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

OVEREXPRESSION OF SlAIM1 GENE ENCODING R2R3 MYB TRANSCRIPTION FACTOR IN POTATO UNDER DROUGHT CONDITION

IREM AYCAN SIREL

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MASTER THESIS

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Supervisor

Asst. Prof. Dr. Allah BAKHSH

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The study entitled "**Overexpression of SlAIM1 Gene Encoding R2R3 MYB Transcription Factor in Potato under Drought Condition**" and presented by Irem Aycan SIREL under the supervision of Asst. Prof. Dr. Allah BAKHSH has been accepted as Master thesis by the jury at the Department of Agricultural Genetic Engineerig of Niğde Ömer Halisdemir University, Graduate School of Natural and Applied Sciences.

Head :Prof. Dr. Muhammad ASIM
Sivas University of Science and Technology

Member :Asst. Prof. Dr. Emre AKSOY,
Niğde Ömer Halisdemir University,

Member :Asst. Prof. Allah BAKHSH,
Niğde Ömer Halisdemir University

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MÜDÜR

THESIS CERTIFICATION

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

Irem Aycan SIREL

SUMMARY

OVEREXPRESSION OF SlAIM1 GENE ENCODING R2R3 MYB TRANSCRIPTION FACTOR IN POTATO UNDER DROUGHT CONDITION

SIREL, Irem Aycan
Nigde Ömer Halisdemir University
Graduate School of Natural and Applied Sciences
Department of Agricultural Genetic Engineering

Supervisor : Asst. Prof. Dr. Allah BAKHSH

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Potato is one of the most widely used crop in agriculture around the world. However, during cultivation, potatoes encounter many biotic and abiotic stress factors. All these stresses can disrupt the normal growth functions of the plant and eventually cause a decrease in tuber yield. Plants have developed various defense mechanisms at the molecular level against stressors. One of these defense mechanisms is changes in gene expression. Engineering of gene(s) encoding transcription factors to plants is an important approach to enhance and develop stress tolerant crop plants. MYB protein genes have a role as a transcription factors which are induced or suppressed in reply to the different abiotic stresses. In this study, tomato (*Solanum lycopersicum*) *SlAIM1* (an ABA-induced MYB transcription factor) gene engineered in potato to evaluate its effect under drought tolerance. Agrobacterium strain *AGL1* harboring *pEarleyGate 100* vector containing *SlAIMI* gene under the control of constitutive promoter (35S) was used to infect leaf, nodal, micro tuber and internodal explants of potato Agria cultivar. The results indicated the overexpression of *SlAIMI* gene under *in vitro* drought condition. These results are expected to potentially contribute to the efforts to increase the tolerance to drought in potatoes through the MYB transcription factor.

Keywords: Potato, drought stress, overexpression of SlAIM gene

ÖZET

MYB TRANSKRİPSİYON FAKTÖRÜNÜN R2R3 AİLESİNİN ÜYESİ OLAN SlAIM1 GENİNİN KURAKLIK KOŞULLARINDA PATATESTE İFADE EDİLMESİ

SIREL, Irem Aycan
Niğde Ömer Halisdemir Üniversitesi
Fen Bilimleri Enstitüsü
Tarımsal Genetik Mühendisliği Anabilim Dalı

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Patates, dünya çapında tarımda en çok kullanılan mahsullerden biridir. Bununla birlikte, yetiştirme sırasında patates birçok biyotik ve abiyotik stres faktörüyle karşılaşır. Tüm bu stres koşulları bitkinin normal büyüme fonksiyonlarını bozabilir ve sonunda yumru veriminde düşüşe neden olabilir. Bitkiler, stresörlere karşı moleküler düzeyde çeşitli savunma mekanizmaları geliştirmişlerdir. Bu savunma mekanizmalarından biri gen ekspresyonundaki değişikliklerdir. Bitkilere transkripsiyon faktörlerini kodlayan gen (ler) in aktarımı, strese dayanıklı mahsul bitkilerini geliştirmek ve geliştirmek için önemli bir yaklaşımdır. MYB protein genleri, farklı abiyotik streslere yanıt olarak indüklenen veya bastırılan transkripsiyon faktörleridir. Bu çalışmada, kuraklık toleransı altındaki etkisini değerlendirmek için patatese domates *SlAIM1* (ABA kaynaklı MYB transkripsiyon faktörü) geni aktarılmıştır. 35S promotörü kontrolü altında, *SlAIMI* geni içeren *pEarleyGate 100* vektörünü barındıran *Agrobacterium AGL1*, patates bitkisinin yaprak, nodal, mikro yumru ve internodal eksplantlarını enfekte etmek için kullanılmıştır. Sonuçlar, in vitro kuraklık koşullarında *SlAIMI* geninin ifadesinin arttığını göstermiştir. Bu sonuçların, MYB transkripsiyon faktörü yoluyla patates bitkisindeki kuraklığa toleransı artırma çabalarına potansiyel olarak katkıda bulunması beklenmektedir.

Anahtar Kelimeler: Patates, kuraklık stresi, SlAIM geninin aşırı ekspresyonu

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SYMBOLS AND ABBREVIATIONS

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

Abbrevations	**Descriptions**
ABA	Abscisic Acid
ABFs	ABRE Finding Factors
AREBs	ABA Responsive Binding Protein
BAP	6-Benzylaminopurine
bp	Base Pair
bZIP	Basic Leucine Zipper
cm	Centimeter
ddH_2O	Double-distilled Water
DF	Degree of Freedom
DNA	Deoxyribonucleic Acid
DREB	Drought-responsive Element-binding Protein
EDTA	Ethylenediamine Triaceticacid
FAOSTAT	Food and Agriculture Organization Statistical Databases
GA3	Giberellic Acid
TBE	Tris/Borate/EDTA
TFs	Transcription Factors
Ti plasmid	Tumor Inducing Plasmid

Kb	Kilobase
LB	Luria-bertani Medium
LEA protein	Late Embryogenesis Abundant Protein
MeJA	Methyl Jasmonate
MS	Mean Square
MS0 medium	Murashige and Skoog Medium
MTE	Mean Transformation Efficiency
MYB	Myeloblastosis
MYC	Myelocytomatosis
NAA	1-Naphthaleneacetic Acid
Ng	Nano Gram
nptII	Neomycin Phosphotransferase
OD	Optical density
pH	Power of Hydrogen
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSM	Regeneration Selection Medium
rpm	Rounds Per Minute
SlAIM1	Solanum Lycopersicum L. Abscisic Acid-Induced MYB
SS	Sum of Squares
UV	Ultraviolet
ZF-HD	Zing-Finger Homeodomain

CHAPTER I

INTRODUCTION

Plants have great importance to human life. Oxygen and nutrients on Earth are also obtained directly or indirectly from plants. Increasing the yield of plants has become one of the most important goals in the agricultural sector by the reason of the rapid increase in the world population and the shrinkage of agricultural areas. However, it is estimated that 805 million people in the world are constantly undernourished (FAOSTAT, 2015). It is stated that one of the most common causes of food deficiency is drought, in other words water shortage.

Potato *(Solanum tuberosum* L.*)* is one of the world's most important main food crops (Vincent et al., 2013). Potato is one of the most essential food item for many families and it is known that consumption of potato is around 36 kg per person (Kart et al., 2017). According to FAOSTAT data in 2020, potato is grown in almost 70 province with 140.766 hectares. The production of potato was 4.9 million tons, and the average yield was 33.60 t/ha in Turkey. The consumption of potato is around 47.9 kg per person (Anonymous, 2019). Nigde is biggest potato producer city which supplies 14.18% of the production whereas 10.37% in Konya and 8.57% in İzmir (Kart et al., 2017).

Potato is considered as high value crop and contributes to poverty minimization. Also, potato is high yielding carbonhydrate-rich crop and other important features are it's high-quality protein and abundance of antioxidant (Brown, 2005). Global warming is estimated to have an effect on potato production, so it is very significant to increase production. In this regard, it is essential to procure quality seeds with high quality but there are some difficulties for potato quality and quantity. Unfortunately, potatoes are affected from environmental conditions or living organisms. These adverse conditions affect plant growth, productivity, yield and quality (Obidiegwu et al., 2015).

Scientists are developed stress tolerant varieties to supply enough food as the population increases in the whole world. The traditional breeding techniques have been used to improve crops from ancient times. Main problem is that the conventional breeding techiques require many years to develop a desired plant.

The conventional breeding has supported with molecular techniques to create resistant potato lines since 1980s. (Raman and Palacios, 1982). Commonly used potato cultivars are auto-tetraploid, heterozygous and out-breedig species that suffer from inbreeding depression. Classical techniques are not efficient due to narrow base of potato (Kumari et al., 2018). That is why researchers have used modern approaches to discover effective and reproducible techniques by transferring economically important genes between species. These approaches ensure the fast and presumable association of desirable characters in target species. The agricultural industry has seen a consistent increase in cultivation of crops being sown and harvested each year around the world after the discovery of first GM crop plants in mid 1990s. Potato is an optimum crop for the transformation of new traits by molecular approaches. Developing drought tolerance crop plant is one of the most important application in genetic engineering (Bakhsh et al., 2015). In 1992, virus-resistant tobacco was developed in China; in 1994, FlavrSavr tomato was produced by U.S and potato was the one which modified genetically, and potato was grown as NewLeafTM by Monsanto in 1995 commercially (Halterman et al., 2015). In the past, developed countries used this technology to increase production over the years. Today, developing countries produce transgenic plants on the production of developed countries by using modern biotechnology methods (ISAAA, 2017).

There are many plant genetic transformation techiques such as *Agrobacterium*-mediated method, microinjection, particle bombardment, protoplast transformation, electroporation, embryonic suspension culture and silicon carbide whiskers-mediated plant transformation (Joung et al., 2015). *Agrobacterium*-mediated transformation is the most promising and reliable method in plant genetic transformation. The protocol of this method is clear, simple, and economical. This method results in adjection of single transgene (Albert et al., 1995) Desired traits can be introduced in plants by using *Agrobacterium*-mediated transformation. While this method can be used in dicotyledonous plants, it is not suitable for monocotyledonous plants and thus direct methods for gene transfer have been developed. However, direct gene transfer is not a highly recommended method by scientists due to the insertion of multiple copies in transgene (Christou et a., 1997).

Potato is a sensitive plant against abiotic stresses because it is moderate climate crop and mainly affected by drought stress (Monneveux et al., 2013). Drought stress effects potato crops physiologically and morphologically but scientists have developed many different strategies to survive under stress conditions. As a general, in response to abiotic stress, plant causes any signaling and these signals cause the change in gene expression which means some genes can be activated whereas some can be inactivated. After gene expression, specific proteins which controls defensive mechanism and responsive mechanism are produced (Zhu, 2016). So, exposure of plants to various abiotic stress factors is mostly related with systematic gene expression at transcriptional level and thereby transcription factors. Transciption factors have a potential to improve abiotic stress resistant plants. Many transcription factos have been studied under abiotic stress conditions. They have role as positive and negative regulators of stress responsive genes in stress signaling (Phillips and Hoopes, 2008). And among them, MYB transcription factors have been studied in response to abiotic stress.

Scientists have overexpressed or have used RNA interference technique to knock down the MYB gene in many different crops such as rice, apple, wheat, maize, cotton and soybean (Li et al., 2014). However, there are less reports about the drought tolerance in potato by MYB transcription factors. To cope with the problem on potato under drought conditions, we designed a strategy with specific MYB gene in this thesis work. We used tomato *SlAIM1* (ABA-induced MYB transcription factor) gene to encode drought tolerance in potatoes. In previous work, it was studied that *SlAIM1* gene was induced by both biotic (biological) and abiotic (environmental) stresses (pathogens, plant hormones, oxidative and salt stress) (Abuqamar et al., 2009).

The gene, *AIM1*, have not been studied before in potato and there is no report about drought tolerance. This research is original since it contains the overexpression of *SlAIM1* gene and characterization of its biological role on drought stress in potato. This is the very first study of potato harboring with tomato *AIM1* gene encoded by R2R3 MYB transcription factor against drought stress.

1.1 Aims and Objectives

In this thesis work, biological role of ABA-induced MYB gene, *SlAIM1*, was characterized by engineering it on a potato cultivar.

In order to achieve this objective, the following parameters were studied;

1) Isolation of *AIM1* gene from tomato cultivars (Tiny Tim and Cherry).
2) Cloning of *AIM1* gene in plant expression vector (pEarleyGate 100).
3) Agrobacterium-mediated transformation of potato cultivar (Agria) with plant expression vector harbouring the target gene.
4) Confirmation of gene integration and expression in transformants by PCR and qRT-PCR, respectively.

CHAPTER II

REVIEW OF LITERATURE

2.1 Importance of Potato

The world population was increasing day by day and expected to be 12.3 billion in 2100 that will require enough food for the survival of up growing population (Dockterman 2014).

Potatoes (*Solanum tuberosum*) have high yield and nutritional value and known to be an essential food item to fight against undernourishment. For this reason, sustainable potato production is significant in terms of human nutrition and food security (Thiele et al., 2010).

Potato can be used both as an industrial raw material and as a food stuff, which regarded as a solution to the problems of increased population around the world and insufficient food resources with its rich nutritional composition. Table 2.1 shows the chemical composition of potatoes (Li et al., 2006). Potato is the fourth most widely cultivated crop in the world after rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum aestivum*) (Obidiegwu et al., 2015). Potato is a valuable food that can be easily digested and is consumed mostly by frying, cooking, salad and puree. Potato is an abundant source of stach that used as animal feed and raw material in the production of some chemicals such as alcohol and glucose. Today, there are more than 700 recipes made with potatoes. In addition, about 170 kg of starch and 110 liters of alcohol are obtained from a ton of potatoes. Potato tubers contain 17-20% starch, 1-2% protein, and tubers are rich in vitamins in the C, A, B, K, groups (Beals et al., 2019).

Table 2.1. Chemical compounds of potatoes based on fresh weight (Li et al., 2006)

Component	**Content**
Dry matter	15-28%
Starch	12.6-18.2%
Dietary fiber	1-2%
Sucrose	0.13-0.68%
Glucose	0.01-0.6%
Fructose	0.01-06%
Lipid (fat)	0.075-0.2%
Protein	0.6-2.1%
Asparagines (free)	110-529 mg/100 g
Glutamine (free)	23-409 mg/100 g
Proline (free)	2-209 mg/100 g
Other amino acids (free)	0.2-117 mg/100 g
Polyphenols	123-441 mg/100 g
Carotenoids	0.05-2 mg/100 g
Tocopherols	Up to 0.3 mg/100 g
Thiamin (B1)	0.02-0.2 mg/100 g
Riboflavin	0.01-0.07 mg/100 g
Vitamin B6	0.13-0.44 mg/100 g
Vitamin C	8-54 mg/100 g
Vitamin E	~ 0.1 mg/100 g
Folic acid	0.01-0.03 mg/100 g
Nitrogen (total)	0.2-0.4%
Potassium	280-564 mg/100 g
Phosphorous	30-60 mg/100 g
Calcium	5-18 mg/100 g
Magnesium	14-18 mg/100 g
Iron	0.4-1.6 mg/100 g
Zinc	~0.3 mg/100 g
Glycoalkaloids	< 20 mg/100 g

The high regions of the Andes in South America are considered the homeland of cultivated potatoes, and the most important places in this regard are on the borders of Peru and Bolivia. However, wild-type tubers of the Solanaceae family have spread over a wide area from central Mexico to 45 ° south latitude in Chile (Harris, 1992). In the last two centuries, major areas of potato have spread to various parts of the world with a temperate climate. Therefore, it is an indispensable food product in many countries and thus ranked among the most important food crops worldwide. Eight species of the Solanum genus are suitable for consumption as human foodstuffs, but the most widely produced and widely known type is *Solanum tuberosum* (Rowe, 1993).

