to -8 MPa) and are capable of maintaining their full rehydration capacity even after losing almost 40% of their tissue water (Rhizopoulou *et al*., 1991). These adaptations ensures both a high water potential between leaves and roots and water uptake from a water potential of up to -2.5 Mpa.Water deficit stress leads to a reduction in growth and photosynthesis (Bongi and Palliotti, 1994). Olive plants have the capacity to exhibit a net assimilation (10% of well-water plants) rate under very low (-6.0 MPa) pre-dawn leaf water potential (Xiloyannis *et al*., 1999) and prevent water loss water by stomata closure (Fernandez *et al*., 1997).

Investigations on diurnal course of leaf water and gas exchange parameters of olive plants exposed to varying levels of water stresses revealed that plants attained their maximum net photosynthetic and stomatal conductance potential early in the morning both in well-watered and in stressed plants, but decreased drastically and faster in plants exposed to stress conditions. Furthermore, the total disruption of photosynthetic processes during extreme water deficit influenced the non-stomatal aspect of photosynthesis thereby inducing a light-dependent inactivation of the primary photochemistry aspect of photosystem II (PSII) (Angelopoulos *et al*., 1996).

Salinty stress is beginning to pose huge problems on low water quality irrigation areas due to the accumulation of salt in those soils over the years. In the coastal regions of the mediterranean basin, olive plants can go through temporary salinity stress during the dry period of the growing season when irrigation is intensified in order to protect plants against the harmful effects of severe drought stress. Olive is classified as an intermediate salt tolerant speci (Rugini and Fedeli 1990), but salinity resistance varies from cultivar to cultivar.

Tattini and colleagues in 1997 demonstrated that there were huge differences in the salt tolerance abilities of Frantoio and Leccino cultivars of olive. Growth in plants exposed to alternating cycles of salinity stress (50, 100, and 200 mM NaCl) and relief periods was significantly inhibited. Olive experienced growth recovery once salt stress was relieved, but the rate of recovery was dependent on the level of salt to which plants were subjected. Relief from low (50 mM) or intermediate (100 mM) NaCl treatments resulted in a full recovery, which was not obtained in olive plants exposed to more severe salinity stress (200 mM) (Tattini *et al*., 1997).

Finally, despite the fact that the existing olive genotypes have the genetic potential to tolerate freezing, the degree of resistance is not enough to ensure their survival under extreme frost conditions (Bartolini *et al*., 1994).

1.2 Common bean

Common bean (*Phaseolus vulgaris* L.) is one of the widely cultivated plants in the world. It is considered as one of the most important legumes for human consumption (CIAT, 2001; Emam *et al*., 2010). According to the FAO, the production of dry beans (Phaseolus species) had recahed about 23 million tonnes in 2012, occupying a land area of 29 million ha (FAO, 2013). The vegetable protein in beans can easily be a substitute for animal protein such as meat and poultry and is affordable for most small-scale households. Legumes in association with rhizobium bacteria fix atmospheric nitrogen in the soil (Kay, 1979). The nitrogen that is fixed into the soil improves the soil nitrogen level thereby benefiting the crops that are grown thereafter and ultimately leading to the reduction in production costs.

Residues of common bean left on the field also improve the soil structure (Barrett, 1990). The production of bean like any other plant is hampered by biotic and abiotic stress factors (Jaleel *et al*., 2009). With the constant changes in the weather patterns due to the global phenomenon of climate change, these stress factors are anticipated to exert an increasing negative effect on crop productivity (Man *et al*., 2011). Water deficit stress is perhaps the most damaging abiotic stress to crop production worldwide (Jones and Corlett, 1992). Water stress is the second most serious stress factor after pest and diseases in that it causes a 60 % reduction in the total yield of grain produced worldwide. Bean production in most parts of the world is rainfall dependent, consequently insufficient or unpredictable rainfall may limit yields (Acosta-gallegos and Domingo, 2009).

Despite the fact that improved management practices such as supplementation with irrigation during drought periods can bring about improvement in yields in water-deficient environments, irrigation facilities are costly for most small scale farmers. It is certain improvement in yields can be achieved through genetic improvements since the improved genotype would be resilient to drought without the farmer providing extra inputs (White *et al*., 1994; Singh, 2001). The development of common bean varieties with tolerance to water deficit is therefore of critical importance especially if bean production in areas prone to intemittent or terminal drought is to be sustained.

1.3 Dehydrins

Despite the fact that water deficit stress causes a reduction in general protein production, there is an overproduction of certain specific proteins (Lahibilili *et al*., 1995). Plants react to abiotic stresses such as water deficit, salt stress and extracellular freezing biochemically by increasing the production of dehydrins. Dehydrins are a distinct family involved in the stress defence mechanism of plants charcterised by a consensus amino acid sequence (EKKGIMDKIKELPG) near the carboxy terminus known as the "K" segment (Close *et al*., 1993). The number of K segments present varies from one to 11 copies. Generally, angiosperms have a highly conserved K segment (Campbell and Close, 1997). Another characteristic feature of most dehydrins is the "S" segment which composed of a block of serine residues. Another consensus amino acid sequence (V/T) DEYGNP, known as the "Y" segment, may also be present near the amino terminus. Dehydrins are expressed in different plant tissues including roots, leaves, coleoptiles, seeds and crowns (Houde *et al*., 1992) and some were found to be associated with dehydrative stress tolerance.

1.4 Problem Statement And Justification

Even though at present environmental stresses pose no significant negative effects to the country's olive population, with the continuous deterioration of the climate due to the problem of climate change, the probability of the nation's olive population facing harsh extreme weather conditions in the near future is very high.

Moreover extensive genetic studies have been carried out on most temperate fruit crops but very few such researches have been carried out on olive plants (Scuola *et al*., 2002). This research therefore seeks to stimulate further improvement studies on olive plants.

Like olive, the development and productivity of common beans is adversely affected by many abiotic stress factors whose effects are expected to intensify with the global worsening phenomenon of climate change. It is therefore of utmost importance that studies be conducted on candidate genes that confer resistance to extreme stress conditions especially drought in bean plants.

1.5 Objectives Of The Study

The objectives of this study is to successfully clone and investigate the functions of two novel dehydrin-like genes; *LEA-dehydrin* (late embryogenesis abundant-dehydrin) and *OeSRC1* identified during the transriptome profiling of common bean (*Phaseolus vulgaris* L.) and olive (*Olea europaea* L.) respectively in tobacco plants.

2 LITERATURE REVIEW

2.1 Cold Stress In Plants

Every plant has an optimum temperature at which it exhibits proper growth and development. For example most tropical plants, exhibit injury symptoms when exposed to low non-freezing conditions (Lynch., 1990). Plants such as soybean (*Glycine max*), cotton (*Gossypium hirsutum*), tomato (*Lycopersicon esculentum*) and maize (*Zea mays*) are especially sensitive to temperatures under 10–15 °C and show injury symptoms when exposed to such temperatures (Guy *et al*., 1990).

In these plants, symptoms of stress related injuries begin to show from 48 to 72 h, however, this duration depending on a plant's sensitivity to cold stress varies from plant to plant. Plants exhibit the following visible signs when exposed to chilling stress: wilting, reduction in the rate of leaf expansion and death of tissues (necrosis) resulting from the yellowing of leaves (chlorosis). The reproductive capacity of plants are also seriously affected by chilling. For example, flowers become sterile when rice plants are exposed to chilling temperatures at the floral opening stage (Jiang *et al*., 2002).

Freezing exhibits its major harmful effect on plants by inducing severe membrane damage to plant cells (Steponkus, 1984). This damage is mostly as a result of the severe dehydrative effect of freezing. The lipid or fatty acid component of membranes is essentially of two kinds: saturated fatty and unsaturated fatty acids. Saturated fatty acids possess a single bond between its carbon atoms (CH_2 CH_2). Unsaturated fatty acids on the other hand possess one or more double bonds between two carbon atoms (CH CH). Generally, saturated fatty acids solidify faster than unsaturated fatty acids. Thus, the fluidity of a membrane is determined by the relative proportion of its unsaturated fatty acid component (Steponkus *et al*., 1993).

The transition temperature of a membrane is defined as the temperature at which a membrane moves from a semi fluid to a semi crystalline state. Plants that are sensitive to chilling normally possess a higher amount of saturated fatty acids and

correspondingly higher transition temperatures whereas chilling resistant plants are characteristically high in unsaturated fatty acids and therefore possess lower transition temperatures. Most crops are able to attain their full genetic potential because of their capacity to withstand the extreme cold conditions of late spring or early autumn frost. Freezing tolerance is therefore of utmost importance with regards to sustaining agricultural production.

The main cause of injury resulting from freezing in plants is the ice formation rather than low temperatures. Ice begins to form in the apoplastic space of plants due to its relatively lower solute concentration. Due to the fact that the vapour pressure of water at any given temperature is higher than ice, the formation of ice in the apoplast causes a vapour pressure gradient to be established between the apoplast and its neigbouring cells. The unfrozen cytoplasmic water causes an enlargement of the existing ice crystals, mechanical strain and cell wall rupture by moving down the gradient from the cell cytosol to the apoplast (Mckersie and Bowley, 1997). Cellular dehydration resulting from freezing causes different kinds of membrane damage such as expansion-induced-cell lyses, fracture lesions and lamellar-to-hexagonal-II phase transition (Uemura *et al*., 1997).

Although the principal mechanism by which freezing causes damage to membranes is through severe cellular dehydration, there are other mechanisms by which freezing induced injuries may occur. ROS produced as a result of freeze stress plays a role in membrane damage. Plants that are sensitive to chilling develop structural injuries and may also suffer metabolic dysfunctions (Kacperska *et al*., 1989). Ultimately chilling results in the destruction of membranes and consequently leads to solute leakage. The other effects of chilling are the loss of compartmentalization, reduction in protein assembly, photosynthetic impairment and the disruption of general metabolic processes resulting from the loss of integrity of intracellular organelles.

2.1.1 Mechanisms of freezing tolerance

Considering the important role membranes play in freezing-injury, it comes as no surprise that various mechanisms seem to be involved in enhancing the cryostability of membranes during cold acclimation. Steponkus and colleagues in 1993 reported that plasma membranes of plants that underwent cold acclimation did not suffer expansion-induced lysis whereas plasma membranes of nonacclimated plants did.

The elimination of these forms of membrane damage involves various changes in the lipid composition, such as an increase in the rate of fatty acid desaturation of membrane phospholipids (Steponkus *et al*., 1993; Uemera *et al*., 1997). Moreover, the build up of sucrose and other simple sugars that are mostly produced in response to cold acclimation appears to contribute to stabilizing membranes, as these molecules can protect membranes from freeze-induced damage in vitro (Strauss *et al*., 1986; Anchordoguy *et al*., 1987).

Other mechanisms such as those that help prevent or reverse freeze-induced denaturation of proteins or lessen the direct physical damage to cells caused by the build up of extracellular ice have been demonstrated to play potential roles in freezing tolerance. Molecular chaperones have also been demonstrated to accumulate during cold acclimation. Examples of these are *Hsp70* (Neven *et al*., 1992, Anderson *et al*., 1994) an *Hsp70* from soyabean (Caban *et al*., 1993) and an *Hsp90* from *Brassica napus* (Krishna *et al*., 1995). Furthermore, it has been demonstrated that "freeze-inhibitor" sugars may lessen cellular damage by preventing the attachment of extracellular ice to the cell wall (Olien *et al*., 1995). It has come to light through recent studies that antifreeze proteins are accumulated in many plants during cold acclimation and some of these proteins are present in the apoplastic fluids (Duman *et al*., 1994). These proteins could possibly play a role in freeze tolerance through the modification of ice crystal structure and/or the prevention of ice recrystallization.

2.1.2 Cold-responsive genes and freezing tolerance

In 1985, Guy and colleagues demonstrated that there were changes in gene expression during cold acclimation. Since then, the aim of researchers has been to ascertain whether or not cold-responsive genes play roles in freezing tolerance. To do this, researchers have isolated and characterized a number of genes that are induced during cold acclimation (Thomashow *et al*., 1994). Many of these genes produce proteins with known activities that could play potential roles in freezing tolerance. For instance, the *Arabidopsis FAD8* gene (Gibson *et al*., 1994) and barley *blt4* genes, (White *et al*., 1994) which encode a fatty acid desaturase and a putative lipid transfer protein respectively, are stimulated under low temperatures. These genes might possibly play some roles in freezing tolerance by changing the composition of lipids (Neven *et al*., 1992).

2.2 Drought Stress

Water stress may be caused either by water deficit or excess water. Flooding, an example of excess water stress, essentially leads to the reduction of oxygen supply to roots which consequently leads to the breakdown of important root functions such as limited respiration and nutrient uptake. The water deficit stress also referred to as drought stress is the most common water stress experienced by plants. The normal bilayer structure of a membrane is destroyed and becomes porous when it loses water through desiccation. Stress within the lipid bilayer also displaces membrane proteins and thereby contributes to the destruction of membrane structure, disruption of cellular compartmentalization and a loss of the activity of enzymes found in the membrane. Furthermore these stress factors may result in the reduction of the activity of enzymes in organelles and the cytosol and in some cases their complete inactivation due to dehydration. The high concentration of protoplasmic electrolytes resulting from dehydration may also lead to the breakdown of cellular metabolism. The mechanisms of drought and salt stresses are similar to each other as both stresses result in cellular dehydration and osmotic imbalance (Mahajan and Tutejah, 2005).

2.2.1 Effects of drought on plants

The effects of drought on plants can be observed at both the morphological and molecular levels and are clearly seen at all the developmental levels of plants' growth and at whatever point in the the plant's lifecycle it experiences drought stress (Farooq *et al*., 2009).