Potatoes were brought to Europe from South America by the Spanish. While the Spaniards were trying to find gold in South America, they met delicious mealy potato tubers through the locals. The Spaniards brought this unique plant to other people around the world by bringing the potato tubers from South America to Europe (Harris, 1992). Potatoes were brought to European countries in the XVI century but became popular with it’s taste and started to be planted in the XIX century and began to spread throughout the world (Baayen et al., 2006). Then, potatoes came to the top among the plants used in agriculture in a short time.

Today, potatoes are produced in about 130 countries and 300 million tons of tubers are produced every year (FAO, 2008). The leaders of the world's potato production are China, Russia, India, the USA and Ukraine. In the last few years, the amount of potatoes produced has increased by 41% (Byshov et al., 2017).

In 2017, 388 million 190 thousand 674 tons of potatoes were produced in a total area of 19 million 302 thousand 642 hectares in the world. Half of these potatoes were produced in Asia, followed by Europe (31.4%), America (11.4%), Africa (6.4%), and a small portion of Oceania (0.4%) (FAOSTAT, 2017). Potato is a notable crop that provides raw material for the agricultural industry in Turkey. According to the TSI (2017), 37 million 992 hectares of land is used for agriculture. Potato production share of these agricultural areas is 1 million 428 thousand 835 decares. 4 million 800 thousand tons of revenue was obtained from these areas where potatoes were planted. Potato yield produced was determined as 3359 kg / decare. In this respect, Nigde shares around 61% of potato production.

2.1.1 Potato yield losses by abiotic stresses

Potato is grown by tubers. Carbonhydrate synthesis by photosynthesis, translocation, and sucrose conversion to the starch of stolon are important physiological process for initiation of tuber and its growth. These processes specify tuber quality. Unfortunately, abiotic stresses have adverse effects on these physiological processes which can prevent tuber initiation and growth resulting with lower tuber quality and yield. Water makes up the bulk of potato tubers. In dry areas, low soil moisture reduces the yield of potatoes, particularly during the tuber stages and affects the organs development (Harris, 1978).

The losses in yield depend on the potato growth stage, duration, and severity of drought (Keshav et al., 2019). Biotic and abiotic stresses cause 40% crop losses in potato fields and in storages (Oerke, 2006).

Potato crop is sensitive to high temperature, drought, salinity, as well as diseases and insects. Thereamong abiotic stress factors, drought is one of the significant abiotic constraint in the production of potato (Obidiegwu et al., 2015). The yield of potato crop in Turkey is lower than other countries due to drought stress. Because water availability is essential for tuber formation and its growth (Calıskan et al., 2010). Compared to crops such as corn, wheat and paddy, potato produces more energy per unit area under sufficient rainfall and irrigation conditions. Potato is more sensitive to water deficiency due to its exposed root structure. Because of fibrous root system, water and nutrients use efficiency is low in potatoes (Storey, 2007). Generally, potato production in the world is carried out in countries where dry conditions occur. Lack of water causes significant yield losses in potato production (Levy, 2013). It is estimated that tuber yield decreases by 117 kg / ha as a result of 1 mm reduction from optimum water need in potato farming (Schafleitner, 2009). According to Obidiegwu et al., 2015, drought caused potato yield by 18-32% all the world. It has been estimated that the yield of potato will decrease considerably by 2055 by the reason of drought and global warming (Holden et al., 2003). Drought stress mainly inhibits photosynthesis and this affect potato performance and productivity. Drought affects tuberaziation, bulking and tuber yield in early growth stage of potato crop.

If drought occurs during tuber initiation, stolon number reduces and resulting with yield losses. When the potato crop encounter drought during tuber bulking, potato will procure smaller sized tuber. The most critical stage of drought stress on potato is during the initiation of stolon and the formation of tuber (Keshav et al., 2019).

It is thought that the decrease in water resources that can be used in agriculture and the increase in air temperature worldwide may adversely affect potato agriculture in the future. Keeping the productivity declines in potatoes due to drought to a minimum or altogether preventing them will both eliminate significant yield losses and allow the cultivation of potatoes in regions with less water resources. Although there are many methods to prevent yield loss caused by drought, the most effective method with a consensus is to use drought-tolerant varieties in potato cultivation. Researchers are researching to find out the mechanisms of potato drought tolerance and to develop more tolerant, high yielding potato genotypes using these mechanisms (Obidiegwu et al., 2015).

On the other hand, potato is a cool weather crop and high temperature decrease growth and resulting with tuber yield (Levy and Veilleux, 2007). The sensitivity of potato crop to the high temperature depends on stress duration, stage of growth and potato cultivar varieties. The optimal temperature is the range of 20-25°C for tuberization and 15-20 °C for tuber growth (Rykaczewska, 2015). Burton, 1972 was found that higher than 30 °C decrease net photosynthesis in potato.

Potato is known to be delicate to salinity stress. It affects growth and tuber development, resulting with lower tuber yield (Ghosh et al., 2001). The sensitivity of potato crop to the salt stress depends on salt levels and potato cultivar varieties (Keshav et al., 2019).

2.2 Abiotic Stress

“Stress” in plants is an external factor which influences the growth of the plants, it’s production efficiency and survival of a plant. This can be divided into two different categories: biotic (biological) and abiotic (environmental) stresses. Both stresses cause a yield loss in plants especially in potato. As it has been presented in Table 2.2, abiotic stress can be onset by the environmental factors, e.g. high temperature, drought, limited temperature, salinity, water flooding, high or low intensity, high or low nutrient which causes a loss of yield worldwide (Rhodes et al., 2011).

Table 2.2. Major stress factors (abiotic and biotic stresses) effecting plant growth

Abiotic stresses	**Biotic stresses**
1. Drought conditions 2. Excess salt 3. High temperature 4. Less temperature (cold) 5. Flooding (excess water) 6. Ultra-violet and high intensity of visible light 7. Pollutants and chemicals 8.Oxidative agents (reactive oxygen species, ozone) 9. Wind 10. Nutrient deprivation in soil	1. Pathogens 2. Insects 3. Herbivores 4. Rodents

Since the plants cannot move, they are regularly exposed to abiotic stress factors such as high temperature, drought and frost. Environmental stresses differ in that they affect plants. While high and less temperature can damage plants in minutes, some other stresses can take even months to give damage. Biological, morphological and molecular level changes occur in plants exposed to abiotic stress and thus plants tolerate stress. Mainly, in physiological level; respiration, photosynthesis and nitrogen fixation are affected. Also, germination of seeds and the stages of maturation and senescence in plant growth are affected differentially (Bakhsh et al., 2015).

More than 50% of agricultural production is adfected by environmental stresses all around the world (Minhas et al., 2017). Mostly, drought conditions effect badly on potato yields in many regions (Thiele et al., 2010).

Abiotic stress factors cause a decrease in the yield of cultivated plants and create a big problem in agriculture. The development of abiotic stress resistant plants is among the crucial fields of study of molecular genetics in recent years. Through molecular genetics studies, it is aimed to determine the genes responsible for drought tolerance, to regulate the expression of the specified genes with the changes made in the plant cell, and thus to make plants more tolerant to drought. In order to develop such plants, it is necessary to first understand the drought tolerance mechanisms (Monneveux et al., 2013; Obidiegwu et al., 2015; Mahajan & Tuteja, 2005).

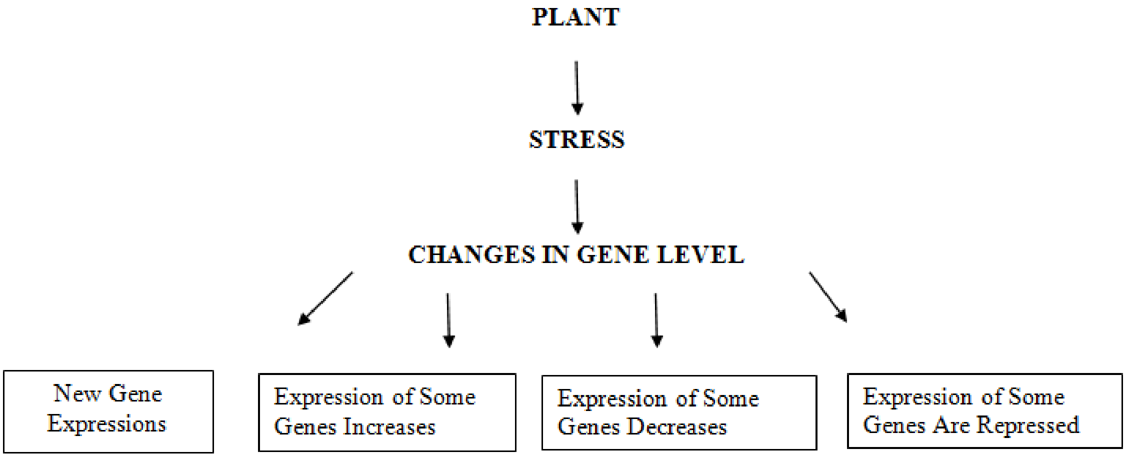


Figure 2.1. Changes of gene expression in the plant cell under stress conditions

In stress conditions, plants have to make some changes at the gene level to decrease the negative effects of stress. Some changes may occur in plants grown under stress conditions, as shown in Figure 2.1 (Atkinson, 1977). The response of plants to drought stress requires restructuring at the metabolic level. Although studies to understand the changes in the mechanisms of cell, gene expression and protein synthesis in plants experiencing water shortage have been ongoing for many years, it is difficult to say that significant evolution has yet been made in increasing drought stress tolerance of agriculturally important plants (Nakashima et al., 2014). The most important reason for this is the complexity of the mechanism called the drought stress response of plants and the intertwining of other abiotic stress mechanisms such as salinity, temperature and cold. In all four conditions, the plant's response to abiotic stress begins with the stress perception and continues with signal transmission that activates stress-specific regulatory mechanisms.

2.2.1 Drought stress

Scientists have stated that with the increase of global temperatures, the productivity in agriculture will decrease significantly, but on the other hand, the world population is increasing rapidly. Besides that, the availability of irrigation water is also reduced for agricultural practices. For example, there is a serious decrease in the amount of water in 80 countries where 40% of the world population is located. Almost a quarter of the damage caused by natural disasters in developed countries is due to damage in the agricultural sector. FAO explains that 22% of total damage occurred in the agricultural sector due to 78 natural disasters (drought, flood, storms or tsunamis) that occurred in 48 developed countries between 2003 and 2013 (FAO, 2015). These natural disasters give great concern and unfortunately abiotic stress factors as drought, erosion and frost are causing significant damage to the agriculture. Potential harmful effect on agriculture means a decrease in food products, an increase in prices of food products and therefore hunger in some countries because people in those countries with low financial affluency spend more than half of their salary on food (Bradley, 2009).

It's known that water is the most significant constituent of a herbaceous plants that makes up about 90% of the fresh weight. When the water uptake is not enough for plants, the plants face with water deficit, drought. Also, high salinity and low temperature causes drought (Passioura et al., 1996).

2.2.2 General defense mechanisms of plants against abiotic stress

Plants have to adapt to different stress conditions and develop some specific mechanisms of tolerance for their development and productiveness. These tolerance mechanisms are mainly depend on the gene manipulations which protect and sustain the structure and functions of cellular components. Unlike most monogenic features of engineered resistance to herbicides and pests, genetically complicated responses to abiotic stress conditions are more tougher to control and regulate. The complexity of the abiotic stress response that involves responsible genes and molecular mechanism is shown in Figure 2.4 (Wang et al., 2003).

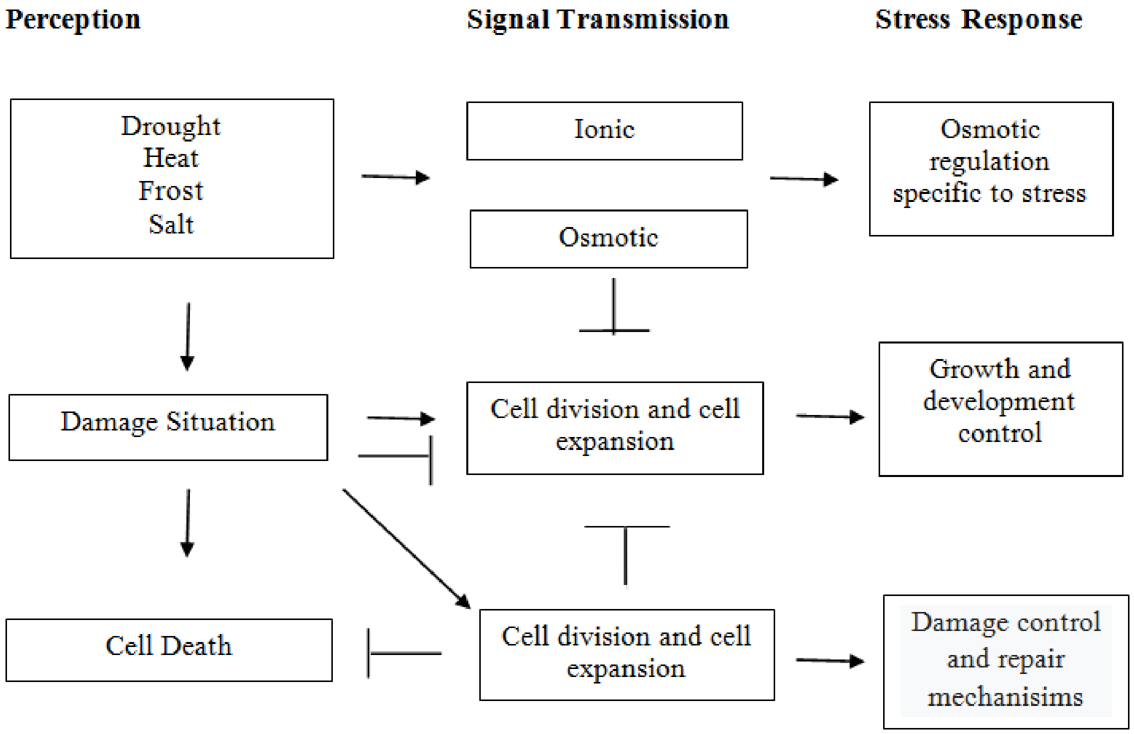


Figure 2.2. General stages of the plant abiotic stress response

The response to abiotic stress factors always starts with the perception of the stress (Figure 2.2) (Ozturk, 2015). The receptors perceive the stress and they are located on the plant cells membrane. Then, the signal is delivered downstream. Thus, second messengers are generated such as reactive oxygen species, calcium, and inositol phosphates (Figure 2.3 A). The calcium level can be increased by the second messengers in the cell. In cytosolic Ca2+ level, Ca2+ sensors detect changes in calcium binding proteins. Ca2+ sensors connect to their relevant interaction partners and then initiate a phosphorylation cascade. Stress-responsive genes or transcription factors are the targets to regulate stress- responsive gene expression (Mahajan & Tuteja, 2005). Many genes have been identified that are stimulated when exposed to abiotic stresses. The studies for the adaptation of these genes against drought stress are important to determine specific functions in the mechanism.

Salinity, drought, heat, cold, and chemical pollution are primary stresses and they are often interdependent. These stresses cause some damages in cell and osmotic and oxidative stresses called as secondary stresses in plants. Initial stress signals such as temperature or osmotic and ionic effects, changes in the membrane fluidity stimulate the downstream signaling process and transcription controls that activate stress-sensitive mechanisms to repair homeostasis and protect and restore damaged membranes and proteins. Insufficient response in one or more steps in signaling and gene activation can eventually lead to certain changes in cellular homeostasis and cell death by destroying structural and functional proteins and membranes (Figure 2.4). The ionic content of plant leaves may guide plants on their tolerance to the drought stress. It’s known that stems of tolerant varieties under dry environments accumulate less ion than sensitive varieties (Ashraf et al., 1996; McKimmie and Dobrenz, 1991).