2.2.1.1 Effects of drought on plant growth and yield

The principal end result of water deficit stress is that it impairs germination and results in poor stand establishment (Harris *et al*., 2002). It came to light in a study conducted on pea that water deficit caused late seedling growth and led to an impairment of the germination of the seeds of five different cultivars tested (Okcu *et al*., 2005). Moreover, alfalfa (*Medicago sativa*) showed reduction in germination potential, hypocotyl length, shoot and root fresh and dry weights but an increase in root length when water deficit stress was induced with polyethylene glycol (Zeid and Shedeed, 2006). However, when water deficit stress was applied at the vegetative stage, rice plants experienced stunted growth (Tripathy *et al*., 2000). The quality and

quantity of plant growth is determined by cell enlargement, cell division, differentiation, genetic, physiological, ecological and morphological events and their complex interactions. These factors are affected by water deficit stress. One of the most water deficit-sensitive physiological process in plants is cell growth (Taiz and Zeiger, 2006). Cell elongation in higher plants can be limited by the disruption of the flow of water from the xylem to neighbouring elongating cells under extreme drought stress (Nonami, 1998). Water deficit stress leads to impaired mitosis, reduced cell elongation and expansion which consequently causes reduction in plant growth, leaf area and plant height (Nonami, 1998). Most of the physiological factors that determine yield are affected by water deficit stress and are integrated by plants in a complex manner in yield determination. Thus, it is difficult to comprehend the mechanisms by which plants manage and display these ever-changing and indefinite physiological factors over their entire life cycle. Severity, duration, timing of stress, post stress behaviour of plants and interactions between stress and other factors are the main important factors that influence drought stress (Plaut, 2003). For example, drought stress applied at pre-anthesis in triticale genotypes shortened time to anthesis, while drought when applied after anthesis hastened grain-filling (Estrada-Campuzano *et al*., 2008). Samarah in 2005 reported that barley subjected to drought stress recorded a reduction in grain yield as a result of reductions in the number of tillers, spikes and grains per plant and individual grain weight. He further demonstrated that regardless of the stress severity, post-anthesis drought stress was harmful to grain yield (Samarah, 2005).

Drought normally results in infertility when it coincides with the flowering period of plants. This effect can be attributed to a host of factors such as the reduction in the flow of nutients to the developing ear below the level required for optimum grain growth (Yadav *et al*., 2004). Water deficit stress at the time of pollination resulted in increased rate of kernel abortion in maize (*Zea mays*) (Morgan, 1990). Drought stress also resulted in a 40–55% loss in seed yield of pigeon pea when it coincided with its flowering stage (Nam *et al*., 2001). Overall, drought stress interferes with plant growth and development thereby leading to hampered flower production and grain filling which intend results in smaller and fewer grains formation. The formation of fewer and smaller grains can be attributed

to the reduction in nutrient sharing and sucrose and starch synthesis enzymes' activities.

2.2.1.2 Effects of drought on the water content of plants

The principal factors that influence plant water relations include relative water content, leaf water potential, and stomatal resistance, rate of transpiration, leaf temperature and canopy temperature. Relative water content of wheat leaves was observed to be higher at the early stages of leaf development but decreased with the accumulation of dry matter and at maturity (Siddique *et al*., 2001). Water-stressed wheat and rice plants experienced significant reductions in relative water content as compared to non-stressed ones. Water deficit stress resulted in drastic decrease in the leaf water potential, relative water content and transpiration rate, with an accompanying increase in the leaf temperatures of plants (Siddique *et al*., 2001). Nerd and Nobel in 1991 reported a 57% decreament in the water content of *Opuntia ficus-indica* cladode when exposed to drought stress. A greater amount of water was lost in the water-storage parenchyma cells of cladodes compared to the chlorenchyma therefore resulting in a lower turgor potential. It was also demostrated in another study on *Hibiscus rosa-sinensis* that relative water content, turgor potential, transpiration, stomatal conductance and water-use efficiency dropped under drought stress (Egilla *et al*., 2005).

2.2.1.3 Photosynthesis

One of the principal effects of drought is the reduction in photosynthesis due to a decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence and consequently a reduction in food production (Wahid and Rasul, 2005). Water deficit stress caused changes in photosynthetic pigments and components (Anjum *et al*., 2003), damaged photosynthetic apparatus (Fu and Huang, 2001) and diminished activities of calvin cycle enzymes, thereby leading to a reduction in yield (Monakhova and Chernyadèv, 2002). The imbalance between the production of reactive oxygen species and antioxidant defense is another important factor that affects the photosynthetic capacity and growth of plants (Fu and Huang, 2001), leading to the build up of ROS which induces oxidative stress in proteins such as enzymes and hormones, membrane lipids and other cellular components.

2.2.1.2 Oxidative damage

Reactive oxygen species, such as superoxide anion radicals ($O^{-}{}^{2}$), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), alkoxy radicals (RO) and singlet oxygen (O_2) (Munné-Bosch and Penuelas, 2003) are often produced in response to certain environmental stresses. These molecules may react with proteins, lipids and deoxyribonucleic acid, resulting in oxidative damage and impairment of normal cell functions (Foyer and Fletcher, 2001). Among the various cell compartments, the chloroplast is a potentially important source of reactive oxygen as excited pigments in thylakoid membranes may interact with O_2 to produce strong oxidants such as $O^{-}{}^{2}$ or $O_{1}{}^{2}$ (Niyogi, 1999). Other ROS such as H_2O_2 and OH^{-} are produced as a result of further downstream reaction processes. The reaction of O_2 with reduced components of the electron transport chain in mitochondria can lead to the formation of ROS (Möller, 2001), and peroxisomes produce H_2O_2 when glycolate is oxidized into glyoxylic acid during photorespiration (Fazeli *et al*., 2007).

2.2.2 Drought resistance mechanisms

Plants respond, adapt and survive under drought stress through different kinds of morphological, biochemical and physiological responses. The ability of plants to perform and display economic yields under sub-optimal water supply is referred to as drought tolerance. Water deficit stress affects the water relations of plants at all levels of its organisation leading to damage, specific and unspecific reactions as well as adaptation reactions (Beck *et al*., 2007).

2.2.2.1 Morphological mechanisms

Plants are able to escape drought by shortening their life cycles or growing season or by undergoing reproduction before the arrival of drought conditions. Flowering time is an important trait in drought escape mechanisms that involves a short life cycle (Araus *et al*., 2002).

Drought avoidance mechanisms involve processes that limit water loss from plants, through stomatal control of transpiration, and also ensures the uptake of water through a well established root system (Turner *et al*., 2001; Kavar *et al*., 2007). The most important drought avoidance trait that influence the final yield of plants under severe water deficit conditions are root characters such as biomass, length, density

and depth (Subbarao *et al*., 1995). An extensive and thick root system helps in extracting water from considerable depths (Kavar *et al*., 2007).

2.2.2.2 Physiological mechanisms

Osmotic adjustment, osmoprotection, antioxidation and scavenging defense system have been identified as the most important factors responsible for drought tolerance (Farooq *et al*., 2006)

2.2.2.3 Molecular mechanisms

Plant cells may lose water when grown on soils with low water content. Under these conditions, changes in gene expression (up and down-regulation) occur. Different kinds of genes whose products are thought to function in drought tolerance are induced at the transcriptional level in response to water deficit stress (Kavar *et al*., 2007). Gene expression may be induced directly by stresses and/or injury responses. Nonetheless, research has proven that drought tolerance is a complex phenomenon involving many genes acting together (Agarwal *et al*., 2006).

2.3 Salinity Stress

Salinity stress seriously affects agricultural crop productivity. Salinity exhibits its influence by negatively affecting germination, plant vigour and crop yield. According to Munns and Tester 2008, the use of brackish water on many irrigated areas has resulted in the accumulation of salts in tthose soils. They further stated that over 45 million hectares of irrigated lands have been destroyed by salt whilst an additional 1.5 million hectares continue to be lost to high salinity each year (Munns and Tester, 2008). High salinity exhibits its effect by inducing water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Hasegawa *et al*., 2000). Together, these effects contribute to limiting the growth, development and survival of plants.

2.3.1 Salinity's effects on plants

Soil salinity strongly affects yields of agricultural crops by limiting the capacity of agriculture to support the ever growing human population (Flowers, 2004). Yields

are less affected or not affected at all at low salt concentration (Maggio *et al*., 2001). The yields of most plants including glycophytes move towards zero with increasing salt concentration since these plants fail to grow under high salt concentrations and are seriously adversely affected or even killed by 100-200 mM NaCl. The reason being that they have not adapted to low salt conditions and therefore are not salt tolerant (Munns, 1986). On the other hand halophytes can withstand salinity in excess of 300-400 mM. Halophytes can grow on the saline soils of coastal and arid regions because they have developed salt tolerance mechanisms in the course of their development (Parida and Das, 2005). Zhu in 2007 measured the amount of ion of both halophytes and glycophytes under salt stress. His results revealed that halophytes accumulated salts and glycophytes excluded them (Zhu, 2007). The effect of salinity on plants can be expressed either in the form of high concentrations of salts in the soil interfering with the root's capacity to absorb water or high salt concentration in the plant itself resulting in the inhibition of vital processes such as nutrient uptake and use (Hasegawa *et al*., 2000). The cumulative effect of salt stress is the reduction of plant growth, development and survival.

2.3.2 Salt tolerance

The molecular processes invoved in plants' salt tolerance mechanism have not yet been fully understood because of its complex nature. In various species, there are actually a host of genes with strong environmental interactions that control salinity, that is, genetic variation can only be assessed indirectly by determining the stress response capacities of different genotypes. Probably, the most appropriate parameter to assess is growth or yield, especially under moderate salinities (Allen *et al*., 1994). Salt tolerance can actually be measured as the percentage biomass of production in saline versus control conditions over an extended period or in terms of survival, which is quite suitable for perennial species (Munns, 2002). Salt tolerance may vary with genetic characteristics in a considerable manner. The salt tolerant ability of a given plant will be suspended if that plant is suddenly exposed to salt stress, even if the species is a halophyte (Albert, 1975). Plants adapt in a gradual manner to salt stress through a variety of processes known as acclimation. During the course of development, a given species' sensitivity to salinity stress may change. Salinity tolerance may fluctuate depending on the plant species and/or the existing environmental factors. For example, whiles certain plants may be more sensitive to

salt stress at germination, others may experience great sensitivity at the reproductive stage (Howat, 2000). Various salt stress acclimation mechanisms have been developed by plants. These mechanisms can be classified into three cartegories: a) the tolerance to osmotic stress, b) the Na^+ exclusion from leaf blades and c) tissue tolerance (Munns and Tester, 2008).

2.3.2.1 Osmotic tolerance

Irrespective of their capacity to exclude salt, the growth of plants under salt stress is mostly inhibited by the osmotic effect of salinity which consequently leads to the reduction in the the rate of growth and stomatal conductance (James *et al*., 2008). Osmotic tolerance depends on the plant's capacity to withstand the water deficiency that results from salt stress and its capacity to maintain leaf expansion and stomatal conductance (Rajendran *et al*., 2009).

From a study conducted on 50 international durum varieties and landraces to determine their genetic variation with respect to their osmotic stress tolerating abilities, it came to light that a positive relation exist between stomatal conductance and relative growth rate in salt treated plants and that higher CO_2 assimilation rate can be attributed to higher stomatal conductance (James *et al*., 2008) but the accumulation of salts beyond lethal levels results in the death of old leaves (usually old expanded leaves) and young ones becoming either stunted or new ones failing to grow as a result of inadequate supply of nutrients due to a severe reduction in photosnythesis. Therefore increased osmotic tolerance depends on the plant's enhanced ability to continously produce and develop fresh and greater leaves as well as maintian higher stomatal conductance. Only plants with sufficient soil water such as those in irrigated food production systems with regular supply of water would benefit from the resulting increased leaf area (Munns and Tester, 2008).

2.3.2.2 Sodium exclusion

Sodium appears to reach lethal levels before chlorine in most plants cultivated under saline conditions (Munns and Tester, 2008). Another important tolerance mechanism employs the plant's capacity to reduce ionic stress by reducing the amount of accumulated cytosolic Na^+, especially those in leaves undergoing transpiration. This

mechanism, just like tissue tolerance, involves up and down regulation of the expression of specific ion channels and transporters thereby allowing the regulation of Na^+ transport throughout the plant (Munns and Tester, 2008). Plants such as rice, durum wheat, bread wheat and barley employ the Na^+ exclusion mechanisms in their leaves in dealing with salinity stress (Richard *et al*., 2011). Na^+ exclusion from leaves is a result of the net Na^+ uptake by the root cortex cells and the tight regulation of net loading of the xylem by parenchyma cells in the stele (Davenport *et al*., 2005). Na^+ exclusion by roots prevents Na^+ from accumulating to lethal levels in leaf blades. The failure of the plant to exclude Na^+ leads to premature death of older leaves. These lethal effects can be observed after few days or weeks depending on the plant (Munns and Tester, 2008). An example of an efficient cytosolic Na^+ exclusion system can be observed in the operation of vacuolar Na^+/H^+ antiports that transports potentially harmful ions from the cytosol into large, internally acidic, tonoplast-bound vacuoles. These ions, then, function as water flow facilitating osmoticum within the vacuole of the cell and enable plants to grow in highly saline soils. The proton-motive force generated by vacuolar H^+ translocating enzymes, H^+ adenosine triphosphatase (ATPase) and H^+ inorganic pyrophosphatase (PPiase) is applied by antiports to ensure the downhill movement of H^+ (down its electrochemical potential) with uphill movement of Na^+ (against its electrochemical potential). AtNHX1 is the Na^+/H^+ antiporter, located in the tonoplast, suggested to be involved in the regulation of vacuolar osmotic potential in *Arabidopsis* (Apse *et al*., 1999).

2.3.2.3 Tissue tolerance

Tissue tolerance results in increase in the survival rate of old leaves. It involves the separation of Na^+ and Cl^- at the cellular and intracellular levels in order to prevent salts from reaching toxic levels, especially within mesophyll cells of leaves (Munns and Tester, 2008) and the production and build up of cytoplasmic compatible solutes. Compatible solutes play several important roles in osmotolerance in plants such as the protection of enzymes from denaturation, the stabilisation of membrane or macromolecules or playing adaptive roles in the mediation of osmotic adjustment (Ashraf and Foolad, 2007). Compatible solutes not only play vital roles in osmotic balance but are also hydrophilic and function as low molecular weight chaperones by replacing water on proteins or membranes' surfaces (Hasegawa *et al*., 2000).