Plants make osmotic balancing in their cells through osmolytes to reduce the effect of water deficiency. Osmolites are soluble substances, their synthesis increases in plants under drought stress and they play an important role in the cell's turgor balance. If stress conditions are persistence, osmolite accumulation is not enough to prevent water losses (Ozturk, 2015).Proline amino acid is one of the important osmolytes and protects the protein structures in the cell through the compounds it creates, reduces lipid oxidation, and prevents membrane damage. Proline has a higher metabolic advantage over other amino acids. Proline plays a major role in the phases of adaptation to or recovery from plant stress.

Because the value of a proline oxidation is equal to 30 ATP, which means that proline is a great source of energy (Hayat et al., 2012).

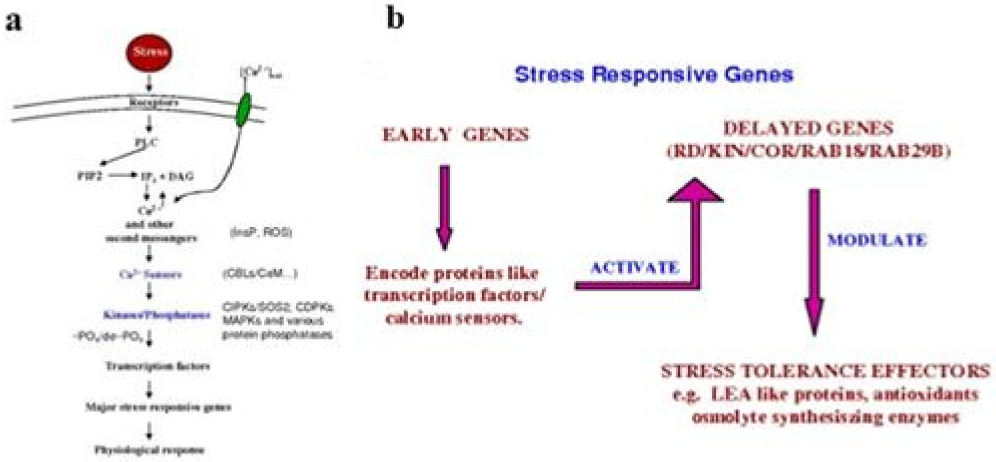


Figure 2.1. Genetic signal transmission pathway (a) and early and late genes concerned in abiotic stress signaling (b) (Mahajan & Tuteja, 2005)

The synthesis of hormones such as ABA, ethylene and salicylic acid can be affected by stress-induced changes in gene expression. ABA has many important function in plants i) promotes stomatal closure; ii) induces seed dormany and delays its germination; iii) maturation of embryo; iv) the activations of stress responsive genes. During stress adaptation, ABA helps to activate the genes that are involved in osmotic adjustment and ion compartmentation. Absisic acid (ABA) is a plant hormone (phytohormone) which has an important role in the stress signaling and responses of stress. That's why ABA is called as a 'stress hormone' (Tuteja, 2007). Plants should be regulated ABA level under changing conditions and it is know that ABA level induce under abiotic stress conditions. It's basically about the induction of the genes in ABA biosynthesis. Stress responce mechanism include ABA-dependent and ABA-independent processes. (Yamaguchi-Shinozaki & Shinozaki, 2006). Abiotic stress responsive genes are classified by early and late induced genes (Figure 2.3 B). When the plants percept the stress signal, early genes are expressed in short time. Signaling components of some TFs have already been arranged so they do not require new protein sythesis. Due to this reason, transcription factors are declared as early genes. LEA proteins (late embryogenesis abundand proteins), osmolytes and antioxidants are involved in late induced gene that are responsible for the change in protein synthesis. By comparision to early genes, late induced genes are activated more slowly (Mahajan & Tuteja, 2005).

Molecular control mechanisms of abiotic stress tolerance is depends on specific stress-related gene expressions that may result the usage of molecular tools to engineer more tolerant plants. These genes classified in three main categories; (1) genes which comprising in ion and water uptake transport (e.g. ion transporters and aquaporins); (2) genes directly involved in the protection of proteins and membranes (e.g. LEA proteins, osmoprotectants, chaperones and heat shock proteins.); (3) genes which comprising in transcriptional control and in signaling cascades (e.g. transcription factors such as HSF, DREB, ABF and MYB families, phospholipases, SOS kinases and MAP kinase).

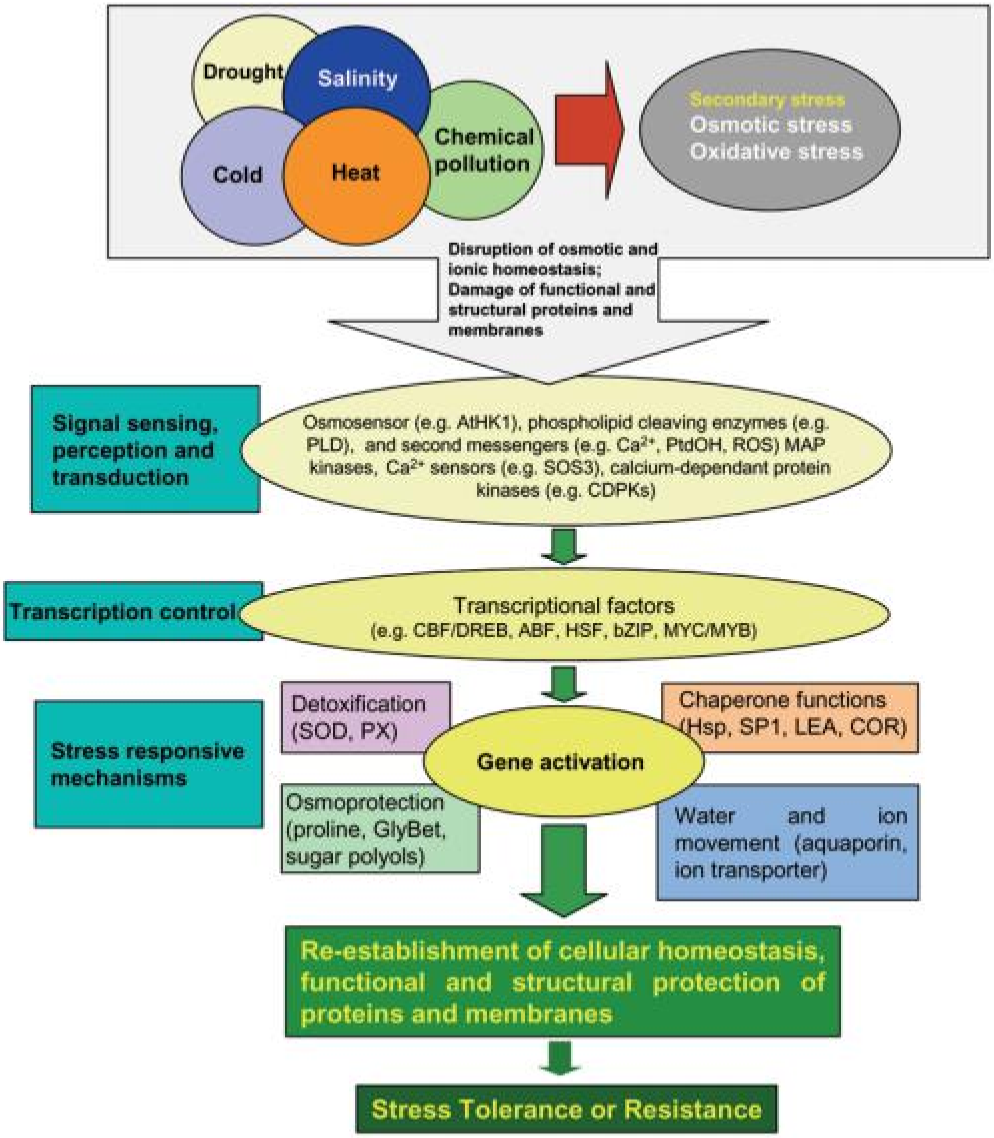


Figure 2.4. Plant responses to abiotic stresses

Abiotic stresses are allied with the ROS formation such as O2, H2O2, and OH that bring damages to membranes and macromolecules (Mittler, 2002). Plants have non-enzymatic and enzymatic antioxidant systems to combat oxidative stress. Antioxidant enzymes become more active under drought stress. Drought stress causes the accumulation of reactive oxygen compounds in plants, which leads to a separate oxidative stress (Terzi et al., 2009). The main aim of antioxidants or plant antioxidant defense system is to suppress or reduce the damage caused by reactive oxidant molecules accumulated in the plant under stress conditions by preventing or reducing the accumulation of reactive oxygen compounds (Cadenas and Davies, 2000).

One important strategy is reducing reactive oxygen derivaties formed by drought stress and preventing their accumulation (Ozturk, 2015). Because the formation of ROS such as single oxygen, superoxide anion and hydrogen peroxide are involved in the mechanisms of drought adaptation of plants. (Anjum et al., 2011).

Anthocyanins, carotenoids, ascorbate and glutathione are non-enzymatic antioxidants and superoxide dismutase (SOD), peroxidases (POD), glutathione peroxidase (GPx), glutathione reductase (GR), ascorbate peroxidase (APX), glutathione-S-transferase (GST), catalase (CAT) are the enzymatic antioxidants (Tındaş, 2015). Superoxide dismutase (SOD) takes the first place among antioxidants whose activity increases under arid stress conditions (Bowler and Van Montagu, 1992).

2.2.3 Defense mechanisms of plants against drought stress

Plants are sensitive organism and drought stress effects plant at physiological level, morphological level and molecular level (Table 2.3.) (Tuteja, 2007).

Table 2.3. Responses of plants drought stress at morphological, physiological, and molecular level (Tuteja, 2007)

Morphological Responses to Drought	**Physiological Response to Drought**	**Molecular Response to Drought**
Reduction in leaf area Leaf Shedding Decreasing in leaf number Decreasing in stem growth Enhancing Root Extension into Deeper, Moist Soil	Decrease in turgor pressure Stomatal closure Increase in abscisic acid (ABA) synthesis Decrease in Photosynthesis Increase in Photorespiration Increase in ROS accumulation Increase in accumulation of compatible osmolytes	Up-regulation of the expression of drought-responsive gene Down-regulation of the expression of key photosynthetic gene Reduced key photosynthetic enzymes activities Increased starch degrading enzymes activities

Plants have some strategies to overcome drought stress through the changing of gene expression and metabolic activities. The strategies help plants to enhance adaptation mechanisms to the low amount of water. Plant cultivars, growth stages of plant, genetic potential and severity of drought stress are the factors that determine drought adaptation strategies (Bray, 1997).

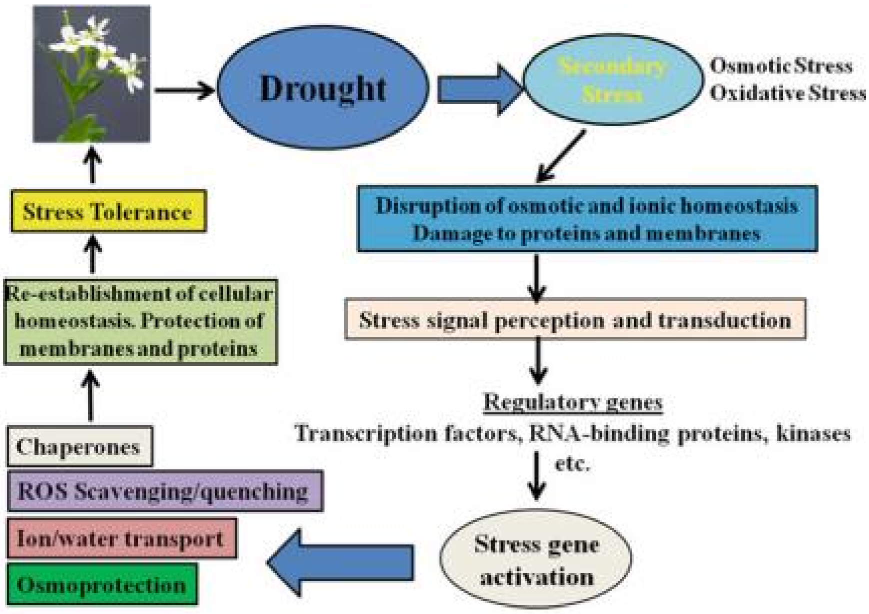


Figure 2.5. Response mechanism of plant to the drought stress (Khan and Zinta, 2016)

Plants are more susceptible to drought stress in the generative period, and the drought stress occurring in this period negatively affects plant yield (Medlyn, 2011). Plants have mainly three different strategies to avoid the drought stress effects: adaptation, avoidance of drought and drought tolerance (Ors and Ekinci, 2015). The most important event that causes yield losses is the decrease in photosynthesis efficiency in plants (Medlyn, 2011). So that, first adaptation occur in drought stress is preventing water loss by closing of stomatas (Osakabe et al., 2014). Water plays a vital role in photosynthesis as known. Plant photosynthesis rate from open stomata is related with the amount of carbon dioxide (CO_2) taken into the plant leaf. Keeping the stomata open causes the plant to water loss by respiration. Therefore, plant closes the stomata quickly to minimize water loss. Consequently, the plant photosynthesis rate decreases as the CO_2 uptake decreases (Chavez et al., 2003).

The development of water use efficiency (WUE) is one of the essential strategies by the way of leaf number and leaf area reduction and stomatal conductivity to conserve water in plant leaves (Deblonde and Ledent, 2001).

Dry conditions affect the turgor pressure of plants. The main effect of drought stress is often explained by osmotic pressure. Osmotic balance is important that ensure the plants less affected from drought stress. Osmolits, which have a role in osmotic balance, responsible to balance of leaf water pressure and hence the stomatal conductivity increases, finally this ensures photosynthetic activities. Maintaining water balance and cell metabolic activities can provide a short-term drought tolerance to the plants. Determination of photosynthetic activities in the leaves (Sharma and Hall, 1992), damages in the cell membrane permeability (Blum, 1985), accumulation of organic substances that function as osmolite accumulation are the variables in the selection of drought stress tolerant plants (Ors and Ekinci, 2015).

Drought and salt stress exposure stimulates the common reactions as both ultimately results in cell dehydration and osmotic imbalance. In addition, they cause the formation of ROS that adversely affect metabolism and cell structures. Removal of water from the membrane hinders the integrity and the membrane selectivity, resulting the enzyme activity losses that are mainly membrane-based. In addition to the membrane damage, it can affect to protein activities and when dehydrated the proteins can undergo complete denaturation (Mahajan&Tuteja, 2005; Bray, 1997). As metabolic processes are similar such as increased levels of ABA or a decrease in the rate of photosynthesis; there is one exception in the responses of plant to the salt and drought: ionic complement. High intracellular concentrations of chloride and sodium ions are problem during salt stress faced by plants (Bartels & Sunkar, 2005). The overall strategies lead to regulation of drought-responsive gene expression to ensure plant tolerance.

2.3 Introduction to Transcription Factors

Transcription Factors (TFs) are proteins having domains which bind to the DNA of specific genes (promoter or enhancer regions) to affect gene expression. TFs can be responsible for the plant cell development, differentiation, and growth of plant cells by binding to DNA site or sites and regulates gene expression (Singh et al., 2002). TFs are controller proteins which control where and when transcription happens (Figure 2.6). TFs only works at specific DNA sequences. These sequences are called promoters (Kostrewa et al., 2009). Some of them are general which are found in all cells of plants. Others are specific for some types of cells and development stages. Specific transcription factors are most important for the expression of gene which can result in developmental changes (Gonzalez, 2016).