Moreover these solutes act in protecting cellular structures by ROS scavenging (Zhu, 2001). Compatible solutes are water soluble, small and evenly neutral with respect to the perturbation of cellular functions, even when highly concentrated in a cell (Sakamoto and Murata, 2002). Compatible solutes do not only consist of nitrogenous compounds such as amino acids, amines and betaines, but also organic acids such as sugars and polyols (Mansour, 2000).

2.3 Engineering Stress Tolerance In Plants

2.3.1 Genetic engineering of crop plants

Genetic engineering is a crop improvement tool for producing transgenic plants with superior traits (Dunwell, 2000). Genetic material can be found in three different organelles of the plant cell: the nucleus, plastids and mitochondria (Maliga and Small, 2007). Plant transformation involves gene(s) transfer into any organelle genome of the plant tissue. Plant transformation has only been successful for the nucleus and for plastids (Dunwell, 2000).

2.3.1.1 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a gram negative soil bacterium that causes crown gall disease in plants. In nature, tumour formation is initiated by the conjugative transfer of a DNA segment (T-DNA) from the bacterial tumour-inducing (Ti) plasmid, where the T-DNA by means of the activity of a range of virulence (*Vir*) gene products integrates semi-randomly into the genome of the host cell and causes the formation of crown galls by expressing tumour inducing (Ti) genes (Zupan *et al*., 2000; Gelvin, 2003). Due to this special feature of *Agrobacterium*, the Ti plasmid has been engineered in such a way as not to cause any harm to plants while simultaneously carrying foreign genes into plant cells (Zupan *et al*., 2000). *Agrobacterium* and plant tissue specific factors such as plant genotype, explant, vector-plasmid, bacterial strain and many other factors determine the ease with which T-DNA is transferred and integrated into the plant's genome. Despite the fact that direct transformation methods such as PEG-mediated transfer, microinjection, protoplast, intact cell electroporation and gene gun technology have been developed (Gelvin, 2003), the *Agrobacterium*-mediated transformation method still remains the most widely used and most advantageous method (Opabode, 2006).

2.3.1.2 PEG-mediated transformation

The polyethylene glycol (PEG)-mediated method of gene transfer into plant cells involves isolating protoplast and subsequently exposing it to PEG solution for the insertion of foreign DNA (Koop *et al*., 1996). Protoplasts, plant cells without cell walls, serve as unique biological resources for highlighting several aspects of modern biotechnology (Davey *et al*., 2005). PEG-mediated transformation is particularly used in transforming plants that cannot be successfully transformed by other transformation methods such as *Agrobacterium*-mediated transformation (Rakoczy-Trojanowska, 2002). It has also been used to achieve successful integration of genes into nuclear and chloroplast genomes (Davey *et al*., 2005). The underlying mechanisms of PEG-mediated DNA transformation of nuclear and chloroplast genomes however still remain unclear (Kofer *et al*., 1998).

2.3.1.3 Biolistics (particle bombardment)

Biolistics is done by shooting DNA-coated tungsten or gold particles into the plant tissues where the DNA dissociates from the gold or tungsten particles and integrates into the plant genome (Altpeter *et al*., 2005). Transformation by particle bombardments can also be achieved by using gold particles coated with *Agrobacterium* instead of naked plasmid DNA (Rasmussen *et al*., 1994). Biolistics is not only used for the transformation of the nucleus and chloroplast genomes but also mitochondrial genomes. As at now there has not been any report on mithochondrial genome transformation (Altpeter *et al*., 2005). The advantages of the biolistic method over other transformation methods are that: it does not have any biological limitations, it does not depend on any particular target cell type and multiple genes can be transferred into a single cell. There have however been reported cases of mechanical shearing of large plasmid DNA due to reaction with tungsten during particle preparation or delivery and plasmid DNA modification (Heifetz, 2000).

2.4 Dehydrins

A lot of interest has been shown in understanding plants' dehydration survival mechanisms with the aim of protecting crops and increasing arable lands. A broad spectrum of proteins overproduced under dehydration stress such as the Late Embryogenesis Abundant (LEA) proteins were discovered in a study conducted on

cotton seeds (Galau *et al*., 1986). As can be inferred from their name, these proteins are very common during seed development and enables the seeds to withstand water deficit stress. They were identified to be available in many plants and various plant tissues through subsequent studies. LEA proteins possess different conserved sequence motifs and are highly rich in Ala, Gly, and Ser residues, with very few hydrophobic residues. Their lack of significant secondary structure can be attributed to their highly hydrophilic nature. Dehydrins belong to the D-11 or group 2 of the Late Embryogenesis Abundant class of proteins (Close, 1997). Dehydrin expression can be stimulated by abiotic stress factors including water deficit stress, salt stress and cold stress. Abscisic acid (ABA) treatment can also elicit osmotic and cold stress (Talanova and Titov, 1994), where this relationship has been investigated at both the protein and mRNA level. A set of five dehydrins from arabidopsis were characterised by Nylander and colleagues in 2001 using western blotting. It was discovered that the upregulation of three set of dehydrins was stimulated by cold and one set upregulated only in response to abscisic acid treatment. The fifth dehydrin, despite being constitutively expressed was upregulated by abscisic acid, cold and salt stress. Danyluk and colleagues discovered that the expression of wcor410 were highly responsive to cold at both transcript and protein levels (Danyluk *et al*., 1998). Despite the fact that not all dehydrins are overexpressed by the same stresses, there are many that increase at the mRNA levels as a result of abiotic stressors (Cellier *et al*., 1998). It has not been determined yet the specific role of dehydrins but various physiological reactions have been attributed to the presence of dehydrins (Ismail *et al*., 1997). Hara and colleagues in 2003 observed that liposomes oxidation was prevented by the citrus dehydrin *CuCOR19* (Hara *et al*., 2003). Xie and colleagues in 2012 also demonstrated that the *MtCAS31* dehydrin functioned by interacting with the ICE1 transcription factor to reduce stomatal density (Xie *et al*., 2012)

2.4.1 Dehydrin sequences

2.4.1.1 Cellular localization of dehydrins

Dehydrins can be located in various parts of the cell but are commonly found in the cytoplasm and the nucleus. They can also be found in the mitochondria, chloroplasts and near the plasma membrane. Two SKn dehydrins (Danyluk *et al*., 1998) were located close to the plasma membrane, while a KnS dehydrin was found in the

mitochondrion (Hara *et al*., 2003). No other dehydrin architecture was observed at a membrane. This implies that the Y-segment is not involved in protecting membranes. No specific localization pattern was observed for the remaining architectures. Phosphorylation of the S-segment can result in the relocation of a dehydrin from the cytosol to the nucleus (Goday *et al*., 1994). However, a Kn dehydrin (Houde *et al*., 1995) and two YnKn dehydrins (Wisniewski *et al*., 1999) were found in the cytoplasm and the nucleus. It is possible that an unidentified sequence element is responsible for these differences in localization. There is a more defined relationship between the YSK architecture and abiotic stress. It is likely that the stress response pathway (signaling and transcription control) can possibly determine the expression patterns of dehydrin in response to various abiotic stresses (Wang *et al*., 2003).The study of the relationship that exists between the YSK architecture and dehydrin localization is aimed at providing potential information on the role each segment plays in plant protection. Despite the fact that several investigations have been done, in many cases on individual dehydrins, the patterns are not very clear (Eriksson and Harryson, 2011). One reason that can be attributed to this is that the YSK-naming system does not consider the minor observable motifs or the vari-able φ-regions that may potentialy have an undetermined role in localization. This challenge is compounded by the fact that comparisons are made between dehydrins from different plants with different protective needs based on their structure. In a recent study on dehydrin localization in *Vitis vinifera* (Yang *et al*., 2012) and in *Triticum aestivum* (Wang *et al*., 2014) the dehydrins with different YSK architectures in one species were compared using one stress protocol. Four dehydrins were discovered in *Vitis vinifera* and the mRNA levels in tissues under stress investigated (Yang et al., 2012). These dehydrins were then named according to the YSK-nomenclature as follows *DHN1* (Y2SK2), *DHN2* (SK2), *DHN3* (SK3), and *DHN4* (Y3SK2). All four dehydrins were observed during seed development. The expressions of *DHN1* and *DHN2* were upregulated by cold and heat stress whereas only *DHN1* was upregulated in response to water deficit stress. Dehydrin expression in *T. aestivum* seedling leaves and roots were examined during dehydration, cold, and salinity stress. It was observed that YSK2 transcription responded to dehydration rather than low temperatures whiles Kn dehydrins only responded to cold in wheat seedlings (Wang *et al*., 2014) .

2.4.2 Structure and flexibility of dehydrins

An investigation of dehydrin sequences revealed that they had no hydrophobic residues. Protein folding is driven by the hydrophobic core of globular proteins. The presence of mostly polar and charged amino acids prevents proteins encoded by dehydrins from forming a stable structure (Tompa, 2002). The disodered nature of dehydrins confers on it the ability to avoid denaturation. Globular proteins undergo denaturation through the exposure of their hydrophobic residues to the aqueous environment and the subsequent interraction of thier hydrophobic residues on other proteins. The intrinsic disorder of dehydrins protects them from denaturation during desiccation or at freezing temperatures, due to their lack of significant structure and very few hydrophobic residues that could cause aggregation.

3 MATERIALS AND METHODS

3.1 Plant Materials And Tissue Culture Media

Eight week old leaves of *Nicotiana tabacum* cv. Samsun were used as explants for our transformation studies. Tobacco seeds were grown on MS medium containing 30 g/l sucrose, 2.8 g/l phytogel and a pH of 5.8. Shoots were induced from transformed tobacco leaves by growing them on MS medium containing 4.4 g/l MS, 30 g/l Sucrose, 2.8 g/l Phytogel, 1 mg/BA, 0.1 mg/l NAA and 50 mg/ml hygromycin. Well developed shoots were transferred to MS medium prepared without hormones for the generation of transgenic plants.

3.2 Bacterial Growth Media And Culture Conditions

LB and YEB media were respectively used to grow *Escherichia coli* cells and *Agrobacterium tumefaciens* (AGL1) cells.

3.3 Chemicals And Enzymes

Chemical used in this experiment were purchased from Duchefa, Sigma, Merck and Applichem Chemical Companies. All solutions were prepared with distilled water. Polymerases and cloning kits were also obtained from New England Biolabs and Thermo Fisher Scientific Corporation respectively.

3.4 Primer Design

The following primers were designed with the aid of Vector NTI, GENTLE and Primer 3 softwares programs.(http://www.bioinformatics.nl/cgi-bin/) for *LEA-dehydrin* and *OeSRC1* based on sequences obtained from NCBI.Forward primer for Lea-dehydrin:5’- CACCGAAATTGTAATGGCTGAAGC-3’. Reverse primer for Lea-dehydrin: 5’-TTTTCAGTGGTTGTGGTGCCC-3’. Forward primer for OeSRC1:5’ CACCATGGCAGGAATCATTCAC-3’. Reverse primer for OeSRC1:5’-CTAATCGCTATCGCTGCT-3’.

3.5 Growth Of Common bean Plants

Seeds of Ispir (a salt tolerant variety of common bean) were surface sterilized by incubating in 5% sodium hypochloride for 5 minutes after which seeds were repeatedly washed with sterile water for 5 times and then transferred to plastic pots. Plantlets were grown in vermiculite and incubated afterwards in growth chamber at 25 °C under 16 h/8 h photoperiod. During the course of their growth, plantlets were subjected to salt stress by watering with 200mM NaCl concentrated Hoagland solution. Finally, leaf and root samples were collected for RNA isolation.

3.6 *Agrobacterium* Strain And Plasmid.

The AGL1 strain of *Agrobacterium tumefaciens* engineered to contain the hypervirulent Ti plasmid, pTiBo542 possessing extra *Vir* genes was used in our experiment.

3.7 Gene Cloning And Plasmid Confirmation.

3.7.1 Isolation of total RNA

All equipments were double sterilised by autoclaving prior to RNA isolation. All solutions used in the RNA isolation process were prepared with distilled water and autoclaved before use. Mortars, pestles and eppendorf tubes were chilled in liquid nitrogen before use. 50 mg of olive and Ispir leaf samples were ground to a fine powder in liquid nitrogen, transferred into centrifuge tubes and chilled in liquid nitrogen. 1 mL TRI reagent was added to the reactions and mixing done by vortexing for 10 minutes followed by centrifugation at 10000 g at room temperature for 5 minutes. After centrifugation, 900 µl of the supernatants were transferred to new tubes without disturbing the pellet. 200 µl of chloroform was added to the reaction, mixed for 15 seconds by shaking vigorously and then incubated at room temperature for 3 minutes. The tubes were again centrifuged for 15 minutes at 4°C at a speed of 10000 g. 400 µl of the upper phases of the samples were transferred into new 1.5 ml centrifuge tubes and the chloroform treatment step repeated. The reactions were then mixed by shaking vigorously with a vortex for 15 seconds, incubated at room temperature for 3 minutes and centrifuged for 5 minutes at room temperature at a speed of 10000 g. Without contacting the interphase, 400 µl of the upper phases of

the reactions were again transferred into new 1.5 ml eppendorf tubes and 1 volume isopropanol added to each tube. Mixing was done by inverting the tubes several times and the samples incubated for 10 minutes at room temperature. Afterwards, RNAs were obtained in pellets by centrifuging for 10 minutes at room temperature at a speed of 10000 g. The supernatants were then decanted and the pellets washed with 1 ml of 70% ethanol at room temperature for 3 minutes. After centrifugation for 5 minutes at room temperature at maximum speed, the supernatants were again discarded. An additional 15 second spin was done by centrifugation to collect the pellets at the bottom of the tube in order to do away with any remaining liquid and the pellets air dried afterwards for 10 minutes. Finally, 50 µL of distilled water was added to the center of the tubes and incubated for 15 minutes at 65ºC with occasional vortexing to dissolve pellets. The resulting RNAs were then stored at -80°C.