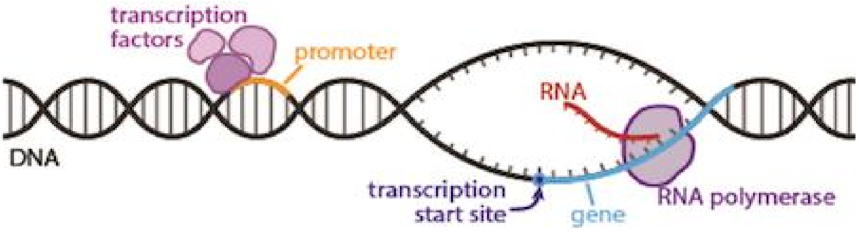


Figure 2.6. Transcription factors that regulate transcription of genes

Firstly, it is crucial to understand transcription mechanism because transcription factors regulate changes of gene transcription. In all eukaryotes, transcription is performed by the RNA Polymerase 2 (RNAP II) enzyme. TFIIB is an an essential component of the RNA polymerase II initiation complex (Figure 2.7). The RNA polymerase is almost in place to start reading the gene with TFIIB. TFIIB protein and the DNA sequence help RNA polymerase recognize where to start reading DNA (Kostrewa et al., 2009). Precisely, RNAP II cannot move alone. This enzyme's activity is controlled by cis-regulatory region which is the sequences of DNA that are in the gene and by proteins (trans-acting factors) called TFs. In that case, to transcribe DNA, TFs are required for the enzyme RNAP II. The cis-acting elements are the DNA segments which regulates transcription (Phillips and Hoopes, 2008). The TFs are involved in an interaction with *cis*-elements in the regions of promoters of several stress-related genes and hence up-regulation of many downstream gene expression result in imparting tolerance of abiotic stress (Kimotho et al., 2019; Agarwal & Jha, 2010).

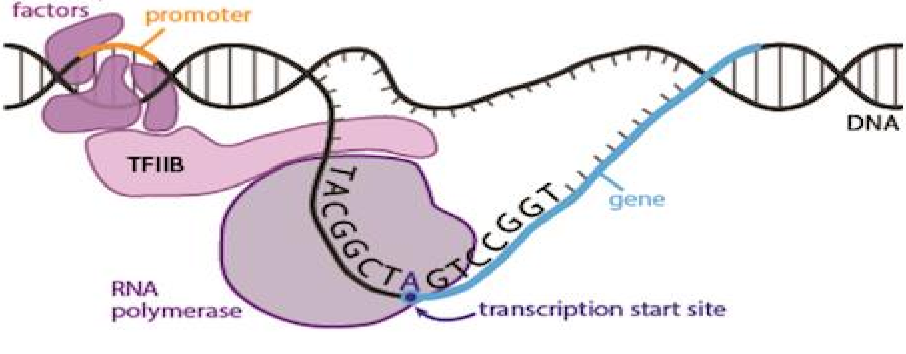


Figure 2.2. The role of tfIIB and the dna sequence in transcription

Plant genomes appoint about 7% of the TFs coding sequence of providing the transcriptional regulation complexity. In *Arabidopsis thaliana,* for instance, approximately 1500 TFs are described in the genome that are related to the gene expression in stress (Lata et al., 2011). As described before, the stress response mechanism includes ABA-dependent and ABA-independent processes.

The MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene), AREB/ABF (ABA-responsive element-binding protein/ ABA-binding factor) are ABA-dependent regulator TFs that require stress hormone (ABA). ABA-independent regulator TFs do not require stress hormone (ABA) and they are NAC (NAM, ATAF and CUC) and ZF-HD (zinc finger homeodomain) and CBF/DREB. Besides, several studies identified that some of the TFs have both ABA-dependent and independent pathways in stress response (Figure 2.8).

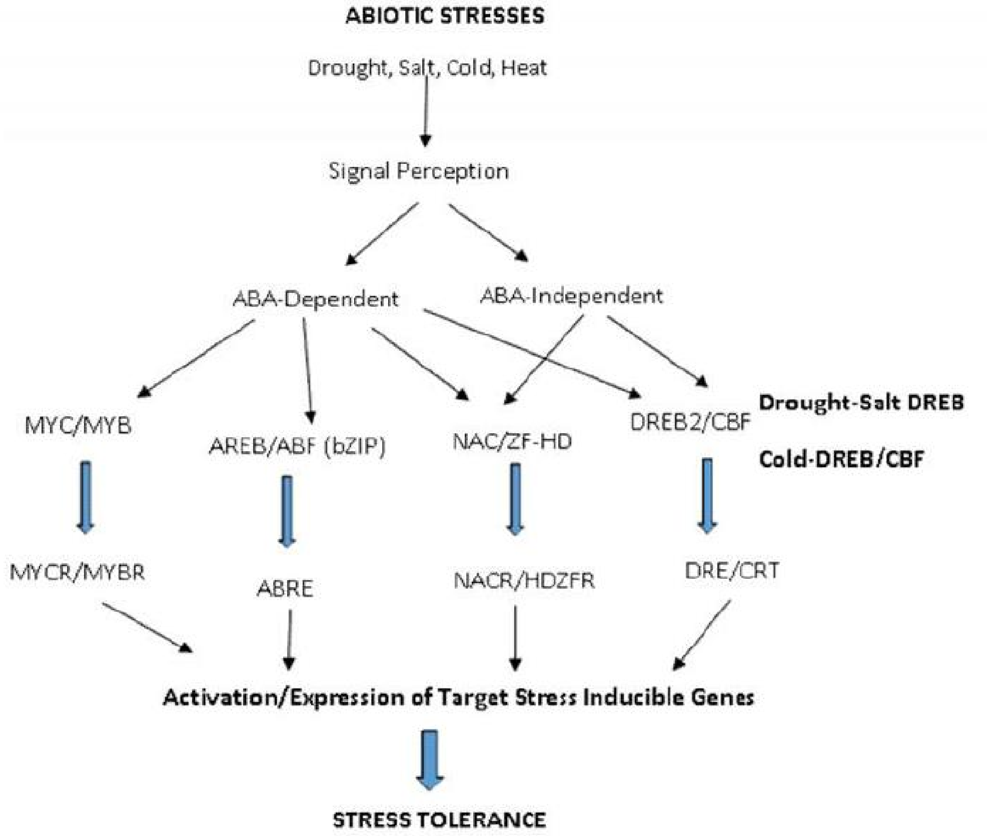


Figure 2.8. Abiotic stress tolerance mechanism by transcription factors **(**Bakhsh and Hussain, 2015)

2.3.1 Regulatory functions of transcription factors in abiotic stress response of plants

Plants are developing many different methods to come out alive under abiotic stress conditions. Exposure of plant to abiotic stresses related with gene expression and transcription factors. As it is known that abiotic stresses may require the several genes regulation including the TFs (Kimotho et al., 2019).

Under stress conditions, TFs control the gene expressions and TFs regulate the genes which are related with plant stress tolerance. The overexpression of TFs can increase the abiotic stresses tolerance in plants (Agarwal & Jha, 2010). Researchers have worked on abiotic stress-related genes, some TFs and regulatory sequences in plant promoters because; transcription factors are one of the excellent applicants for genetic engineering to obtain stress-tolerant plant crops (Kimotho et al., 2019).

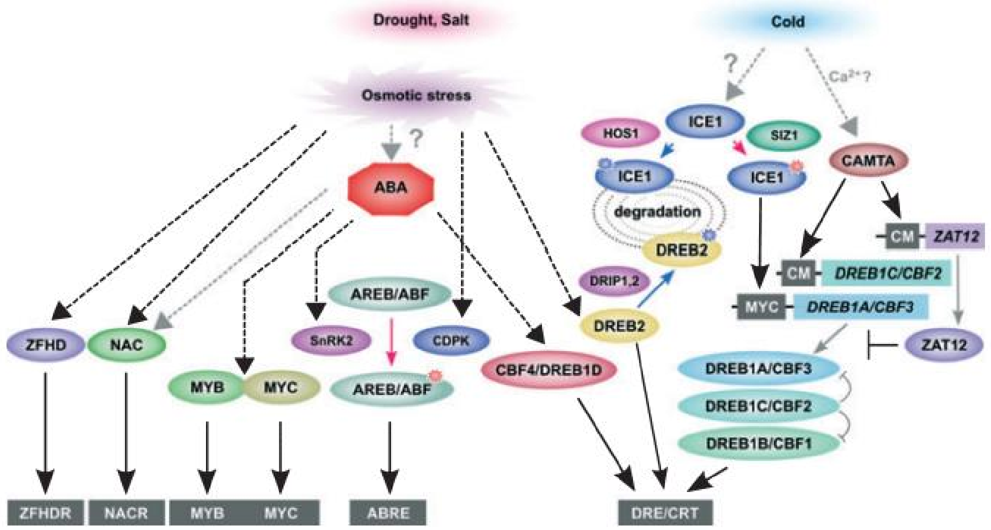


Figure 2.9. The network of transcriptional regulators that function in salinity, drought and cold stress responses. Functional proteins are shown in elliptical boxes and cis elements are shown in grey boxes. Straight lines point out direct links whereas dotted lines point out indirect links

The responses of transcriptional regulation in drought, salt and cold stresses is shown in Figure 2.9 (Hirayama & Shinozaki, 2010). The TF families have been studied in many important food crops with detail including *Arabidopsis thaliana, Solanum tuberosum,* *Oryza sativa, Hordeum vulgare, Triticum aestivum, Vitis vinifera, Brassica napus, Zea mays, Glycine max, Gossypium hirsutum* and *Sorghum bicolor*.

TFs play a significant role in abiotic stress and the potential of TFs are used in the molecular breeding to improve different crop varieties (Kimotho et al., 2019). Many studies have been identified and the role of TFs in stress has been characterized in plants. Table 2.4 shows some of those studies on the tolerance of abiotic stress.

Table 2.4. Transcription factors that help plants tolerance to various abiotic stress

Family	Gene	Species	Abiotic tolerance	References
bZIP	*ABF1*	*Arabidopsis thaliana*	Cold	Choi et al., 2000
	OsbZIP71	*Oryza sativa*	Salinity, drought,	Liu et al., 2014
	GmbZIP62	*Glycine max*	Cold, Salt, Drought	Liao et al., 2008
	Wlip19	*Triticum aestivum*	Cold, Drought	Kobayashi et al., 2008
	ZmbZIP4	*Zea mays*	Salinity	Ma et al., 2018
bHLH	*AtMYC2*	*Arabidopsis thaliana*	Osmotic stress	Abe et al., 2003
	OsbHLH148	*Oryza sativa*	Drought	Seo et al., 2011
	MYC2	*Arabidopsis thaliana*	Oxidative	Dombrecht et al., 2007
NAC	*ANAC042*	*Arabidopsis thaliana*	Heat	Shahnejat-Bushehri et al., 2012
	TaNAC29	*Triticum aestivum*	Salinity	Xu et al., 2015
	OsNAC6	*Oryza sativa*	Drought	Lee et al., 2017
	GhNAC4 NA	*Gossypium hirsutum*	Cold, Drought, Salt	Yang et al., 2017
WRKY	*OsWRKY45*	*Arabidopsis thaliana*	Salinity, Drought	Qiu and Yu, 2009
	GmWRKY21	*Arabidopsis thaliana*	Freezing	Zhou et al., 2008
	OsWRKY89	*Oryza sativa*	UV irradiation	Wang et al., 2007
	TaWRKY33	*Triticum aestivum*	Drought	He et al., 2016
CBF/DREB	*OsDREB2B*	*Oryza sativa*	Heat, Cold	Matsukura et al., 2010
	DREB2C	*Arabidopsis thaliana*	Salt,Cold, Mannitol	Lee et al., 2010
	WDREB2	*Triticum aestivum*	Salt, Drought, Cold	Egawa et al., 2006
	HvDREB1	*Hordeum vulgare*	Salt, Drought, Cold	Xu et al., 2009
	GmDREBb	*Glycine max*	Salt, Drought, Cold	Li et al., 2005
	SlERF5	*Solanum lycopersicum*	Drought, salinity	Pan et al., 2012
	ZmDREB2A	*Zea mays*	Salt, Drought, Cold, Heat	Qin et al., 2007
	AtDREB1A	*Solanum tuberosum*	Salt	Behnam et al., 2006

Transcription factors such as MYB/MYC, NAC, DREB2 (drought-responsive element binding and AREBs (ABA-responsive binding protein)/ABFs (ABRE binding factors are involved in both ABA-independent and ABA-dependent pathways which are mostly activated by drought and salt stress. The AREBs and ABFs are bZIP (basic leucine zipper). TFs which activate ABA-dependent gene expression (Lata et al., 2011).

CBF/DREB transcription factors are involved in ABA-independent pathways which are regulated by cold stress. The promoter region of salt, drought and cold-responsive gene comprise DRE (drought-responsive element) and ABRE (ABA-responsive element). DREBs have two groups that mediate abiotic stress tolerance in plants. First group is DREB1/CBF which are induced by cold and the second group is DREB2 which are induced by dehydration (Agarwal & Jha, 2010). Also, they can be responsible in heat stress (Table 2.4).

Plants have more than 100 NAC TFs. They are regulated by abiotic stress and Table 2.4 shows that they are good targets to develop tolerance plants against abiotic stress. The genomes of *Glycine max*, *Oryza sativa* and *Arabidopsis thalina* have about 197, 103, 74 WRKY TFs. Many of WRKYs have been found that they have involved in plant abiotic stress responses (Yoon et al., 2020). Besides, the other stress-responsive bHLH TFs includes *OsbHLH148* and *MYC2* that are involved in ABA-mediated stress tolerance (Table 2.4).

2.4 MYB (Myeloblastosis) Family of Proteins (MYB TFs)

As a general, a single TF can regulate different abiotic stress responsive genes. Many TFs which belong to different families have found to be important in stress signalling (Lata et al., 2011). And MYB transcription factor is one of them that play a role in stress signalling system.

ABA is a plant-stress hormone and plants should be regulated ABA level under changing conditions and it is known that ABA level induces under abiotic stress conditions. It’s basically about the induction of the genes in ABA biosynthesis.

MYB TFs regulate ABA-responsive gene expression because MYB proteins are synthesized after ABA accumulate (Tuteja, 2007).

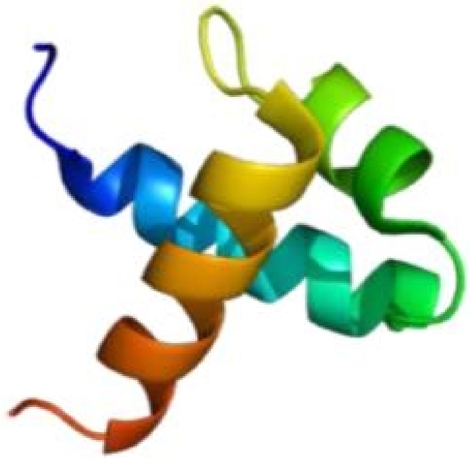


Figure 2.10. The structure of myb (myeloblastosis) gene

All TFs contain related protein families that share a homologous DNA binding domain, the signature motif of TF families (Agarwal & Jha, 2010). MYB TF family have conserved DNA binding domain (MYB domain) containing around 50-55 amino acids and forms a helix-turn-helix. Most of them have a function as transcription factor because of MYB domain's ability to bind DNA (Li C. et al., 2014). Firstly, *MYB* gene (*v- myb* oncogene) is identified from avian myleblastosis virus (AMV) and *MYB* gene is myeloblastosis. As concerns to plants, the first identified *MYB* gene is *COLORED1* (*C1*) gene in plants which has role in anthocyanin synthesis in the aleurone layer of kernels of maize (Martin and Paz-Ares et al. 1987).

When the first MYB TF was cloning from plants, the *C1* gene of maize, showed that plants use TFs to control gene expression. *C1* showed notable structural homology to the vertebrate cellular proto-oncogene c-MYB. The MYB-related proto-oncogenes invertebrates are a small family that controls development of cells and cellular proliferation (Martin and Paz-Ares, 1997).

All eukaryotes have the MYB protein family. Plants have more *MYB* genes as compared to animals and fungi (Katiyar et al., 2012). MYB proteins have many functions in seed development, cell shape, hormone signal transduction, the regulation of primary and secondary metabolism and also stress defences mechanism in plants (Dubos et al. 2010).

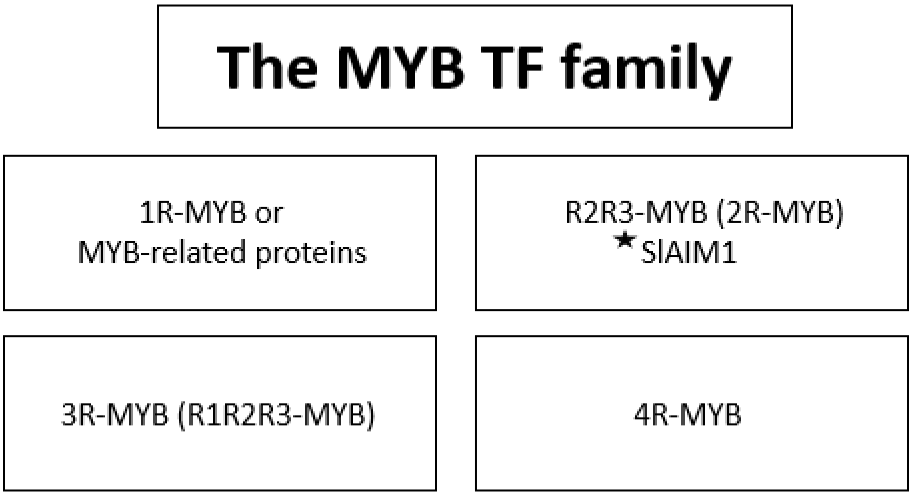


Figure 2.11. The classification of the myb transcription factor family

The structure of the MYB domain (Three-dimensional) evinced that the DNA recognition site α-helix interacted with the main thread of DNA. However, outside of the aminoacid sequences in MYB domain is quite different (Katiyar et al., 2012). The main property of MYB domains are composed of one to four imperfect repeats (Figure 2.12). As it is shown in the figure 2.11, the MYB TF family consists of four subfamilies which differs in the number repeat of DNA binding domains. These are MYB-related proteins or 1R-MYB, R2R3- MYB family or 2R-MYB R1R2R3- MYB family or 3R-MYB and 4R-MYB based on the number and position (Martin and Paz-Ares, 1997).

The smallest subfamily is 4R-MYB in MYB family. Each member have four R1/R2 repeats (Figure 2.12). The functions of this subfamilies are still unknown well and only one 4R-MYB gene was found, At3g18100, in Arabidopsis (Li et al., 2014).

The 3R-MYB subfamily (R1R2R3-MYB) is conserved in plants evolutionarily. This subfamily has three repeats as it has been presented in Figure 2.12. Among all MYB proteins, few 3R-MYB proteins are found. For instance, four genes encode 3R-MYB proteins in Rice whereas 5 genes in tobacco and Arabidopsis (Ito et al., 2001; Katiyar et al., 2012). R1R2R3-type MYB domain proteins are dominant in animals whereas R2R3-type MYB domain proteins are dominant in plants.

The 2R-MYB (R2R3-MYB) is the biggest subfamily in MYB family. It's estimated that R2R3-type MYB genes could be evolved from R1R2R3-type MYB gene by losing R1 repeat or it could be evolved from the duplication of R1 repeat in R1-type MYB gene R2R3-MYB proteins have DNA-binding domain at N terminus and a C-terminal. In Arabidopsis, there are more than 120 genes in this family and rice, there are around 90 genes (Katiyar et al. 2012). These proteins have an essential role in stress responses of plants.

The MYB-related subfamily (1R-MYB) have proteins with partial and intact repeats (Figure 2.12). It is the second biggest subfamily in MYB family. In rice, there are 70 genes and in arabidopsis, there are 64 genes. Firstly, *MybSt1* gene was identified in 1R-MYB subfamily in potato (Li et al., 2014).

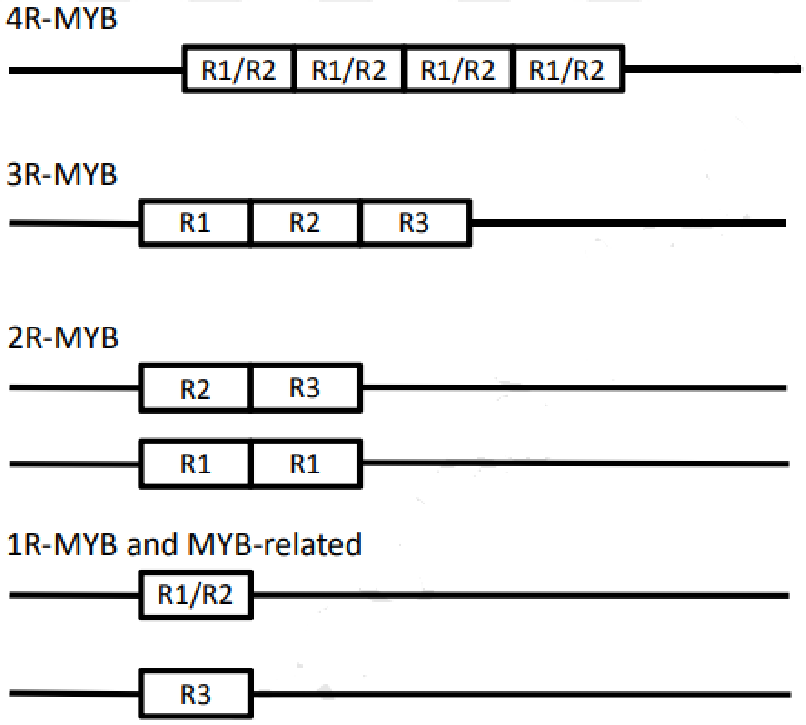


Figure 2.12. The structure and the number of myb transcription factor repeats (r) (Li et al., 2014)

2.4.1 The role of MYB transcription factors in abiotic stress on plants

MYB TFs have an important role in abiotic stresses on many different plants. To response to abiotic stresses, the MYB family is involved in many stress-related genes regulation. Many different elements have regulated by MYB families. Stress-related genes are target genes of MYB proteins against abiotic stresses. The information is essential to understand the mechanism behind the role of MYB families in abiotic stresses (Roy, 2016).

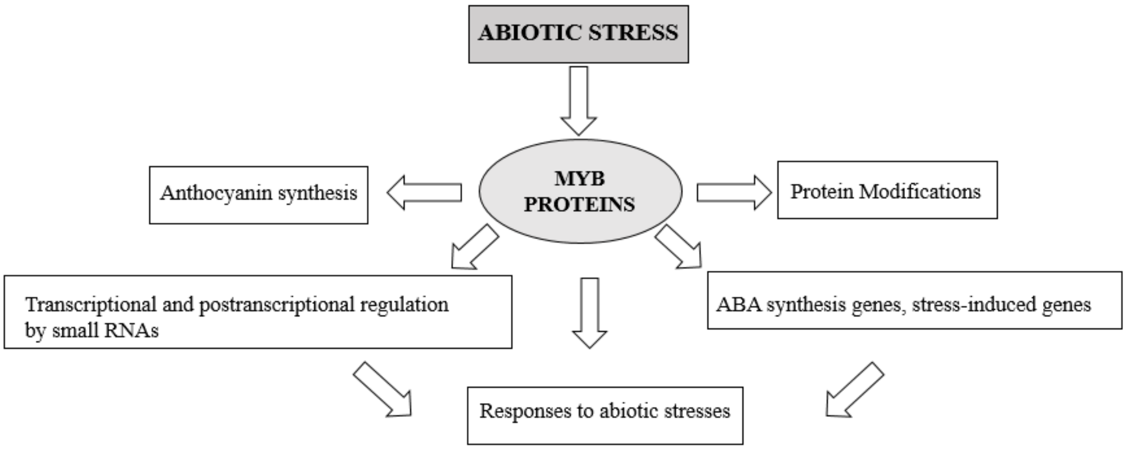


Figure 2.13. The abiotic stress response of MYB transcription factors (Li et al., 2014)

As it is known, plants accumulate some secondary metabolites to adjust itself for environmental conditions e.g, anthocyanins. Anthocyanins are flavonoids pigments which have a role in the protection of plants from abiotic stresses (Shi et al., 2019). MYB proteins can regulate the expression of anthocyanin biosynthesis genes. These proteins bind to anthocyanin-related genes and have a role of promoting anthocyanin synthesis (Figure 2.13). As a general, flavonoid synthesis is vital for the tolerance of abiotic stress and it involves in the complex of MYB (MBW complexes) in arabidopsis, maize, cotton, apple and rice (Li et al., 2014).

In addition to this information, When MYB family involve in abiotic stress responses, it targets the small RNAs. MYB protein regulations is essential at the protein level which includes phosphorylation, ubiquitination and sumoylation for their functions. Protein interactions are imperative for the regulatory activation of MYB proteins (Figure 2.13).

Many MYB TFs have found to be responsible to the abiotic stress tolerance in many crop plants (Table 2.5). MYB TFs help to develop abiotic stress-tolerant plants using overexpression and knock-out systems (Yoon et al., 2020). Many different studies have been done with the Arabidopsis plant. Many R2R3-MYB proteins in Arabidopsis are essential for drought responses. Also, other families have a role in abiotic stress. Genome of Arabidopsis has about 1500 TFs which involve in abiotic stress tolerance. And out of 198 genes are MYB genes. Results prove that MYB TFs accumulate with the presence and accumulation of ABA. Jung et al., 2008 reported that overexpression of *AtMYB44* gene increases ABA-sensitivity which led to drought stress tolerance by alteration of ABA-dependent stomatal closing. They have also reported that this gene regulates cold and salinity tolerance. In another experiment, Cui et al., 2013 showed that suppression of *AtMYB20* gene regulates salt tolerance in plant.

Although more than half studies are from Arabidopsis, *MYB* genes have identified in wheat, soybean, maize, rice, apple and cotton (Ambawat el al, 2013). 524 *MYB* genes in *Gossypium hirsutum* L., 70 MYB genes in *Beta vulgaris* L., 55 *MYB* genes in *Cucumis sativus* L., 184 *MYB* genes in *Pyrus bretshneideri* were identified at the genome-wide level (Sun et al., 2019).

Studies on other plants except Arabidopsis have similar roles. For example, Mao et al. 2011 reported that wheat *MYB2A* expression is up regulated by cold, salinity and drought. Also, there is a report about *N. bethamiana*. Huang et al. 2013 found that silencing of *NbPHAN* induce to stress-related genes expression of which is highly expressed under drought conditions. So, the results have shown that *NbPHAN* plays a role in the drought stress. There is a limited study about potato MYB transcription factors. The MYB TF (1R-MYB subfamily) role in drought stress response have identified in potato is *StMYB1R-1*. Overexpressing of *StMYB1R-1* in potato showed drought tolerance (Shin et al., 2011). Sun et al., 2019 checked the expression level of some potato *MYB* genes. *StMYB6* decreased under high temperature and hormone treatments, *StMYB116* and *StMYB27* decreased by high temperature treatment, *StMYB133* decreased under high-temperature and drought stress. Under drought treatment, *StMYB116* and *StMYB119* had highest expression levels. Lin et al., 2013b reported that suppression of *lbMYB* help wounding responses in sweet potato (Ipomoea batatas).

156 *GmMYB* genes are identified in soybean and 43 genes were regulated by drought, salt, cold stress and exogenous ABA application. Overexpression of *GmMYB177*,

GmMYB92, *GmMYB76* increased salt tolerance in transgenic Arabidopsis (Liao et al. 2008).

Rius et al., 2012 studied that R2R3-type *ZmP1* gene controls UV-B tolerance in maize and more expressed in leaves. Also, there are many reports from different plants about MYB transcription factors. For example, Wang et al., 2014 showed the expression of apple *MdSIMYB1* is induced by cold, salt and PEG treatments. The apple *MdMYB10* has role to regulates anthocyanin biosynthesis (accumulation of anthocyanin) and osmotic stress response. The pears *PcMYB10* controls low-temperature- induced anthocyanin accumulation. According to these results, it is evident that *MYB* gene proteins are involved in the relationship between cold tolerance and anthocyanin biosynthesis.

VvMYB5 gene was cloned from grape and overexpressed in tobacco to regulate phylproanoid synthesis and metabolism of anthocyanins. (Deluc et al., 2006). There are many reports on rice MYB transcription factors. 183 MYB TFs were identified under abiotic stress until now (Li et al., 2014). Dai et al., 2007 reported the expression of rice *OsMYB3R-2* was induced by cold treatment. And it was observed that overexpressed *OsMYB3R-2* showed more tolerance to cold when compared with controls, non-transgenic plants (Katiyar et al., 2012). The overexpression of *OsMYB3R-2* regulates cold stress tolerance in rice. And they have reported that this helped the developmental process of rice. (Ma et al. 2009).

In maize, 157 R2R3-MYB genes and 72 MYB related proteins (1R-MYB) were found using a comparative genomic analysis (Kimotho et al., 2019). From earlier studies it is revealed that scientists analysed the expression of 46 1R-MYB subfamily genes in maize which are responsible for abiotic stresses. Out of 22 genes responded to the different abiotic stresses whereas 16 of them responded to two stresses. The results indicate that these genes may have role in the signal transduction pathways of abiotic stress responses (Chen et al., 2017).

Table 2.5. MYB transcription factors involved in various abiotic tolerance

Family	**Gene**	**Species**	**Abiotic tolerance**	**References**
MYB	*AtMYB20*	Arabidopsis thaliana	Salinity	Cui et al., 2013
	AtMYB44	*Arabidopsis thaliana*	Drought	Jung et al., 2008
	OsMYB6	*Oryza sativa*	Salinity, Drought	Tang et al., 2019
	OsMYB4	*Oryza sativa*	Freezing	Vannini et al., 2004
	OsMYB3R-2	*Oryza sativa*	drought, cold,salt	Dai et al. 2007
	GmMYB76	*Glycine max*	Salinity, freezing	Liao et al., 2008
	MYB2A	*Triticum aestivum*	Drought, salt, cold	Mao et al. 2011
	NbPHAN	*Nicotiana benthamiana*	Drought	Huang et al. 2013
	StMYB1R-1	*Solanum tuberosum*	Drought	Shin et al., 2011
	ZmP1	*Zea mays*	UV-B	Rius et al. 2012
	MdSlMYB1	*Malus domestica*	Cold, salt and drought	Wang et al. 2014
	IbMYB1	*Ipomoea batatas*	Wound	Lin et al., 2013

2.4.2 Selected gene in the study: abscisic acid-induced MYB (*SlAIM1*) gene

Tomato (*Solanum lycopersicum*) *SlAIM1* gene is the member of R2R3MYB family which is the biggest family in MYB transcription factors. It's studied that both abiotic and biotic stresses (pathogens, plant hormones, oxidative and salt stress) induced *SlAIM1* gene (Abuqamar et al., 2009) where *SlAIM1* gene was silenced by RNA interference, and it caused susceptible to fungus *Botyris cinera*. It increased the sensitivity to abiotic stresses, salt and oxidative stress. In general, previous studies suggested that *SlAIM1* gene regulates ion flux by response to abiotic stress and Solyc12g099130 was identified as *SlAIM1* gene which is regulatory gene of abiotic stress tolerance as well as disease resistance in tomato (Abuqamar et al., 2009).

SlAIM1 shares high identity with some of the Arabidopsis R2R3MYB genes (*MYB108* (At3g06490), *MYB78* (At5g49620), *MYB2* (At2G47190), *MYB116* (At1g25340), *MYB112* (At1g48000), *MYB62* (At1g68320). *MYB108* shows 91.44% identity which is the highest identity with *SlAIM1* whereas *MYB78* shows 84% sequence identity (Abuqamar et al., 2009). Abe et al., 2003 studied that *MYB2* Arabidopsis gene was induced on salinity, ABA and drought. This gene shares 67% identity with *SlAIM1* gene. As it is known, ABA regulates the responses of plants against abiotic stresses such as drought, low temperature, salinity and osmotic stress. On the other hand, two MYB-related proteins (*CPM10* and *CPM5*), from *Craterostigma plantagineum* plant, have high identity with *SlAIM1* gene. These two proteins are known that they have incuded by drought stress and ABA in *C. plantagineum* plant (Iturriaga et al., 1996). *CPM10* has 83% sequence identity with *SlAIM1* in the N-terminal conserved region. Table 2.6 shows that Arabidopsis R2R3MYB genes which are closely associated with *SlAIM1* and their expression in response to pathogens, plant hormones and some of the abiotic stresses.