3.7.2 cDNA synthesis

First strand cDNA was synthesised with DNase treated 1 µg total RNA using MMLV reverse transcriptase (Fermentas) with an 1 µl oligo (dT) 18 primers in accordance with the the standard protocol of Fermentas. After the addition of total RNA and primers, DEPC-treated water was added to bring the reactions to a final volume of 12µL.The reactions were mixed gently and centrifuged for 3 seconds afterwhich the mixtures were incubated at 70°C for 5 minutes and again briefly centrifuged. The reaction tubes were then incubated on ice and the following components added in the order shown: 4 µL of 5X reaction buffer, 1 µL RiboLock™ Ribonuclease Inhibitor (20U/ µL) and 2 µL of 10mM dNTP mix. Afterwards the reactions were mixed gently, centrifuged for 3-4 seconds and incubated at 37°C for 5 minutes. After incubation, 1 µL of RevertAid™ MMuLV Reverse Transcriptase (200U/ µL) was added and the tubes incubated at 42°C for 60 minutes. To end the reaction, the mixtures were incubated at 70°C for 5 minutes and then stored at -20°C.

3.7.3 PCR of *OeSRC1* and *LEA-dehydrin* genes.

Amplifications of *Lea-dehydrin* and *OeSRC1* genes performed with Q5 DNA Polymerase (NEB) and gene specific primers. The primer sequences used for the amplifications were 5'-CACCATGGCAGGAATCATTCAC-3', 5'CTAATCGCT ATCGCTGCT-3' and 5'-CACCGAAATTGTAATGGCTGAAGC-3', 5'-TTTTC AGTGGTTGTGGTGCCC-3' forward primers and reverse primers for *OeSRC1* and

LEA-dehydrin respectively. To achieve optimum PCR conditions for our genes of interest, gradient PCR was performed at different annealing temperatures, such as 46°C, 47.9°C, 49.9°C, and 54 °C.

Table 3.1 PCR conditions for cDNA amplification.

Components	**Amount (µL)**
Q5 reaction buffer	5
10mM dNTP	0.5
Forward primer	1.25
Reverse primer	1.25
cDNA	1
Q5 high fidelity DNA polymerase	0.5
Nuclease free water	11.5

3.7.4 Chemically Competent *E. coli* cell Preparation and Transformation

Chemically competent *Escherichia coli* cells were prepared with rubidium chloride. *Escherichia coli* strain *DH5α* cells were grown overnight on LB plates in an incubator at 37°C. The following day, one colony was inoculated into 2.5 ml liquid LB medium and the culture grown again overnight at 37°C and at an rpm of 200. The next day, the bacterial culture was transferred into 250 ml fresh liquid LB medium and allowed to grow to an optical density OD_{600} of 0.6-0.7. The culture was then incubated on ice for 15 minutes and later centrifuged at 4000 g for 5 minutes afterwhich the supernatant was decanted and the pellet dissolved in 0.4 volume of transformation buffer 1. The bacterial culture was then incubated on ice for 15

minutes, the cells centrifuged at 4000 g for 5 minutes and the supernatant done away with. The pellet was finally resuspended in 0.04 volume of Tfb2 buffer, immediately transferred into centrifuge tubes, and then stored at -80°C till further use.

3.7.5 Cloning of *OeSRC1* and *Lea-Dehydrin* genes into entry vector

Before cloning of the PCR products, the concentrations of PCR products were measured with Nanodrop UV Spectrophotometer (Thermo, US). pENTR™/D-TOPO cloning Kit (Invitrogen) was employed in the insertion of our genes of interest into the Gateway entry clone. 2:1 molar ratio of PCR product: TOPO vector was used in the cloning reaction. The ligation reaction was set up by mixing 4 µL of PCR product, 1 µL salt solution and 1 µL TOPO vector and incubating at 25°C for 10 minutes. The ligated products were then directly transformed into competent *E. coli.* The integration of our plasmid-inserts into *E. coli* cells was confirmed through colony PCR with gene specific primers (given earlier) on colonies grown on 50 mg/ml kanamycin containing LB medium. The PCR products were later loaded on 1% agarose gel for band identification and 2 colonies with expected bands selected for LR reaction.

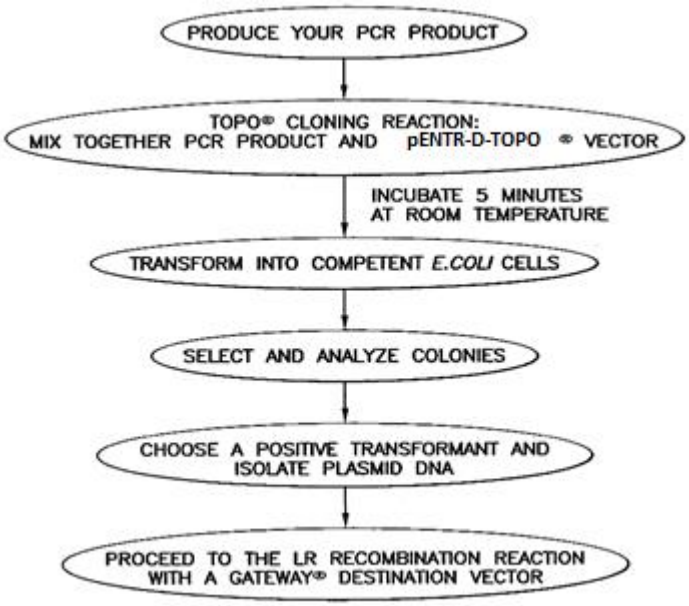

Figure 3.1.Work flow chart of the Gateway Cloning System.

3.7.6 Plasmid isolation

Two positive colonies from our entry clones were selected and grown overnight in 5 mL of LB medium containing kanamycin with shaking at 250 rpm at a temperature of 37°C. The bacterial culture was harvested by centrifugation at 8000 rpm for 2 min at room temperature and the supernatant discarded.

Plasmid isolation was then carried out in accordance with the manufacturer's protocol of the GeneJET Plasmid Miniprep Kit (Thermo Scientific). The harvested cells were resuspended in 250 µL of the Resuspension solution and the cell suspension transferred into eppendorf tubes. 250 µL of Lysis solution was added and the rections mixed thoroughly by inversion until a viscous and slightly clear state was achieved. 350 µL of Neutralization solution was then added, mixed immediately and thoroughly by inverting the tubes 4 times and centrifugation done for 5 minutes to pellet cell debris and chromosomal DNA after which the supernatants were decanted into the GeneJET spin columns. Centrifugation was immediately carried out for 1 min, the flow-throughs discarded and the columns placed back into the same collection tube. 500 µL of the Wash Solution (diluted with ethanol) was then added to the GeneJET spin columns, Centrifuged for 30-60 seconds and the flow-throughs again discarded. The columns were placed back into the same collection tubes and the previous wash step repeated.

Finally, the GeneJET spin columns were put into fresh 1.5 mL microcentrifuge tubes, 50 µL of sterile water added to the center of GeneJET spin column, Incubation done for 2 min at room temperature and centrifuged for 2 min to elute DNA.

The eluted plasmids were later sequenced. Sequence analysis was done by RefGen Company with ABI 310 Capillary DNA Sequencer by using the eluted PCR product.The following primers were used for sequencing : Forward sequencing primer (5'- GTAAAACGACGGCCAG -3') and reverse sequencing primer (5'CAGGAAACAGCTATGAC -3') were used. The sequence results for each plasmid was validated by cross-checking against available sequences in NCBI through the Blastn search.

3.7.7 Construction of *OeSRC1* and *LEA-dehydrin* expression clones using the pIPKb004 overexpression vector

The insertion of *OeSRC1* and *LEA-dehydrin* genes into plant overexpression vector (pIPKb004) was carried out with the Gateway® LR Clonase® enzyme in accordance with the manufacturer's protocol (Invitrogen). pIPKb004 vector possessing the 35S promoter and *HptII* selectable marker gene was used as our expression vector. After LR reaction, the reaction mixture was transformed into chemically competent *E. coli*. cells. Colony PCR was then performed with *OeSRC1* and *LEA-dehydrin* gene specific primers (given earlier) in accordance with standard colony PCR protocols as mentioned earlier with annealing temperatures of 56°C for both *OeSRC1* and *LEA-dehydrin*. Finally, Plasmid isolation was carried out on two positive colonies to be analysed by sequencing.

3.7.8 *Agrobacterium* transformation (AGL1) with *OeSRC1* and *LEA-dehydrin* genes

OeSRC1 and *LEA-dehydrin* genes were transformed into AGL1 using the electroporation method as follows; 1 µL each of the isolated plasmids was mixed with 50 µL aliquot of competent AGL1 cells and electroporated. After electroporation, 200 µL of YEB medium was added to the cells and grown for 3 hours at 28°C at a speed of 200 rpm. After incubation, the cells were grown on a YEB selective medium containing 100 mg/ml spectinomycin for 2 days. Positive colonies were then selected after colony PCR and used for tobacco transformation.

3.7.8.1 Growth of *Agrobacterium tumefaciens* strain AGL1

Agrobacterium tumefaciens strain AGL1 possessing the pIPKb004-*OeSRC1*-pAL154 and IPKb004-*LEA-dehydrin*-pAL154 compexes were grown in YEB medium supplemented with carbenicillin (100 mg/L) , spectinomycin (100 mg/L) and rifambicin (20 mg/L) in a shaking incubator at 250 rpm to an OD_{600}:0.5-0.7.

3.7.9 PCR with *Vir D* specific primers

Two different PCRs were carried out with *Vir D* specific primers (Forward primer 5'-CCGCTTATCAAAGCAGCGAC-3', Reverse primer 5'-TCTGGAGGCACA ACCGATTC-3'). The first PCR was carried out on AGL1 genome at an annealing temperature of 58°C in accordance with our standard PCR conditions. The aim of the experiment was to ascertain whether or not our *Agrobacterium* strain of choice

possessed the necessary set of *Vir* genes to be able to effect plant transformation. The second PCR was performed on DNA samples isolated from the T1 generation of transformed tobacco plants in order to determine whether or not there were any bacterial contaminations in the transformed plants.

3.8 *Agrobacterium*-mediated Transformation Of Tobacco Leaves

6 weeks old tobacco leaves were inoculated with an overnight culture of AGL1 possessing IPKb004-*OeSRC1* and IPKb004-*LEA-dehydrin* complexes (OD_{600}:0.7-0.9) for 10 minutes, blotted semi-dry, and then co-cultivated for 3 days on MS medium (Svab *et al*., 1990). After co-cultivation, explants were washed with liquid MS medium supplemented with 160mg/L timentin for 20 minutes. The explants were then transferred to MS medium containing hygromycin (50 mg/L) and timentin (160mg/L) for transformed shoot selection and *A. tumefaciens* growth inhibition. Shoots that emerged on MS medium after 6-8 weeks of incubation were transferred to rooting medium supplemented with the same antibiotics. After 4 weeks plantlets which had well developed root systems were transplanted into pots and transferred to the green house.

3.8.1 Determination of lethal hygromycin concentration

The IPKb004-*OeSRC1* complex possessed *Hpt II* (*Hygromycin phoshotransferase* gene) gene in the T-DNA region, which enabled the putative transgenic plants to withstand the toxic effects of hygromycin whereas wild type (control) ones could not. In order to use negative controls in the experiments, the lethal hygromycin concentration for tobacco plants had to be determined. Wild type seeds and explants of young healthy tobacco plants were prepared and placed on MS medium containing varying concentrations of hygromycin of 30mg/L and 50 mg/L and photographs taken after 3-4 weeks.

3.8.2 Analysis of putative transgenic tobacco plants

In order to verify the integration, expression and inheritance of the transferred DNA, Putative T1 transgenic plants of tobacco were analyzed by using both molecular and physiological methods.

3.8.3 Molecular analysis of putative tobacco plants

3.8.3.1 DNA isolation from T1 leaves

Genomic DNAs were extracted from trangenic leaves using the manufacturer's protocol of the ZR plant/seed DNA MiniPrep™ kit (Zymo research). 150 mg of finely cut leaves were transferred to a ZR bashingbead™ and lysed in 750 µl Lysis Solution by vortexing for 10 minutes. The ZR bashingbead™ lysis tube was centrifuged in a microcentrifuge at 10000 x g for 1 minute, 400 µl of the supernatants transferred to a zymo-spin™ IV spin filter placed in a collection tube and centrifugation done at 7000 rpm for 1 minute. 1200 µl of plant/seed DNA binding buffer was then added to the filtrate in the collection tube from the previous step and 800 µl of the mixture transferred to a zymo-spin™ IIC column in a collection tube. Centrifugation was immediately carried out at 10000 x g for 1 minute and the flow through discarded from the collection tube. 800 µl of the plant/seed DNA binding buffer and filtrate mixture was again transferred to a zymo-spin™ IIC column in the collection tube and the centrifugation step repeated. Afterwards, 200 µl of DNA pre-wash buffer was added to the zymo-spin™ IIC column placed in a new collection tube and centrifuge at 10000 x g for 1 minute. 500 µl plant/seed DNA wash buffer was then added to the zymo-spin™ IIC column and centrifuged at 10,000 x g for 1 minute. The zymo-spin™ IIC column was transferred to a clean 1.5 ml microcentrifuge tube, 50 µl DNA elution buffer added directly to the column matrix and centrifuged at 10000 x g for 30 seconds to elute DNA. The eluted DNAs were immediately transferred to a prepared zymo-spin™ IV-HRC spin filter placed in a clean 1.5 ml microcentrifuge, centrifuged at exactly 8000 x g for 1 minute and stored at -20°C.

3.8.3.2 PCR with *OeSRC1* and *Hpt II* specific primers

The isolated DNA from T1 transgenic plants and control plants were analyzed with PCR for the presence of *OeSRC1* and *Hpt II* genes with *OeSRC1* and *Hpt II* specific primers.

Table 3.2 PCR conditions for gene amplifications.

	OeSRC1	***Hpt II***	
Initial denaturation	95°C 4min	95°C 4min	
Denaturation	95°C 40min	95°C 40s	
Annealing	56°C 40s	56°C 40 s	35 cycles
Extension	72°C 35s	72°C 45 s	
Final extension	72°C 5min	72°C 5min	

Table 3.3 Composition of PCR reactions.

Reagents	***OeSRC1* (Amount)**	***Hpt II* (Amount)**
dH2O	7µL	7 µL
10 µM Primer Forward	1 µL	1 µL
10 µM Primer Reverse	1 µL	1 µL
Master mix	10 µL	10 µL
DNA	1 µL	1 µL
Total reaction	20 µL	20 µL

Table 3.4 Primers for *OeSRC1* and *HptII* genes.

Gene	Forward Primer	Reverse primer
OeSRC1	CACCATGGCAGGAATCATTCAC	CTAATCGCTATCGCTGCT
HptII	GATGTAGGAGGGCGTGGATA	ATAGGTCAGGCTCTCGCTGA

3.8.3.3 Mendelian segregation analysis.

Mendelian segregation analysis of T1 seeds was done by growing putative transgenic and wild type tobacco plants on MS medium supplemented with 50mg/L hygromycin after surface sterilization with 20% hypochloride for 20 minutes in eppendorf tubes. The number of surviving plantlets on hygromycin medium was counted to determine the Mendelian inheritance pattern after 4 weeks of incubation at 25 °C with 8h/16h photoperiod. The expected 3:1 ratio of Mendelian inheritance was evaluated by Chi-square analysis (goodness of fit) with p ($\chi 2 \leq 3.841$) =0.95.

3.9 Physiological Analysis Of Transgenic Tobacco Plants

To determine the expression of *OeSRC1*, the following physiological studies were carried out on putative transgenic tobacco plants;

a. Seed germination and plant growth characteristics under salt stress conditions.

b. Seed germination and plant growth characteristics under drought (water stress) conditions.

3.9.1 Salt stress studies

3.9.1.1 Determination of lethal salt concentration in wild type tobacco plants.

To determine the level of salt concentration seeds of wild type tobacco could tolerate, tobacco seeds were grown in MS containing varying concentrations of salt (NaCl) of 50 mM, 100 mM, 150 mM, and 200 mM. Photographs were taken in the course of plants' growth and growth performances observed and compared. Finally salt concentrations observed to be lethal to wild type seeds were noted and used for further studies on putative transgenic seeds.

3.9.2 Drought stress studies

Drought stress studies were carried out on putative and wild-type plants by growing seeds on MS medium containing and 200 and 400 mM mannitol. Observations were made on important plant characteristics such as germination capacity, root length and plant growth and general plant performance.

3.10 Statistical Analysis.

Data was analysed using the chi square analysis (goodness of fit method) and with Microsoft excel.

4 RESULTS AND DISCUSSIONS

4.1 cDNA Synthesis And Gradient PCR

OeSRC1 gene was amplified from a single strand cDNA synthesized from olive leaf RNA. The amplifications were performed at different temperatures of 46°C, 47.9°C, 49.9°C, 54 °C and 56°C and the optimum annealing temperature of the *OeSRC1* gene determined to be 56°C. In the case of *LEA-dehydrin* cDNAs were synthesized from RNAs isolated from the roots and leaves of Ispir (a salt tolerant variety of bean) subjected to salt stress. Using the resulting single strand cDNA as template DNA, *LEA-dehydrin* gene was also amplified at a temperature of 56°C. The electrophoregrams below show amplicon sizes of 500bp and 534bp for *OeSRC1* and *LEA-dehydrin* respectively.

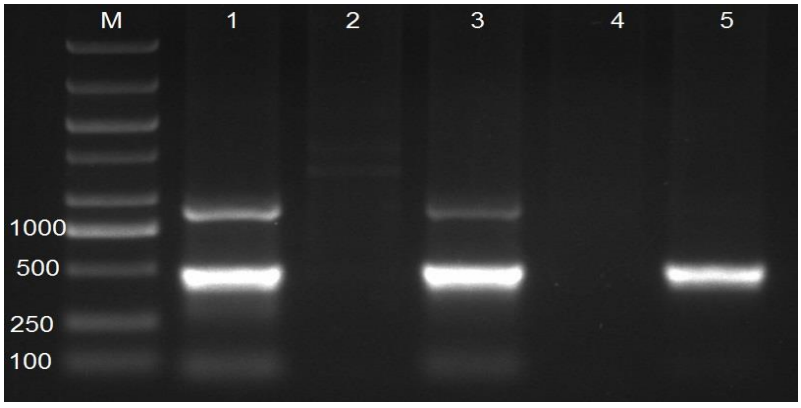

Figure 4.1 Agarose gel (1%) electrophoresis of *OeSRC1* gene. Each lane shows a different annealing temperature (46°C, 47.9°C, 49.9°C, 54 °C and 56°C).

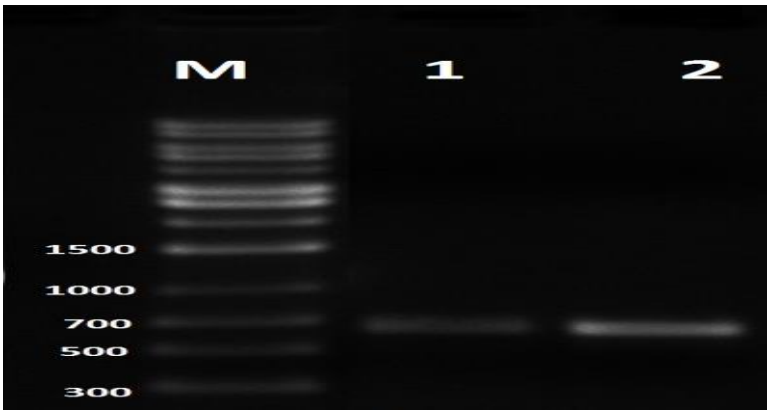

Figure 4.2 Agarose gel (1%) electrophoresis of *LEA-dehydrin* gene. Lane 1 represent ampification of cDNA synthesized from RNA extracted from the leaf of salt stressed Ispir. Lane 2 is a representation of the amplicon amplified from cDNA synthesized from the root of salt stressed Ispir.

4.2 Cloning of *OeSRC1* And *LEA-dehydrin* Genes Into Entry Vectors.

PCR products of *OeSRC1* and *LEA-dehydrin* genes (root cDNA was used as template) were ligated to the pENTR™/D-TOPO vector using the gateway cloning system. To ensure directional insertion of the genes of interest into the entry vector, CACC nucleotides were added to the forward primers of both genes. The ligated products were then transformed into competent *E-coli*. cells. Transformed cells were grown on selective LB medium containing 50 mg/ml kanamycin and colony PCR performed with *OeSRC1* and *LEA-dehydrin* genes specific primers for positive colony identification.

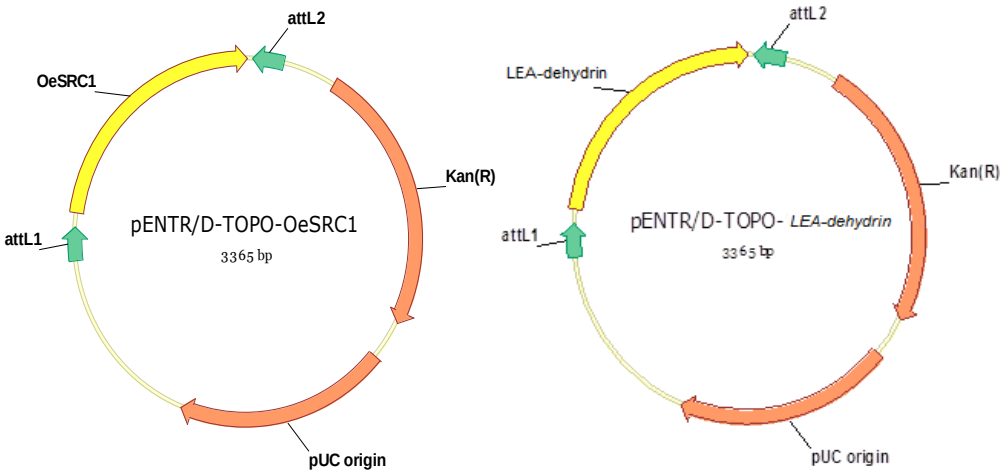

Figure 4.3 Vector maps of the pENTR™/D-TOPO-*OeSRC1* and *LEA-dehydrin* complexes.

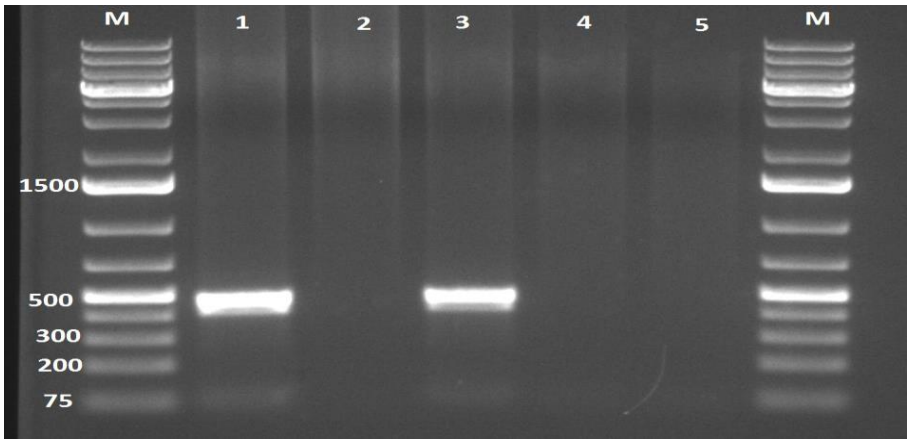

Figure 4.4 Agarose gel (1%) electrophoresis of PCR of *OeSRC1* gene after entry clone creation. Each lane represents amplified bands of plasmids isolated from a different colony used for PCR.

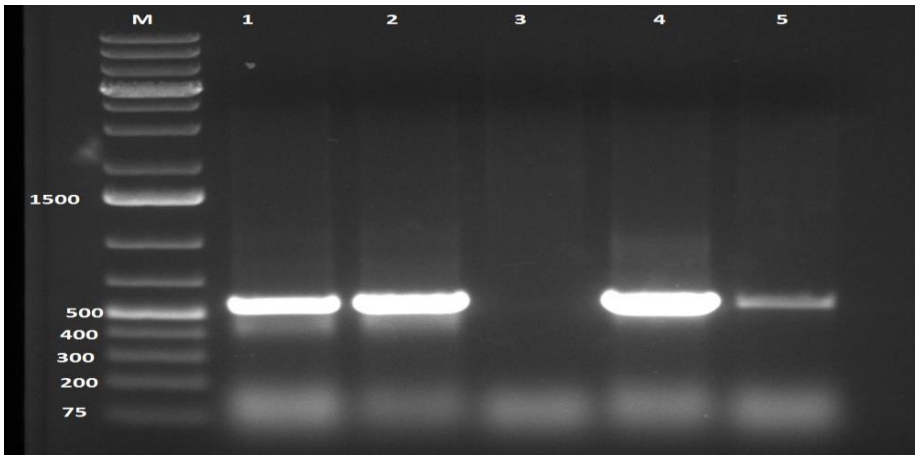

Figure 4.5 Agarose gel (1%) electrophoresis of colony PCR of *LEA-dehydrin* gene after entry clone creation. Each lane represents a different colony used for the amplification of *LEA-dehydrin* gene.

Two positive colonies were selected from each transformation event after PCR for plasmid isolation. After plasmid isolation, *OeSRC1* and *LEA-dehydrin* sequences were confirmed through sequencing after which positive plasmids (plasmids from colony number 3 and 2 came out positive for *OeSRC1* gene and *LEA-dehydrin* respectively) were used for LR cloning with the over-expression vector to produce the final transformation vectors. In this study, the pIPKB004 overexpression vector possessing the maize Ubi1 promoter was used as the destination vector of choice for LR cloning, the final step of the Gateway cloning system. LR reaction was performed by ligating pENTR-DTOPO-*OeSRC1* and pENTR-DTOPO-*LEA-dehydrin* to pIPKB004 to generate final over-expression vectors for *Agrobacterium*-mediated transformation of tobacco. Recombinant products were transformed into chemically competent *E. coli* cells and grown overnight in LB medium supplemented with Spectinomycin (100 mg/L). Colony PCR and electrophoresis runs were then performed on individual colonies for positive colony identification. The results in (fig. 4.7) show that we were able to successfully create expression clones for both *OeSRC1* and *Hpt II* genes as well as successfully amplify and identify these genes on agarose gel.

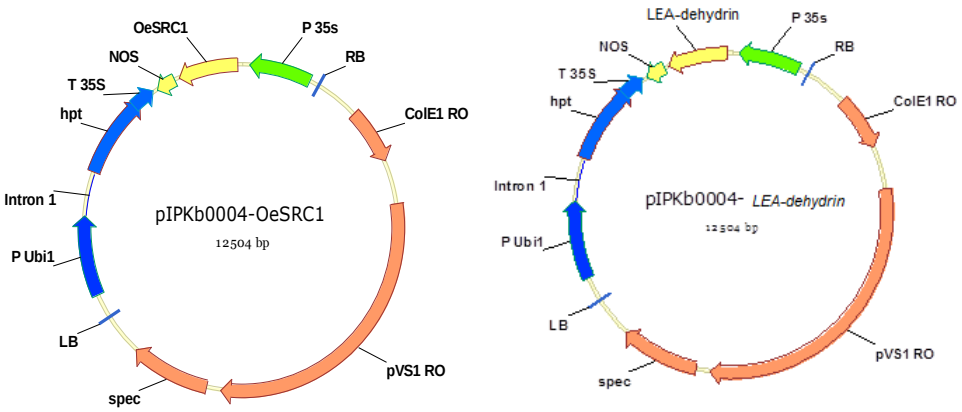

Figure 4.6 Vector maps of the pIPKb0004-*OeSRC1* and *LEA-dehydrin* complexes.