Table 2.6. Closely related genes with *SlAIM1* and their expression to some abiotic and biotic stresses (McGrath et al., 2005; Mengiste et al., 2003; Kranz et al., 1998; Yanhui et al., 2006; Abuqamar et al., 2009)

	Arabidopsis *R2R3MYB* genes						**Tomato**
Abiotic or biotic stresses	***MYB108***	***MYB78***	***MYB2***	***MYB116***	***MYB112***	***MYB62***	***SlAIM1***
B. cinerea	induced	not inducible	induced	not inducible	induced	induced	induced
P. syringae	induced	not inducible	induced	not inducible	induced	not inducible	induced
NaCl	induced	induced	induced	not inducible	induced	not inducible	induced
Osmotic	induced	not inducible	induced	not inducible	induced	induced	No data
Oxidative	induced	not inducible	induced	not inducible	induced	No data	induced
Methyl jasmonate (MeJA)	induced	not inducible	not inducible	not inducible	not inducible	not inducible	induced
Ethylene	induced	not inducible	contradictory data	not inducible	not inducible	not inducible	not inducible
ABA	induced	not inducible	induced	not inducible	not inducible	not inducible	induced
Salicylate	not inducible	not inducible	induced	not inducible	induced	induced	not inducible

Consequently, there have been so many studies about MYB genes in many different plants and understanding the role of this gene is crucial point and it will be an outstanding achevement in this field.

Introduction of further research on regulatory mechanisms involves in abiotic stress responses in plants will remain viable advancement and it would be helpful to develop stress tolerant plants with better yields and qualities. Although several studies have been conducted to introduce stress tolerant plants against abiotic stress in different crop plants but to date, no remarkable study has been launched to with MYB gene in potato against drought stress. Present study with ABA-induced MYB gene *SlAIM1* in potato was characterized as it's biological role and the gene was transformed into potato to develop drought resistant potato lines. Successful introduction of studied gene into potato genome will explore the biological role of *AIM1* gene and its potentiality to combat against drought strees mediated crop losses in potato.

2.5 Development of Drought Resistant Transgenic Plants

2.5.1 Transgenic technology and the strategy of the overexpression of TFs

New organisms that have been developed through the genetic engineering by changing of the gene sequence of a living organism or giving it a completely different non-inherent character are called genetically modified organisms (GMO). The gene transferred during this process is called transgene (Meseri, 2008) and the plants that have been given specific characteristics by transferring genes from any organism other than their nature are called transgenic plants or genetically modified plants.

Since complete genome sequences of many organisms are available, systematic, and genome-wide analysis of gene function made possible (Zhang, 2003). Strategies such as gene silencing or a new gene transfer are popular in strategies to improve the tolerance of plants to biotic and abiotic stresses.

"Overexpression" means that increased in expression. Overexpression began to be used as a screening tool in molecular genetics after yeast transformation techniques were developed (Prelich, 2012). The overexpression of random genes has shown to be helpful to identify many genes involving in the cell cycle of yeast. The provide an alternative strategy to knockdown / knockout analysis affected by functional redundancy. It was not possible to learn the functions of these genes with ressesive knockout approach. There are many examples in plants, knockouts of TF genes were failed to develop phenotypes. As the genes were overexpressed, the gene functions were arised. TFs are overexpressed under a strong constitutive promoter to increase the function of TF alleles. In transgenic plants, overexpressions of TFs play a critical role in transcript profiling and mutant analysis (Zhang, 2003).

In Figure 2.14, ectopic formation of trichomes and embryos were seen by the overexpression of TFs LEAFY CONTYLEDON2 (LEC2) and AtMYB23 under strong constitutive promoter (35S).

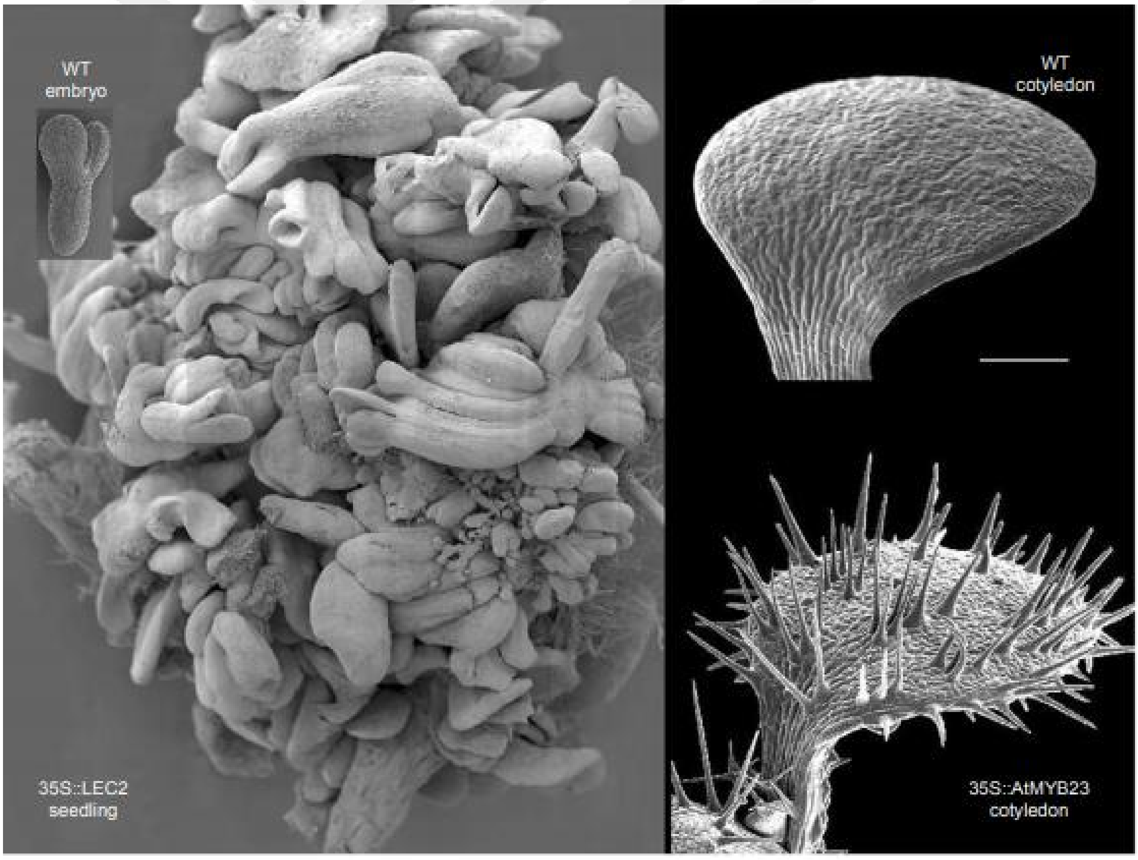


Figure 2.14. The overexpression of the TFs LEAFY COTYLEDON2 (left) and AtMYB23 under 35S promoter (right)

2.5.2 *Agrobacterium tumefaciens* mediated transgenic plant production

The most popular method in transgenic plant technology is done by *Agrobacterium tumefaciens* bacteria. This bacterium was first described by Smith et al. (1907). *Agrobacterium tumefaciens* is an alphaproteobacterium belonging to the *Rhizobiaceae* family that fixates nitrogen in legumes. It is a rod-shaped and gram-negative bacterium that lives in the soil under its natural conditions, does not form a spore. *Agrobacterium tumefaciens* causes tumor (crown gall disease) formation in the root of the dicotyledonous plants (Figure 2.15). Tumor formation is associated with the transfer of T-DNA of *Agrobacterium* to the plant genome and subsequent uncontrolled growth of cells due to an imbalance of hormone synthesis in plant cells. (Matthew et al., 2003; Escobar and Dandekar, 2003).

Figure 2.15. *Agrobacterium* tumor (crown gall disease) (Matthew et al., 2003)

Agrobacterium tumefaciens is mostly used in genetic modification for molecular biology in dicotyledonous and some monocotyledonous plants. For this aim, a foreign gene is transferred to the plant genome because of the transfer of the T-DNA (transfer-DNA) region located in the Ti (tumor-inducing) plasmid of *A. tumefaciens* from the bacteria to the plant (Figure 2.16).

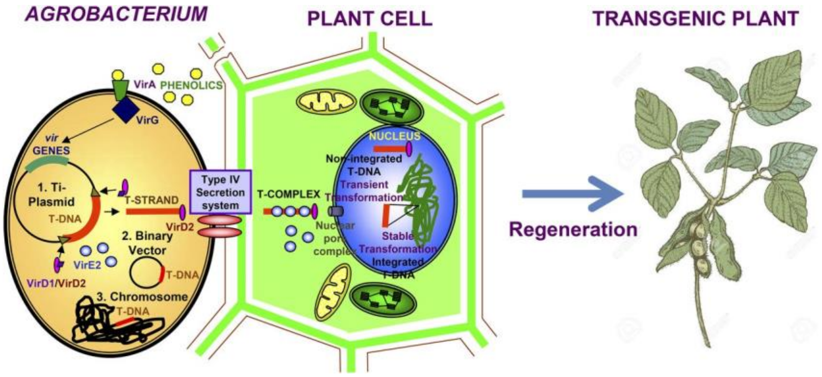


Figure 2.16. Agrobacterium-mediated transformation (Altpeter et al., 2016)

Three important regions of *Agrobacterium tumefaciens* bacteria are required in the genetic transformation of eukaryotic cells; The T-DNA region is the *vir* (virulence) region and the bacterial chromosome (Figure 2.17).

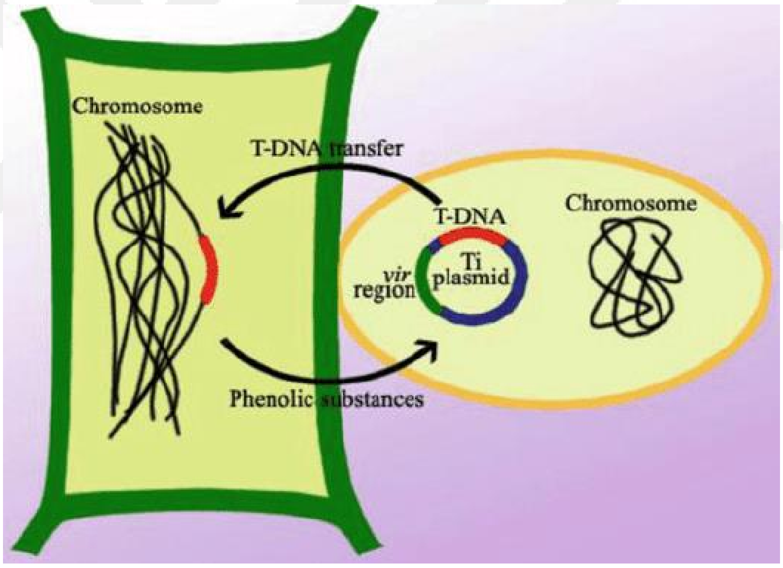


Figure 2.17. Regions required for T-DNA transfer from *Agrobacterium* to plant cells (Ozyigit, 2012)

The T-DNA region is bounded by the right border (RB) and left border (LB) sequences. These two border regions ensure that the target gene is transferred from the bacteria to the plant chromosome. The *vir* region of the Ti plasmid provides the transfer of T-DNA from the bacterial cell to the chromosome of the plant cell. The transfer takes place by cutting the T-DNA region at its borders and combining it with the plant chromosome, thanks to the enzyme produced in the *vir* region (Ozcan et al., 2004).

In conclusion, genetic arrangements can be made on plants through the T-DNA region of the Ti plasmid in *Agrobacterium* bacteria with the development of science and technologies. *A. tumefaciens* bacteria provide us making changes at the molecular level on the plants to obtain a new plant by used in agriculture.

CHAPTER III

MATERIALS AND METHODS

The present research work focuses on understanding the role of MYB transcription factor (*SlAIM1*) tomato gene in potato plants and improve the tolerance against drought stress on potato plants by the overexpression of *SlAIM1* gene. To develop these transgenic plants, following materials and methods were used.

3.1 Experimental Materials

3.1.1 Plant materials (tomato and potato plants)

Tiny Tim and Cherry tomato cultivars were grown in growth chamber for the isolation of *SlAIM1* gene before starting experiment (Figure 3.1). Tiny Tim is a tomato cultivar which was used to ensure salinity tolerance by *SlAIM1* (AbuQamar, 2009). First, the seeds of those cultivars were sown on soil which contains perlite and peat (mixed in the ration 3:1) and were regulary watered (Figure 3.1). Tomato plants were kept in growth chamber at 27± 1 ^{o}C with 16:8 h (Light: Density) photoperiod and at 65% relative humidity.

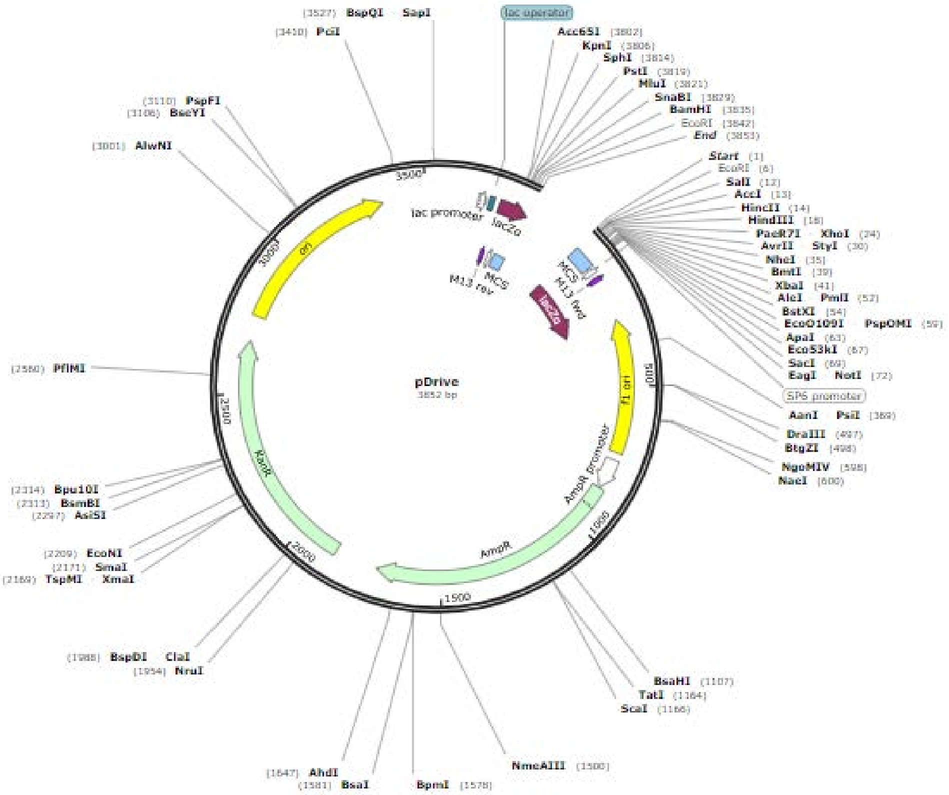
Figure 3.1. Tomato seedling propagation, cherry tomato (left) and Tiny Tim tomato (right) in pots under growth chamber

On the other hand, the potato cultivar Agria was used for the genetic transformation experiments. Agria is commercially cultivated in Turkey which has high productivity but susceptible for drought stress conditions (Hassanpanah D., 2010). Before the experiment, Agria cultivar was cultured in tissue culture conditions (Figure 3.2). To propagate Agria potato cultivar, nodes from already cultured plantlets (maintained in Plant Tissued Lab) were excised and placed on Murashige and Skoog medium (MS0) nutrient medium (Murashige et al., 1962). 4 nodes were cultured in each Magenta box. The cultures were maintained at 27 ± 2ºC with a day length of 16 h light, provided by fluorescent tubes in growth chamber.