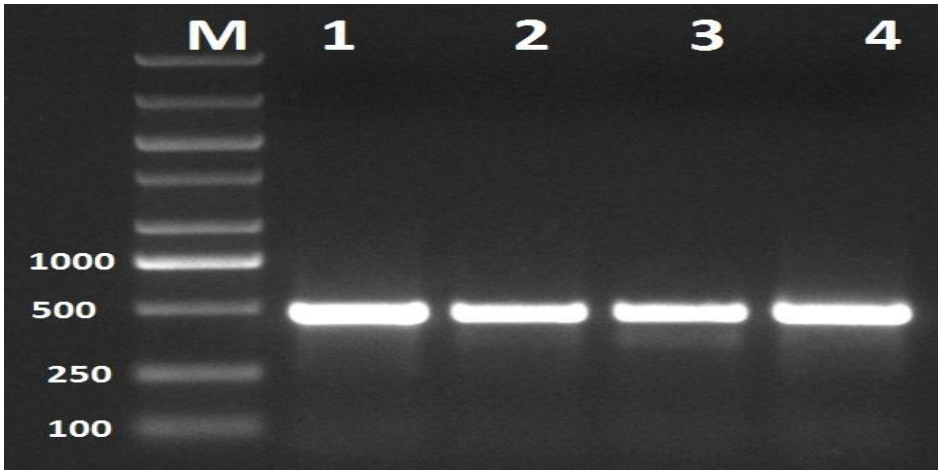

Figure 4.7 Agarose gel (1%) electrophoresis of colony PCR of *OeSRC1* gene after expression clone creation. Each lane represents an independent colony used for the amplification of *OeSRC1* gene.

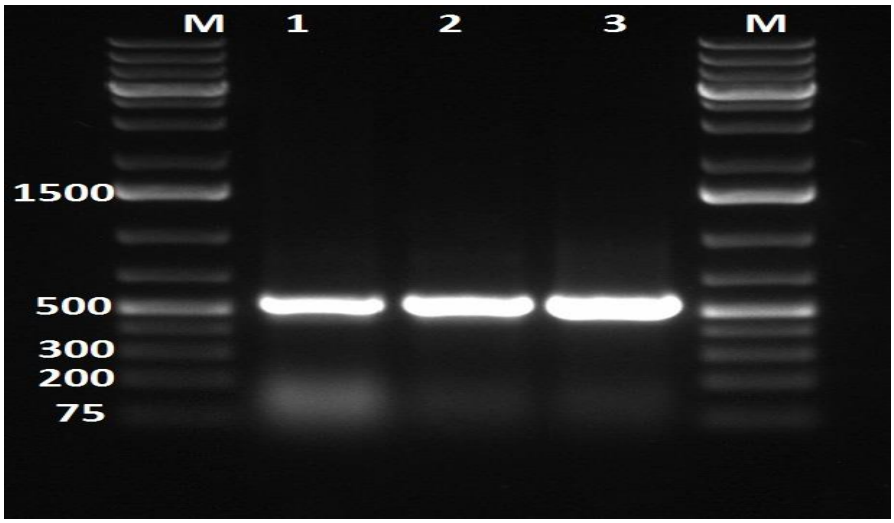

Figure 4.8. Agarose gel (1%) electrophoresis of colony PCR of *LEA-dehydrin* gene after expression clone creation. Each lane represents a different colony used for the amplification of *LEA-dehydrin* gene.

4.3 *Agrobacterium* Transformation (AGL1) With *OeSRC1* and *LEA-dehydrin* Genes

Expression vectors containing *OeSRC1* and *LEA-dehydrin* genes were electroporated into *Agrobacterium tumefaciens* strain AGL1. To ascertain the presence of our genes of interest in *Agrobacterium*, colony PCR and electrophoresis runs were performed. The electrophoregrams below show the successful identification of our genes of interest after colony PCR and electrophoresis runs.

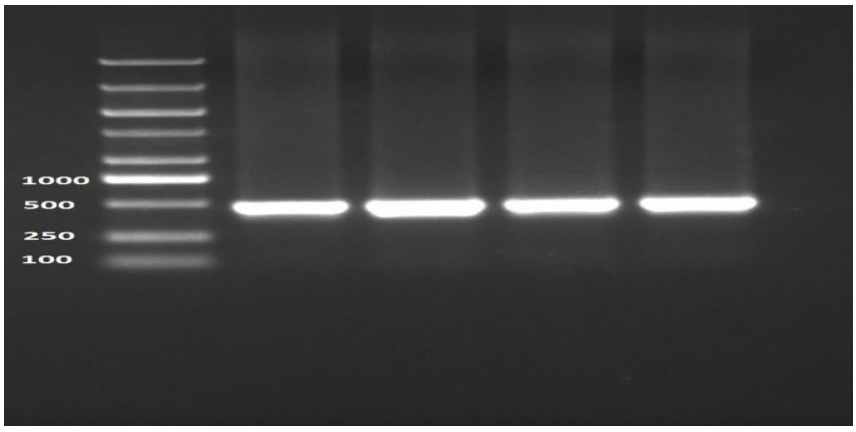

Figure 4.9 Agarose gel (1%) electrophoresis of colony PCR of *OeSRC1* gene after *Agrobacterium* transformation. Each lane represents a different colony used for the amplification of *OeSRC1* gene.

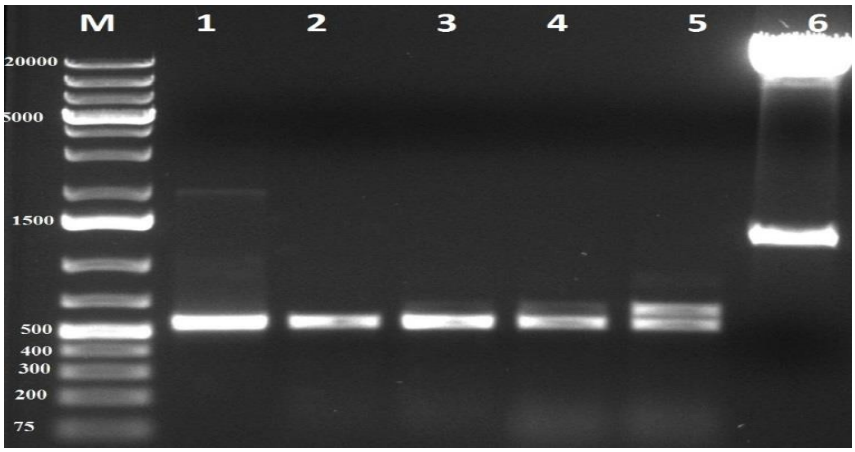

Figure 4.10 Agarose gel (1%) electrophoresis of colony PCR of *LEA-dehydrin* gene after *Agrobacterium* transformation. Lane 1 represents the control experiment, lane 2 to 5 represents different independent colonies used for the amplification of *LEA-dehydrin* gene.Lane 6 shows test digestion of pIPKB004-*LEA-dehydrin* complex with Ecor RV.

One major characteristic of a suitable agrobacterium strain that is the presence of a Ti plasmid with functional *Vir* genes. *Vir* genes are a collection of genes that function in recognising plant signals and excising T-DNA. To ascertain whether or not our agrobacterium strain of choice AGL1 possessed this very important set of genes, PCR was performed on our agrobacterium cells with *Vir D* specific primers. The electrophoregrams below show the successful amplification of the 893 bp *Vir D* gene of the Agrobacterium strain AGL1.

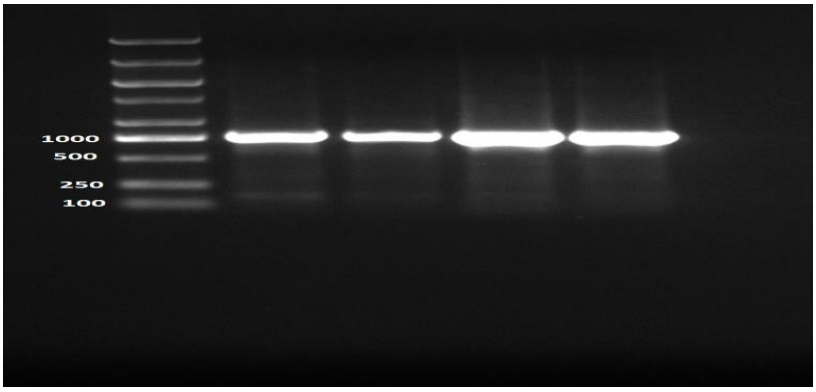

Figure 4.11 Agarose gel (1%) electrophoresis of colony PCR of *Vir D* Agrobacterium strain AGL1. Each lane represents an independent colony used for the amplification of *Vir D* gene.

4.4 Determination of Effective Hygromycin Dosage

Hygromycin B is an aminoglycosidic antibiotic which is usually used as the selection agent of choice for gene transfer studies involving plant vectors that possess the hygromycin resistance gene (*Hpt II gene*). Hygromycin B kill cells by inhibiting protein synthesis. The resistance gene encodes a kinase (*Hygromycin phosphotransferase, Hpt*) that inactivates Hygromycin B through phosphorylation. Depending on the type of cell, media, growth conditions and the metabolic rate of the cell, the working concentration of hygromycin may change. Commonly used concentrations for selection are 200 mg/L for mammalian cells, 20-200 mg/L for plant cells and bacteria cells and 200-1000 mg/L for fungi (AG Scientific, 2005). In a study with orchids, 5 mg/mL was used for selection by (Liau *et al.*,2003). In the present study, both transgenic and non-transformed explants of of tobacco were tested with 30 mg/ml and 50 mg/ml concentrations of hygromycin B. Both concentrations proved effective in selecting against non-transformed plants as can be seen from fig 4.12.

50mg/ml hygromycin containing MS 30mg/ml hygromycin containing MS

Figure 4.12 The growth of seeds and tobacco explants in MS containing 50mg/ml and 30 mg/ml hygromycin.

4.5 *Agrobacterium*-mediated Transformation Of Tobacco Leaves.

Tobacco leaf discs were inoculated with transformed *Agrobacterium* as described in chapter 2. Explants were co-cultivated in darkness for 3 days after which they were washed as described earlier and then transferred to MS medium containing hygromycin (50 mg/L) and timentin (160 mg/L) for transformed shoot selection and *A. tumefaciens* growth inhibition. Shoots that emerged 6-8 weeks after incubation were transferred to rooting medium supplemented with the same antibiotics. After 4 weeks, plantlets with well developed root systems were transplanted into pots and transferred to the green house. In all 30 independent transgenic lines were generated for *OeSRC1* genes and 14 lines for *LEA-dehydrin* gene.

OeSRC1 transgenic lines *LEA-dehydrin* transgenic lines.

Figure 4.13 Photographs of transgenic tobacco lines.

4.6 Analysis Of Putative T1 Transgenic Tobacco Plants Expressing the *OeSRC1* Gene.

The putative transgenic plants were allowed to self pollinate and grow to maturity. Matured seeds were then collected and a series of analysis carried out on them.

4.6.1 Molecular analysis

4.6.1.1 Mendelian inheritance

Seeds from the putative T1 generation of transgenic tobacco plants were surface sterilised in 20 percent sodium hypochloride for 20 minutes and washed five times with sterile water. 10 seeds each of the 30 putative transgenic lines were cultured on a selective MS medium containing 50 mg/ml hygromycin. This experiment was carried out in three replicates. After a period of 8 weeks the mendelian inheritance analysis was evaluated by computing the number of surviving plantlets against non-surviving plantlets for transgenic and non-transformed plants based on the formula $p(\chi 2 \leq 3.841)=0.95$ (Kavas, 2011). Per the formula given it was observed that most of the putative transgenic lines displayed the mendelian segregation ratio of 3:1 despite the fact other transgenic lines such as lines 8, 12 and 20 did not conform to this ratio.

Table 4.1 Mendelian Inheriatance Analysis of T1 progeny, p ($\chi 2 \leq 3.841$) =0.95

Line	Total Number of Seeds	Number of Germinated Seeds	χ^2	Decision
Wild-type tobacco	30	0	300	Reject
1	30	20	3.413	Fail to Reject
4	30	24	1.333	Fail to Reject
5	30	20	3.413	Fail to Reject
7	30	22	0.213	Fail to Reject
8	30	28	17.280	Reject
10	30	24	1.333	Fail to Reject
12	30	26	6.453	Reject
14	30	20	3.413	Fail to Reject
18	30	24	1.333	Fail to Reject
20	30	27	12	Reject
28	30	27	12	Reject

4.6.1.2 PCR analysis of putative transgenic tobacco plants

PCR was carried out on the genomic DNAs of T1 putative transgenic tobacco plants with *OeSRC1*, *hygromycin phosphotransferase II gene* (*Hpt II*) and *Vir D* specific genes. The aims of this experiment were to ascertain the presence or absence of our transgene as well as bacterial contamination in putative transgenic plants. The presence of our transgene and the *Hpt II* genes were not observed in our wild-type plants. The presence of the *OeSRC1* and *Hpt II* genes were observed in all the randomly selected transgenic lines tested. However, none of the tested transgenic lines possessed the *Vir D* gene. The identification of the *OeSRC1* and *Hpt II* genes in putative transgenic platnts is an indication that our transgene was successfully integrated into the tobacco plants. The absence of the *Vir D* gene in transgenic plants however signifies the absence of contaminating agrobacterial cells.