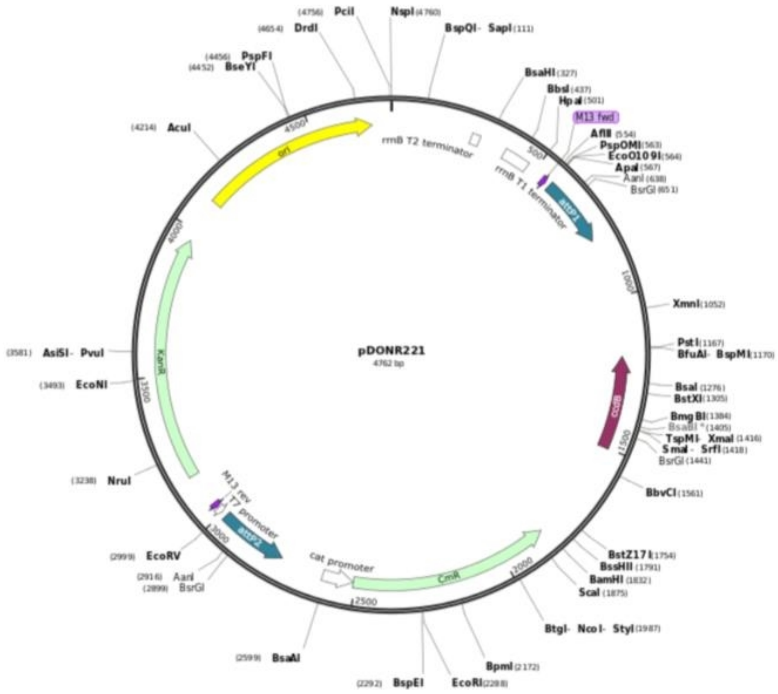
Figure 3.2. *In vitro* propogation of Agria cultivars under tissue culture conditions

3.1.2 Bacteria strains and plasmids

As the bacterial material, *E.coli Top10* bacterial strain was used for multiplication, and *A. tumefaciens AGL-1* strain was used for gene transfer to the plant. Firstly, TA cloning was performed and *pDrive* cloning vector was used in this experiment. For gene transformation to plants, gateway cloning was performed and vectors (*pDNOR221* and *pEarlyGate 100*) were used (Figure 3.3). The maps of both vectors were taken from SnapGene program.

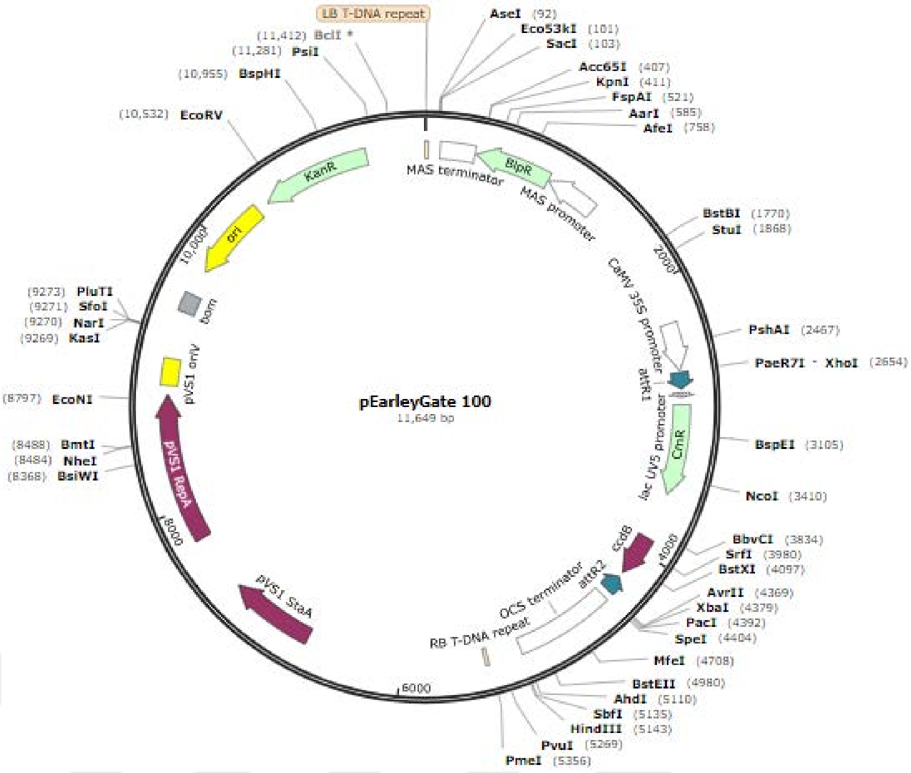


a



b

Figure 3.3. The maps of vectors that are used in experiment (a) *pDrive* (b) and *pDNR221* and *pEarleyGate 100* vectors (c), maps have been created using SnapGENE



c

Figure 3.3. (Continue) The maps of vectors that are used in experiment (a) *pDrive* (b) and *pDNR221* and *pEarleyGate 100* vectors (c), maps have been created using SnapGENE

3.2 Methods

3.2.1 Tomato plant propogation and drought stress application

To isolate and amplify *SlAIM1* gene from tomato, cultivars (Tiny Tim and Cherry) were grown in growth chamber. The both cultivars started germination in 10 days. Three weeks after germination, they were subjected to drought stress. Tomato plants were divided into two application groups as control and drought. While the plants in the control group continued to be irrigated regularly, irrigation was stopped when the plants in the drought group reached the seedling period and no water was given for twelve days (Alp and Kabay, 2017).

3.2.2 Total RNA extraction

Both application groups of tomato plants were subjected to the total RNA isolationfrom the leaves. Trizol protocol was used for RNA isolation (Macedo and Ferreira, 2014).

RNA extaction protocol:

Plant samples were grinded by liquid nitrogen in a mortar with pestle. 1.5 ml of TRI Reagent® (Sigma-Aldrich, T9424) was added onto 0.2 g tissue. After homogenizing by vortex, leaf samples were incubated in Trizol at room temperature for 10 min. The samples were centrifuged at 14,000 rpm and 4 C for 10 min. The supernatants were transferred into new tube and 400 µl chloroform was added. After shaking the samples by hand for 15 second, they were centrifuged at 14,000 rpm at 4 C for 15 min and supernatants were transferred into a new tube. 500 µl of cold isopropanol alcohol was added onto supernatant and incubated for 10 min at room temperature. After incubating for 10 minutes at room temperature, it was centrifuged at 4ºC and 11,000 rpm for 15 minutes.

After centrifugation, the supernatant was discarded and 75% EtOH was added to wash the RNAs. It was centrifuged at 4ºC at 9.000 rpm for 10 minutes. The upper liquid part was poured, pellets were dried in a fume hood for 10 minutes and pellets were dissolved in 50 µl DEPC water.

After RNA isolation, genomic DNA was removed from RNA preparations. This procedure was given Table 3.1.

Table 3.1. Removal of genomic DNA from RNA preparations

Reaction component	**Amount (µl)**
RNA (300ng/µl)	6,5
10× Reaction Buffer (with $MgCl_2$)	1
DNase I, RNase free	1
DEPC water	1,5
Total	10

Reaction was incubated at 37 °C for 30 min. After that, 1 µl 50 mM EDTA was added and incubated at 65°C for 10 min. The quality of RNAs was checked by NanoDrop spectrophotometer. To check RNA integrity clearly on the electrophoresis, a gel cassette and electrophoresis tank were cleaned with 5% hydrogen peroxide (EMSURE® ISO) for 30 min and washed by DEPC water. RNA integrity was checked by electrophoresis using 0.5% TBE (Trisborate EDTA) buffer with 1% agarose gel. Agarose was melted in a 0.5X TBE solution in the microwave oven and ethidium bromide was added after cooling. Then it was poured into a gel cassette covered with two-sided tape. After the solidification of agarose, RNA samples were loaded into the wells, and finally DNA ladder was added on the first well. The RNA samples were run on 90V for 1 hour and observed under UV light.

3.2.3 cDNA synthesis

The extracted RNAs were converted to the 1st strand cDNA by using Thermoscientific Revert Aid First Strand cDNA Synthesis Kit (Cat. No. K1621).

Table 3.2. The content of cDNA synthesis reaction

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

The reaction of cDNA synthesis was given in Table 3.2. The mixture was incubated at 37 °C for 1 hour and 70 °C for 10 minutes in thermocycler machine and cDNA synthesis was performed.

3.2.4 Primer design for insertion of the *SlAIM1* gene into the vector

Specific gene primers were designed by using Primer3 program to amplify by PCR (Polymerase Chain Reaction). *SlAIM1*-F and *SlAIM1*-R primers were designed for TA cloning whereas *SlAIM1*-GTF and *SlAIM1*-GTR primers were designed for gateway cloning (Table 3.3). All the primers which were used in this thesis are listed in Table 3.3.

To design *SlAIM1*-F and *SlAIM1*-R primers, the *BamHI* (GGATCC) recognition sequence was added to the 5′-end of the forward primer. The reverse primer is designed as a reverse complement sequence from the other half (5′-end) of *SlAIM1*. To design *SlAIM1*- GTF and *SlAIM1*- GTR primers, specific attB sites, *att*B1 and *att*B2 sequences added to the forward and reverse primers, respectively.

Table 3.3. Designed primers to be used in TA cloning and Gateway Cloning. *BamHI* sequence was highlighted with red color and attB1 and attB2 sites were highlighted with yellow color. M13 primers were used in BP reaction, BAR and nptII primers were used in LR reaction

Oligo Name	**Primer Sequence (5′ -3′)**
SlAIM1-F	ACAGGATCCATGGATCATCAACATGTT
SlAIM1-R	ACAGGATCCTCAAACAACATTGTTGTTG
SlAIM1-GTF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATAAA TTAATCAATCAAG
SlAIM1-GTR	GGGGACCACTTTGTACAAGAAAGCTGGGT TTAGGACCAAATGTCTTCAAT
M13-F	GTAAAACGACGGCCAGT
M13-R	CAGGAAACAGCTATGAC
BAR-F	GCACCATCGTCAACCACTA
BAR-R	ACAGCGACCACGCTCTTGAA
nptII-F	TTGCTCCTGCCGAGAAAG
nptII-R	GAAGGCGATAGAAGGCGA

3.2.5 The amplification of *SlAIM1* gene from tomato cDNA by PCR

cDNA was used to amplify *SlAIM1* gene fragment by EasyPfu® DNA Polymerase. In order to obtain best amplification, gradient PCR was performed in the range of 50°C and 66°C annealing temperatures. The components are presented below and temperature cycle of the PCR was given in Table 3.4.

PCR components:

dH_2O	8.6µl
10X *EasyPfu* Buffer	2 µl
cDNA (20 ng/ul)	5 µl
2.5 mM dNTP	2µl
EasyPfu DNA Polymerase	0.4µl
Primer *SlAIM1*-GTF (10 µM)	1 µl
Primer *SlAIM1*-GTR (10 µM)	1 µl
Total	20 µl

Table 3.4. PCR cycling conditions for amplification of *SlAIM1* by gateway primers

Step / Segment		**Temperature**	**Duration**	**Cycle**
Initial Denaturation		94ºC	3 min	1
Amplification	Denaturation	94ºC	30 sn	35
	Annealing	50-66°C	30 sn	
	Extension	72 °C	1 min	
Final Extension		72 °C	10 min	1

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

PCR results were confirmed by gel electrophoresis using 0.5% TBE (Trisborate EDTA) buffer with 1% agarose gel. Agarose was melted in a 0.5X TBE solution in the microwave oven and ethidium bromide was added after cooling. Finally, DNA was observed under UV light. Then it was poured into a gel cassette covered with two-sided tape. After the solidification of agarose, the PCR samples were loaded into the wells, and finally DNA ladder was added on the first well. The amplified PCR fragment of *AIM1* gene was run on 90V for 1 hour and observed under UV light.

3.2.7 Purification of *SlAIM1* gene fragment from agarose gel

SlAIM1 gene was amplified with EasyPfu® DNA Polymerase (transGen Biotech) to maintain proof reading ability. Amplified fragment of *SlAIM1* gene was purified from agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific #K0691) in such a way that the required band of *SlAIM1* gene was cut with the help of sterilized blade from the gel, weighted and 1:1 volume of binding buffer was added to the gel slice. Gel mixture was incubated at 55 ^{o}C till gel slice gets dissolved. After incubation, the mixture was then passed through DNA Purification Micro Column and put for 10 minutes at room temperature so that the column may absorb the DNA. The column was then centrifuged for 1 minute at 14,000 rpm. Supernatant was removed after centrifugation and 700 µl wash buffer was added. The column was centrifuged again for 60 seconds at 14,000 rpm. After supernatant was removed. Empty column was centrifuged again for 1 min to remove completely residual wash buffer. Warm elution buffer (65 ^{o}C) was added onto the column and kept for 30 minutes at room temperature. After 30 minutes the column was centrifuged and the supernatant was stored in 1.5 mL tube at -20 ^{0}C. Finally, the concentration of pcr product was measured by nanodrop.

3.3 Subcloning of PCR Product by TA Cloning Method

TA cloning which is one of the convenient methods to subclone PCR products in the linearized vector (Zhou and Gomez-Sanchez, 2000). *pDrive* vector (Figure 3.3), bacterial expression vector, was used to perform TA cloning.

QIAGEN PCR Cloning Kit (Catalog no. 231124) was used for TA cloning. Ligation-reaction mixture was prepared according to the Table 3.5.

Table 3.5. Ligation-reaction mixture

Reaction	**Amount**
pDrive Cloning Vector (50 ng/µl)	1,0 µl
PCR product (30 ng/ µl)	3,0 µl
Distilled water	1,0 µl
Ligation Master Mix, 2x	5,0 µl
Total	10 µl

2x Ligation Master Mix was added lastly and kept immediately at -20 °C. The ligation-reaction mixture was incubated at 16 °C for 12 hours and 4 °C for overnight in thermal cycling block.

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

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

First of all, *Top-10 E.coli* component cells were prepared before starting experiment. The protocol is given below:

Single colony was picked from freshly grown plate of *E.coli* strain on LB plate containing kanamycin for selection at 37 °C overnight. The colony was inoculated in 10 mL LB media without any antibiotic. The culture was grown overnight at 37 °C overnight. Next day, 1 mL from culture was transferred to overnight Top10 culture into 100 mL LB media in 3L flask. Then, they were growed at 37 °C at 250 rpm until OD_{600} was 0.4. The cells were transferred to two 50 mL falcon tubes and placed on ice for 20 min. The tubes were centrifuged at 4 °C at 3,000 g for 10 min. Media was poured and cells were resuspended in 10 mL cold 0.1 $CaCl_2$ and they were incubated on ice for 30 min. The cells were centrifuged at 4 °C for 10 min at 3,000 g. Supernatant was poured and cells were resuspended by pipetting in 2 mL cold 0.1 M $CaCl_2$ containing 15% glycerol. The cells were transferred to the 50 µl aliquots into eppendorf tubes and placed on ice immediately. The cells were freezed at -80 °C.

3.3.1.2 *E.coli* transformation of ligated product

E. coli strain (*Top-10*) competent cells were taken from -80 °C and put on an ice for 10 min. 5 µl from the ligation reaction were transferred in *E. coli* strain (*Top-10*) competent cells (50µl) and mixed by tapping. These cells were chilled on ice for 30 minutes and were then placed in hot water bath set at 42ºC for 50 seconds. After that, the cells were put on ice immediately for 3 min.Luria- Bertani (LB) broth medium (450 µl) was added into the cells and the cells were then incubated at 37ºC for 1 hour in incubator shaker. After 1 hour, cells were spread on LB plates containing 100mg/l of ampicillin and incubated at 37ºC overnight.