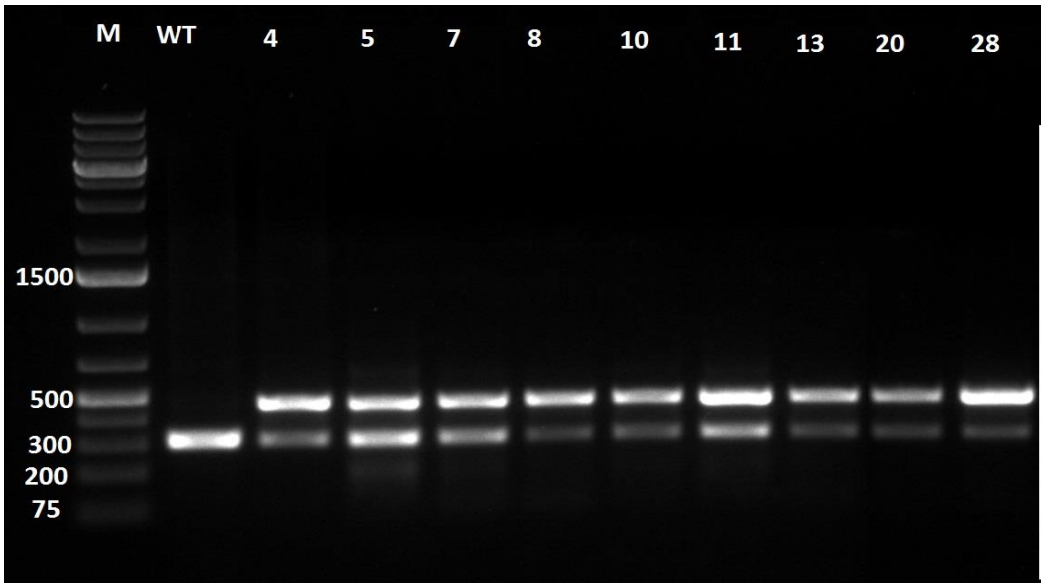

Figure 4.14 Agarose gel (1%) electrophoresis of PCR of *OeSRC1* in Putative transgenic plants. WT represents wild-type tobacco plant. Each other lane representsan independent transgenic line used for amplification of *OeSRC1* gene.

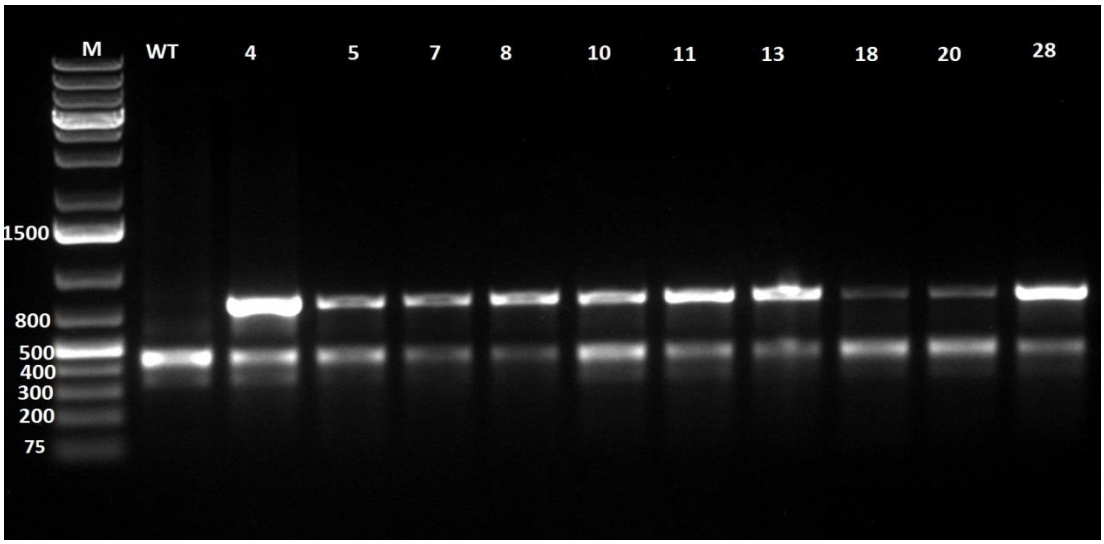

Figure 4.15 Agarose gel (1%) electrophoresis of PCR of *hygromycin phosphotransferase II gene* (*Hpt II*) in Putative transgenic plants. WT represents wild-type tobacco plant. Each other lane represents an independent transgenic line used for the amplification of the *Hpt II* gene.

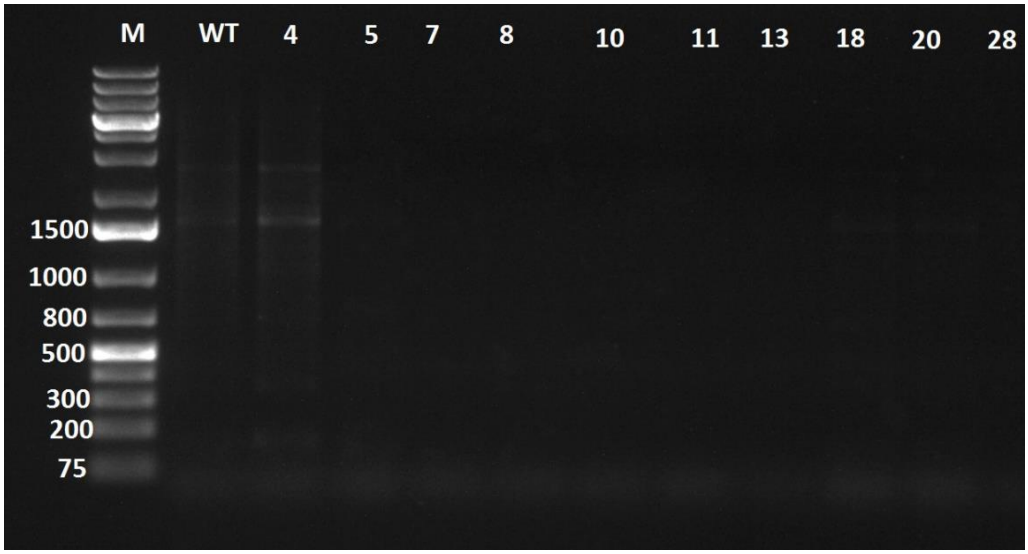

Figure 4.16 Agarose gel (1%) electrophoresis of PCR of *Vir D* gene in Putative transgenic plants. WT represents wild-type tobacco plant. Each other lane represents an independent transgenic line used for amplification of the *Vir D* gene.

4.6.2 Physiological analysis of putative transgenic tobacco plants

The effects of drought, salt and cold on the physiology of wild-type and putative transgenic plants were determined through a series of stress test and observations of important plant characteristics such as germination capacity, root length, plant height and plant general appearances made.

4.6.2.1 Hygromycin test

Seeds of T1 transgenic plants and wild type plants were grown on MS medium containing 50mg/ml hygromycin. After a period of 4 weeks, it was observed that transgenic plants survived whereas wild-type plants died. Transgenic plants were able to maintain normal growth and survive the killing effect of hygromycin B because they possessed the hygromycin resistance gene (*Hpt II gene*).

Wild-type tobacco plants. Transgenic tobacco plants.Line 7.

Figure 4.17 The growth of wild-type and transgenic plants on MS supplemented with 50mg/ml hygromycin.

4.6.2.2 Drought stress test

The effects of drought stress on the physiological characteristics of both transgenic and wild-type plants were tested by growing seeds on MS containing 200mM and 400mM mannitol. The first and foremost effect of drought on plants is usually observed in the form of severe seed germination inhibition leading to reduced plant stand (Harris *et al*., 2002 , Kaya *et al*., 2006).

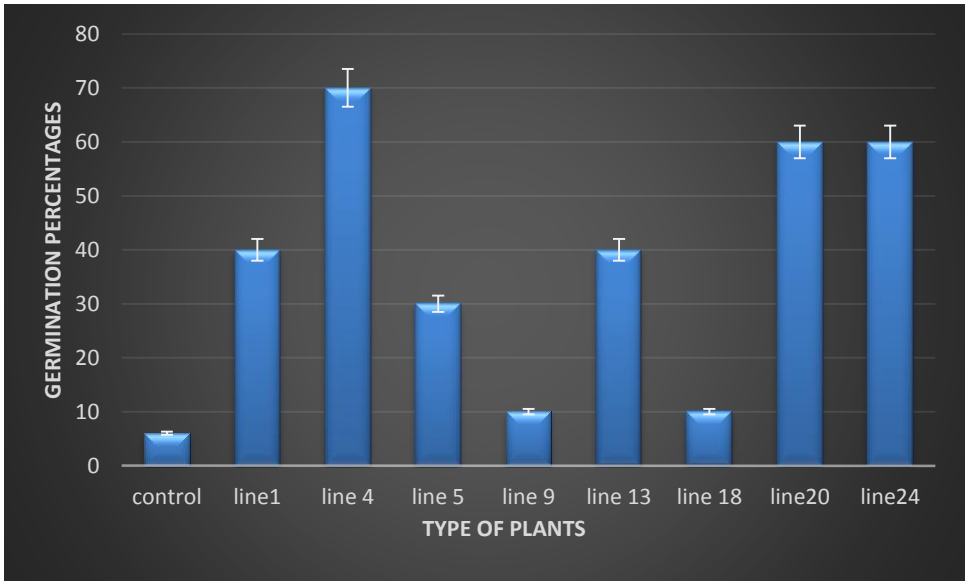

Figure 4.18 Germination percentages of tobacco plants grown on 400mM mannitol after 30 days.

There was no significant difference in the germination capacities of transgenic and non-transgenic tobacco plants grown on 200 mM mannitol. However, germination was highly delayed in both transgenic and wild-type plants grown on 400mM mannitol (extreme drought stress). Despite the delay in germination, transgenic lines recorded higher germination percentages than non-transgenic lines.

Plant growth was also observed to be highly sensitive to drought. This effect just as Taiz and Zeiger in 2006 noted could be due to the reduced turgor pressure occasioned by drought in the plants (Taiz and Zeiger, 2006).

Figure 4.19 Growth of 7 weeks old tobacco plants on 200 Mm mannitol containing MS.

Transgenic lines were however, observed to be healthier, taller and possessed well developed roots and leaves compared to wild-type plants. The observed phenomenon of reduced plant growth and general plant performance in wild-type plants is in line with the observation made by Nanomi about the fact that water deficit stress led to impaired mitosis, reduced cell elongation and expansion which consequently caused reduction in plant growth, leaf area and plant height (Nanomi, 1998).

Figure 4.20 Root lengths of 7 week old tobacco plants grown MS supplemented with 200 mM mannitol.

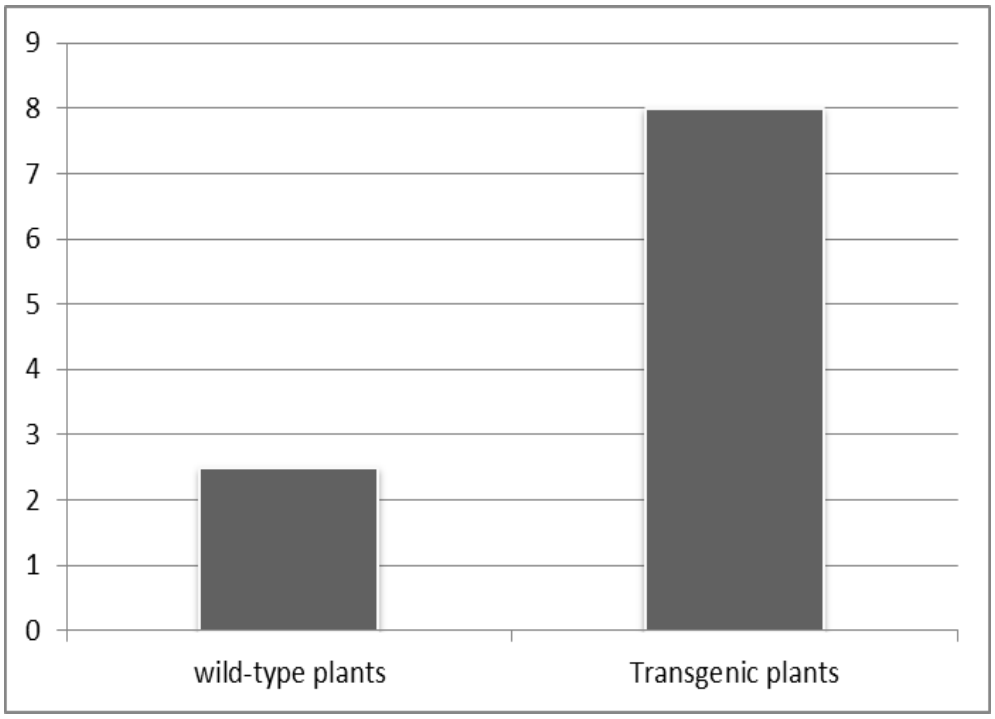

Figure 4.21 Average lengths of tobacco roots after 7 weeks growth on 200mM mannitol

He further stated that growth in higher plants under severe water stress could be inhibited as a result of inadequate water flow from the xylem to neighbouring enlongating cells.

The ability of transgenic lines to maintain high germination capacity, normal growth functions and display healthy growth and root characteristics could also be attributed to the presence of *OeSRC1*, a dehydrin like gene in their genome, a confirmation of Hara *et al*., 2003; Shekhawat *et al*., 2011; Xie *et al*., 2012; Yang *et al*., 2014 who demonstrated that the transgenic expression of dehydrin could lead to drought tolerance in plants.

4.6.2.3 Salt stress test

The effects of salt stress on transgenic and wild-type plants were tested by growing seeds on MS containing 150mM and 200mM sodium chloride.

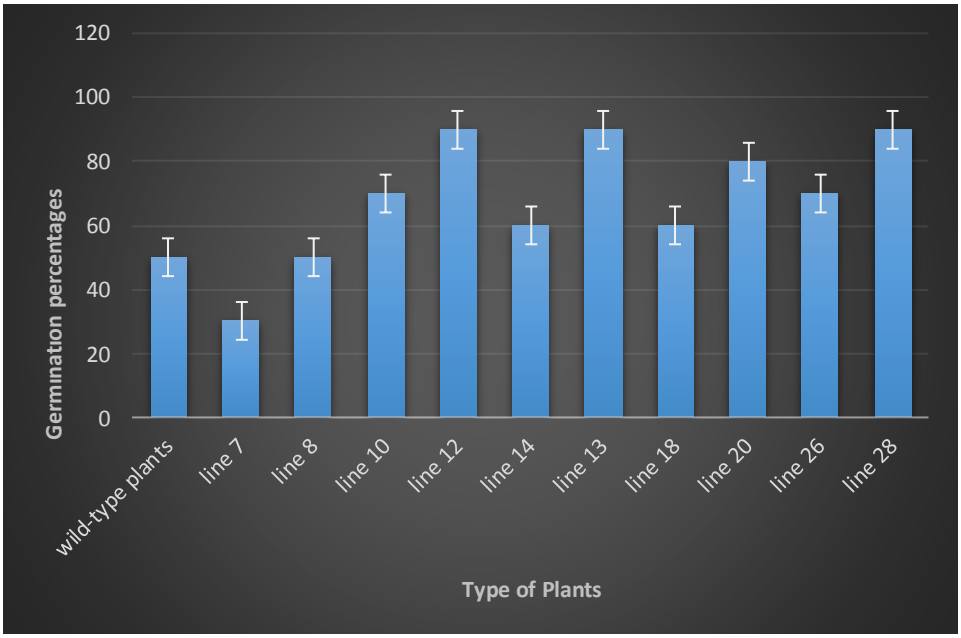

Figure 4.22 Germination percentages of tobacco plants after 21 days of growth in 200mM NaCl.

Generally wild-type plants recorded lower germination rates compared to *OeSRC1* transgenic lines with the exception of plants of transgenic line 7. Dehydrins, the superfamily to which *OeSRC1* belongs, have been demonstrated through a series of experiments such as far-UV circular dichroism technique etc. to possess high levels of hydrophilic amino acids and display changes in their conformation in accordance with changes in their ambient microenvironment (Lisse *et al*., 1996). These characteristics help dehydrins avoid denaturation and thereby protect plants from damage and death.

Wild-type plants Transgenic plants (Line 7)

Figure 4.23 Growth of 8 weeks old transgenic and wild- type tobacco plants on 150mM NaCl containing MS.

Figure 4.24 Root lengths of 8 weeks old transgenic and wild type tobacco plants grown on MS containing 150Mm NaCl.

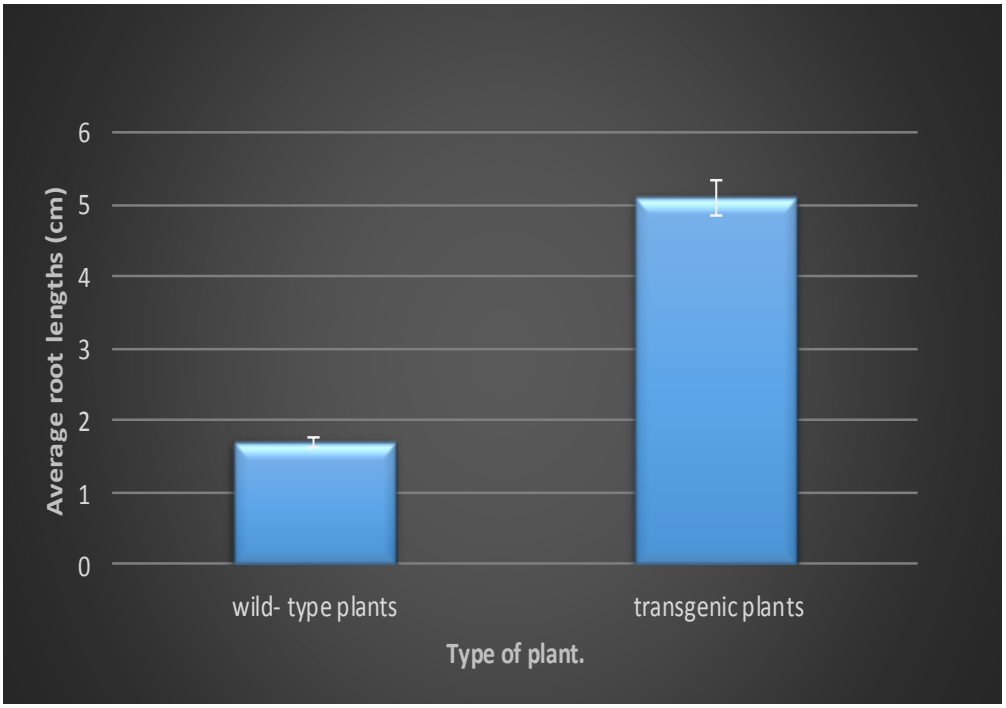

Figure 4.25 Average root length of tobacco plants at 8 weeks growth on 150mM NaCl

Root and shoot length measurements have been observed to be the principal parameters in salt stress studies due to the fact that the root is directly in contact with the soil from where it absorbs water and passes it on to the shoot for transportation to other parts of the plant (Jamil and Rha, 2004). In the present study, shoot and root

development in wild-type plants were observed to be inhibited in both wild-type plants and transgenic lines. We further observed that salt stress inhibited root development more than shoot development. A similar observation was reported by Datta and colleagues in 2009 to the effect that salinity had a more pronounced effect on root length as compared to shoot length because of the direct exposure of roots to the saline environment. However the rate of inhibition was far lower in transgenic plants than in wild-type plants. This effect could be attributed to the lethal influence of the higher levels of NaCl which resulted in the reduction of seedlings' capacity to extract nutrients from the growth media. High salt levels may also have contributed to the stunted growth of roots and shoots due to the negative effects it poses on plants'water uptake capacity (Datta *et al*., 2009). The ability of transgenic plants to maintain better growth and physiological functions compared to wild-type plants could be attributed to the presence of the *OeSRC1*, a dehydrin in their genome. This result has been confirmed by (Xu *et al*., 1996) and (Brini *et al*., 2007) who stated that plant salt tolerance could be improved through the over expression of *HVA1* and wheat dehydrin *DHN-5*.

5 CONCLUSIONS

The aims of the present study was to clone and investigate the functions of *OeSRC1* and *LEA-dehydrin* genes in tobacco plants.

The Gateway cloning system was employed in the cloning of our genes of interest. These genes were first of all cloned into the pENTR/D-TOPO entry vector and later into the pIPKB0004 overexpression vector to create our expression clones. After the creation of expression clones, the next step of the experiment was *Agrobacterium* transformation of our genes of interest. To do this recombinant expression vectors bearing our genes of interest were electroporated into the AGL1 strain of *Agrobacterium*. Tobacco plants were then transformed with our transformed Agrobacterium to produce transgenic lines. In all 30 and 14 independent transgenic lines were generated for *OeSRC1* and *LEA-dehydrin* respectively. Due to the constraint of time, further analyses were only carried out on *OeSRC1* transgenic lines.The analyses carried out included Mendelian inheritance test, hygomycin test, salt stress test and drought stress test

At the end of the experiment, we were able to for the first time successfully clone and transform the *OeSRC1 and LEA-dehydrin* genes into tobacco plants. The result of functional studies carried out on *OeSRC1* transgenic lines revealed that these lines were tolerant to both drought and salt stresses and therefore can be used as candidate genes for breeding against dehydrative stresses in plants.

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CURRICULUM VITAE

PERSONAL DETAILS

Name ADUSE POKU SAMUEL

Marital status: Single

Country of Birth Ghana

Nationality: Ghanaian

Date of Birth: 4th Sept, 1988

Language Spoken: English, Twi and Turkish

Religion: Christianity

Address: Post Office Box 66,

Abuakwa-Kumasi.

Mobile: +905534166729

Email: adusepokusamuel@yahoo.com

PROFILE

A highly effective and committed individual with a creative flair and an enduring positive attitude. I am always ready to learn and determined to make worth contribution to whatever I get involved in. I am a motivated person with excellent communication skills and positive approach to work, goal oriented with a strong ability to work under pressure and own initiative, while at the same time maintaining a calm and reassuring demeanour.

CAREER OBJECTIVE

My interest is to pursue a career of research in the field of biotechnology and breeding with the aim of excelling in academia and applying the acquired knowledge and skill through research and spreading the acquired knowledge through teaching and training.

KEY SKILL

- Ability to work well on my own and a very good team player.
- Able to work on multiple tasks and liaise effectively at all levels.
- Excellent analytical skills and hardworking.
- Excellent communication, interpersonal skills and a creative thinker.
- Experienced in Microsoft packages and the internet.

EDUCATION/QUALIFICATIONS

Sept.2013- Feb-2016 **Ondokuz Mayis university**

Master of Science Agricultural biotechnology.

Sept. 2007 – Dec. 2011 **University for Development studies.**

- Bachelor of Science Agricultural technology (Biotechnology option) First class honours.

Oct. 2003 – August 2006 **Asanteman Senior Secondary School**

- Senior Secondary School Certificate Examination (**General Science option**)

RESEARCH EXPERIENCES AND SEMINARS ATTENDED

University for Development Studies – Third Trimester Field Practical Training Program

Yakubupe, East-Gonja N/R - 2008- 2010

- Developing community profile
- Carried out rural livelihood and development studies.
- Planning and management of rural potentials for development.
- Helping the rural community to identify their strength and Weaknesses.
- Helping create awareness on the importance of Education to development
- Participated in building team strategies and group work plan
- Developing proposal and writing reports for the community

UNIVERSITY FOR DEVELOPMENT STUDIES

- Dissertation: The effects of different levels of petroleum contamination on the growth of soya-beans.

COCOA RESEARCH INSTITUTE. BOLE SUB-STATION DEC-JUNE 2012.

- Project: First phase of cashew hybrid and hybridisation project**.**

18th NATIONAL CONGRESS ON BIOTECHNOLOGY TURKEY 18-19DEC.,2015.

- Cloning of *OeSRC1* gene and functional analysis in tobacco plants**.**

ONDOKUZ MAYIS UNIVERSITY.

- Masters dissertation: Cloning of *OeSRC1* and *LEA-dehydrin* genes and functional analysis in tobacco plants**.**

LABORATORY TRAINING AND SKILLS

- Expert in PCR and DNA techniques such as SSR,RAPD,AFLP etc.
- I am very conversant with bioinformatic tools.
- Highly trained in genetic modification and breeding.
- Highly trained in gene cloning techniques
- 7^{th}-10^{th} March,2011 One week demonstration in bacteria identification, isolation, anti biogram test and milk analysis for safety with Universad Complutense de Madrid (UCM) at the Spanish Laboratory of UDS Nyankpala Campus.
- 28^{th} January-5^{th} February,2011.One week training in DNA extraction, DNA visualization and tissue culture techniques at the Biotechnology and Nuclear Agriculture Research Institute (BNARI) at Kwabenya.
- 22^{nd} -25^{th} June, 2010. Practical attachment on Water quality analysis for total and faecal coli forms in water samples and media preparation for bacteria culturing at the Water Research Institute, Tamale.
- 20^{th}-25^{th} June, 2010.Practical attachment to Zoomlion Ghana Limited on the study of waste management.

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CAREER HISTORY

Oct. 2010- Oct.2011 **NATIONAL SERVICE** **(**Cocoa Research Institute Ghana Bole Sub-station).

- Data entry into data base.
- Yield assessments.
- Recording of meteorological data.
- Research into developing high yielding varieties of cashew through controlled hand pollination.
- Ensuring that the nursery is taken care of.
- Cuttings making and propagation
- Propagator construction and data handling.
- Data generation and scientific report writing.

Aug. 2008/

Sept. 2008 **ATTACHMENT (AKUAFO ADAMFO COCOA MARKETING COMPANY LIMITED)**

- Data processing.
- Quality assessment of cocoa beans.
- Inventory control.

Aug 2009/

Sept 2009

ATTACHMENT (SAVANNAH AGRICULTURAL RESEARCH INSTITUTE)

- Land preparation and field lay-outs.
- Conduction of field trials on cowpea.
- Establishment of experimental fields.
- Scientific report writing.

June 2006/

Sept 2007

TEACHER, DETERMINATION INTERNATIONAL SCHOOL

- Preparation of lesson notes.
- Ensuring discipline among students.
- Provided tuition and guidance for students

ACADEMIC ACHIEVEMENTS

- Overall best student, University for Development Studies 2011/2012 graduating class.
- Valedictorian, 12th congregation of the University for Development studies.
- Best student of the 2011/2012 graduating class of the Faculty of Agriculture.

RESEARCH SKILLS AND TECHNIQUES

- I can recognize and validate problems
- I can demonstrate original, independent and critical thinking, and the ability to develop theoretical concepts
- I have a knowledge of recent advances within my field and in related areas
- I understand relevant research methodologies and techniques and their appropriate application within my research field
- I am able to critically analyse and evaluate my findings and those of others
- I am able to summarise, document, report and reflect on my progress

RESEARCH MANAGEMENT

- I can design and execute systems for the acquisition and collation of information through the effective use of appropriate resources and equipment
- I can identify and access appropriate bibliographical resources, archives, and other sources of relevant information
- I use information technology appropriately for recording and presenting information

PERSONAL EFFECTIVENESS

- I have a willingness and ability to learn and acquire knowledge
- I am creative, innovative and original in my approach to research

- I am flexible and open-minded
- I am self-aware and able to identify my own training needs
- I am self-disciplined, motivated, and thorough
- I recognize my own boundaries and draw upon/use sources of support as appropriate
- I show initiative, work independently and i am self-reliant

COMMUNICATION SKILLS

- I can write clearly and in a style appropriate to purpose, e.g. progress reports, published documents, thesis
- I can construct coherent arguments and articulate ideas clearly to a range of audiences, formally and informally through a variety of techniques
- I can constructively defend research outcomes at seminars and viva examination
- I contribute to promoting the public understanding of my research field
- I effectively support the learning of others when involved in teaching, mentoring or demonstrating activities

NETWORKING AND TEAMWORKING

- I am developing and maintaining co-operative networks and working relationships with supervisors, colleagues and peers, within my institution and the wider research community
- I understand my behaviours and impact on others when working in and contributing to the success of formal and informal teams
- I listen, give and receive feedback and respond perceptively to others

INTEREST

I enjoy listening to Music, Reading and meeting people.

REFERENCES

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