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

After the LB medium was prepared, it was sterilized by autoclaving at 121 ° C and 0.1 MP pressure for 15 minutes. When the mixture was reduced to the tangible temperature (50–55 ºC), Ampilicillin antibiotic was added.

3.3.2 Confirmation of *pDRIVE* plasmid containing *SlAIM1* gene

Next day, the positive clones were confirmed through colony PCR by using M13 primers. Before conducting colony pcr, colonies were taken into the 30 µl of water. And these colonies were incubated at 95 ° C for 10 min. This step helped to break bacterial cell wall.

Colony PCR content:

PCR Master Mix (2×) (contains Taq Polymerase ve dNTP)	10,0 µl
Primer (10mM)	1 µl
M13-F Primer (10mM)	1 µl
M13-R Primer (10mM)	
Colony	8,0 µl
Total	20 µl

Positive clones were again tested by PCR with specific gene primer (*SlAIM1*-F and *SlAIM1*-R). After that, clones were grown in 10 ml LB broth containing ampilicillin and incubated at 37 ºC overnight. The following day, plasmid extraction was performed using plasmid miniprep kit (Thermo Scientific- Cat #K0503). First of all, bacterial cultures were centrifuged at 14000 rpm for 10 min. Then, pelleted cells were resuspended in 250 µl of the Resuspension Solution. After transfering the cell suspension to a microcentrifuge tube, 250 µl Lysis Solution was added and mixed by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. 350 µl of the Neutralization Solution was added and mixed immediately and mixed well by inverting the tube 4-6 times. When the neutralized bacterial lysate became cloudy, mixtures were centrifuged for 5 min to pellet cell debris and chromosomal DNA. After that, the supernatant was transfered to the supplied GeneJET spin column by pipetting. After 1 min centrifuge, 500 µl of Wash Solution was added twice to the GeneJET and centrifuged for 60 sec. After discarding the flow-through, in order to remove residual wash solution, empty column was centrifuged for 1 min. GeneJET spin column was transfered into a fresh 1.5 ml microcentrifuge tube and 40 µl of the prewarmed elution buffer was added to the center of GeneJET spin column membrane to elute the plasmid DNA. Tubes were incubated for 30 min at room temperature and centrifuged for 2 min. Column was discarded and stored the purified plasmid DNA at -20°C. Plasmid concentrations were measured by Nanodrop.

3.3.2.1 Confirmation of clones by restriction analysis

After plasmid extraction, restriction analysis of positive clones which are tested by colony pcr (Table 3.6).

Table 3.6. Restriction analysis protocol with *BamHI* and *SacI* enzymes

1	Plasmid (100ng/ul)	2 µl
2	FD Buffer (Thermo)	2 µl
3	*BamHI* (Thermo)	0,5 µl
4	*SacI* (Thermo)	0,5 µl
5	H_2O	15 µl
6	Toplam	20 µl

The restriction analysis was performed as it is shown in Table 3.6. The reaction was incubated at 37 °C for 30 min and reaction was terminated at 65 °C for 10 min.

3.4 Cloning of *SlAIM1* Gene to Expression Vector (*pEarleyGate 100*)

3.4.1 Amplification of *SlAIM1* gene by gateway primers (*SlAIM1*-GTF and *SlAIM1*-GTR)

To amplify target gene, *SlAIM1*, cDNAs were used (Cherry cDNA, Cherry Drought cDNA, Tiny Tim cDNA, Tiny Tim Drought cDNA). PCR was performed by EasyPfu® DNA Polymerase (transGen Biotech) with optimized annealing temperature, 62°C by gateway primers. PCR results were confirmed by gel electrophoresis using 0.5% TBE (Trisborate EDTA) buffer with 1% agarose gel. The amplified fragment of *SlAIM1* gene was purified from agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific #K0691).

3.4.2 Gateway cloning of *SlAIM1* gene

To transfer *SlAIM1* gene to the plant, gateway cloning method was performed. The Gateway Cloning was performed in two steps.

3.4.3 First step: BP reaction

BP reaction was performed according to Table 3.7.

Table 3.7. Reaction mixture for BP and control reactions

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

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

pEXP7-tet was provided as a positive control for the BP reaction. Invitrogen BP Clone II enzyme was mixed well by vortexing. Other components were centrifuged briefly. BP Clone II enzyme was returned -20 immediately. The mixtures were incubated at 25°C for 1 hour. After that, 1 µl of the Proteinase K solution was added to the mixtures to terminate the reaction. After vortexing briefly, samples were incubated at 37°C for 10 minutes.

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

Prepared *E. coli* strain (*Top-10*) competent cells were taken from -80 °C and put on a ice for 10 min. 1 µl from the reactions (BP and control) were transferred in *E. coli* strain (*Top-10*) competent cells (50µl) and mixed by tapping. These cells were chilled on ice for 30 minutes and were then placed in hot water bath set at 42ºC for 50 seconds. After that, the cells were put on ice immediately for 3 min. Luria- Bertani (LB) broth medium (450 µl) was added into the cells and the cells were then incubated at 37ºC for 1 hour in incubator shaker. After 1 hour, cells were spread on LB plates containing 50 µg/mL of kanamycin and incubated at 37ºC overnight.

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

After the LB medium was prepared, it was sterilized by autoclaving at 121 ° C and 0.1 MP pressure for 15 minutes. When the mixture was reduced to the tangible temperature (50–55 ºC), Kanamycin antibiotic was added.

3.4.3.3 Confirmation of *pDNR221* plasmid containing *SlAIM1* gene

Next day, the positive clones were confirmed through colony PCR using gene specific primers as it described before. Gel eluted fragments of *SlAIM1* gene was used as positive control.

Colony PCR content:

PCR Master Mix (2×) (contains Taq Polimerase ve dNTP)	10,0 µl
SlAIM1-GTF (10mM)	1 µl
SlAIM1-GTR (10mM)	1 µl
Colony	8,0 µl
Total	20 µl

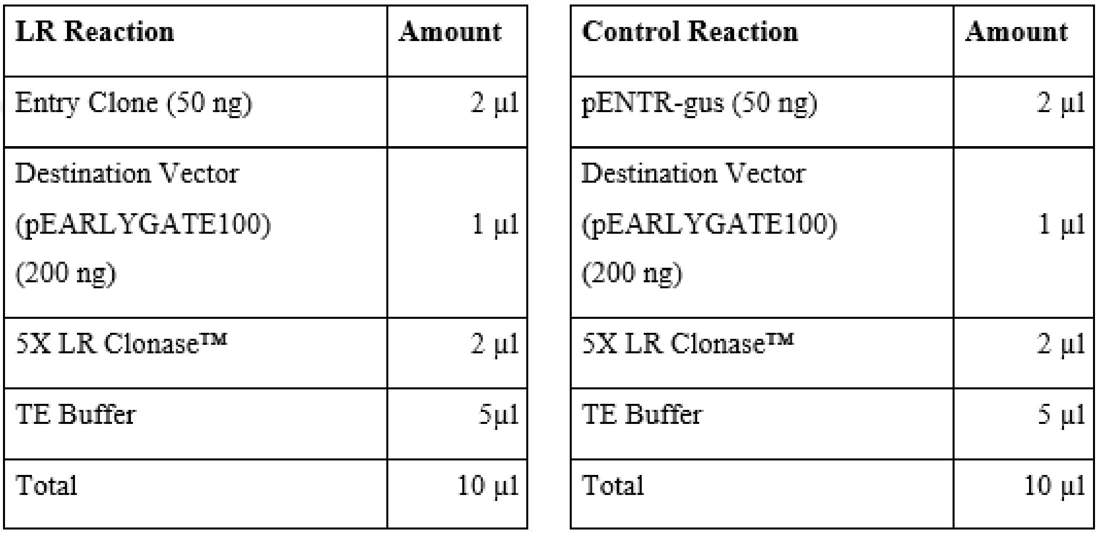
3 of the positive clones were selected. Positive clones were grown in 10 ml LB broth containing kanamycin 50µg/mL and incubated at 37 ºC overnight. The following day, plasmid extraction was performed using plasmid miniprep kit (Thermo Scientific- Cat #K0503. Plasmid concentrations were measured by Nanodrop.

In order to confirm BP reaction, extracted plasmid was checked by PCR reaction. In the first round of PCR, primers were combined (M13 F and *SlAIM1*-GTR) and BP plasmid was checked by gateway gene specific primers (*SlAIM1*- GTF and *SlAIM1*- GTR) as a control. In the second round of PCR, BP plasmid was checked by M13 primers (M13F and M13R), empty *pDONR221* plasmid was used as a control.

3.4.4 The second step: LR reaction

In this stage, attL-containing entry clone (extracted positive plasmids) was transfered to attR-containing destination vector via LR clonase to generate an attB-expression clone (*pEarleyGate 100*). *pEarleyGate 100* allows for rapid recombinational cloning to over-express a DNA sequence of interest (Earley et al.,2006). LR reaction was performed according to Table 3.8.

Table 3.8. Reaction mixture for LR and control reactions



LR Reaction	Amount
Entry Clone (50 ng)	2 µl
Destination Vector (pEARLYGATE100) (200 ng)	1 µl
5X LR Clonase™	2 µl
TE Buffer	5µl
Total	10 µl

Control Reaction	Amount
pENTR-gus (50 ng)	2 µl
Destination Vector (pEARLYGATE100) (200 ng)	1 µl
5X LR Clonase™	2 µl
TE Buffer	5 µl
Total	10 µl

pENTR-gus was provided as a positive control for the LR reaction. Invitrogen LR Clone II enzyme was mixed well by vortexing. Other components were centrifuged briefly (Table 3.9). LR Clone II enzyme was returned -20 immediately. The mixtures were incubated at 25°C for 1 hour. After that, 1 µl of the Proteinase K solution was added to the mixtures to terminate the reaction. After vortexing briefly, samples were incubated at 37°C for 10 minutes. 1 µl of the LR reaction and control reaction were transformed into prepared 50 µl *Top-10 E.coli* components cells as it described before. 3 colonies were picked up and growed in 10 ml LB broth containing kanamycin 50µg/mL and incubated at 37 ºC overnight. The following day, plasmid extraction was performed using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After the measurement of plasmid concentrations, it was transfered to the Agrobacterium components cells (*AGL-1*).

3.4.4.1 Preparation of *Agrobacterium* component cell (*AGL-1*)

AGL-1 Agrobacterium component cells were prepared before starting experiment.
The protocol was given below:

Single colony was picked from freshly grown plate of *Agrobacterium* and inoculated into 10 mL LB liquid medium containing strain on LB plate containing rifampisin antibiotic in 50 mL autoclaved flask and incubated at 28°C for 48 hours with vigorous shaking. 1 mL of the grown culture was added into 100 mL of LB medium and incubated at 28°C until the OD_{600} of cells became 0.6. The cells were transferred to ice cold 50 mL propylene tube and kept cool on ice for 30 min.

The cells were centrifuged at 4 °C for 10 min at 4000 rpm. Supernatant was discarded and resuspended in 50 mL of sterile cold autoclaved water. Again, the cells were centrifuged at 4 °C for 10 min at 4000 rpm. Supernatant was discarded and resuspended in 25 mL of sterile cold autoclaved water. After washing again, the cells were resuspended in 10 ml sterile cold water containing sterilized cold 10% glycerol. This wash was repeated. Finally, the cells were resuspended in 1 mL filter sterilized 10% glycerol. The cells were transferred to the 50 µl aliquots into eppendorf tubes and placed on ice immediately. The competent cells were freezed at -80 °C.

3.4.4.2 Transformation of *SlAIM1-pEarleyGate 100* plasmid in *Agrobacterium* (*AGL-1*)

The prepared *Agrobacterium tumefaciens* strain *AGL-1* was used for transformation of *SLAIM1-pEarleyGate100* plasmid. Two microliters of ligated product were transformed into competent *Agrobacterium* cells (50µl) and mixed by tapping. These cells were chilled on ice for 30 minutes and were subjected to electroporation. The electroporation device was set at 25µF, voltage 2.4kV with a 200 ohms resistance. After electroporation, 450 µl of LB broth was added and the mixture was incubated at 28ºC for 3 hours at 1100 rpm in shaking incubator. Cells were then spread on LB plates containing kanamycin (50 µg/mL) and were then incubated at 28ºC overnight. Following day, colonies were checked and confirmed through colony PCR by gene specific primers, BAR and NPTII primers.

Cultures were prepared from the positive colonies in LB broth containing 50 µg/mL of kanamycin and incubated at 28ºC overnight. The following day, the plasmid DNA were extracted from the overnight grown cultures as per protocol using plasmid miniprep kit (Thermo Scientific- Cat #K0503). After extraction, the plasmid was then run on 1% agarose gel and observed under UV light and glycerol stocks were prepared and stored at -80ºC.

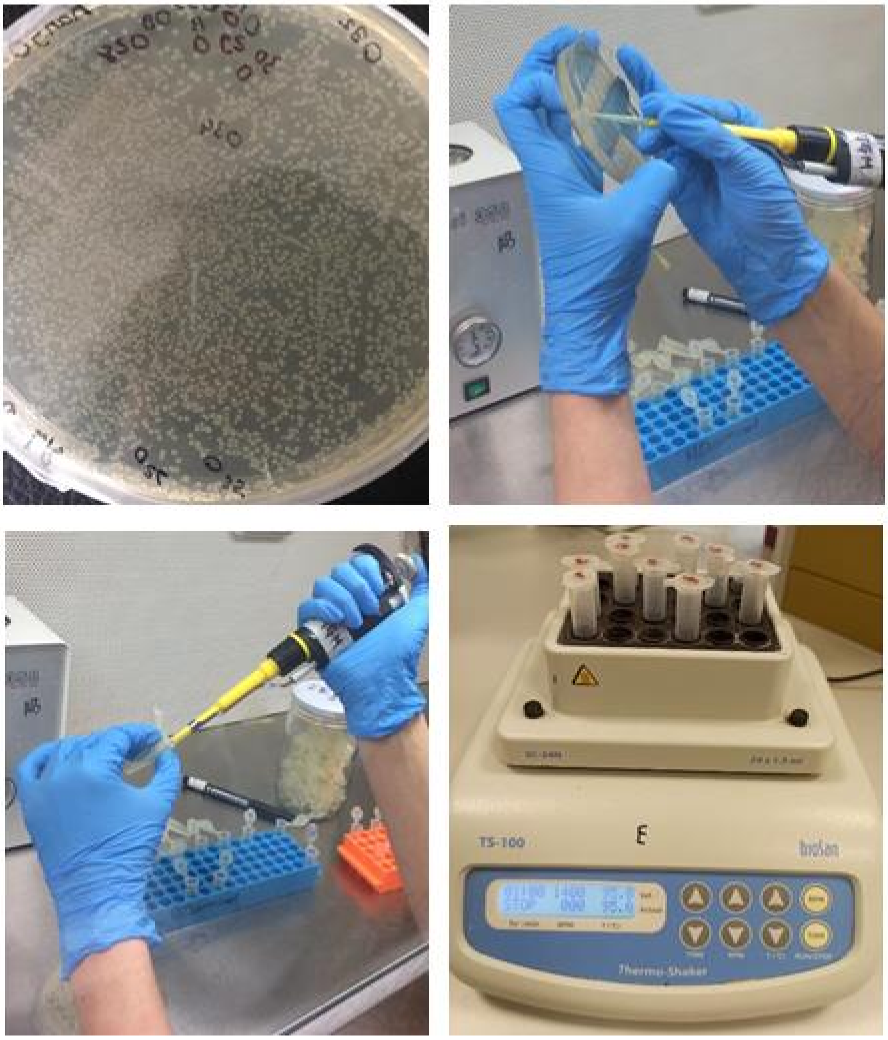


Figure 3.4. Colony PCR of LR reaction, LR reaction colonies (a), taking colonies from plate (b), taking colonies inside sterile water (c) and incubating colonies at 95 °C (d)

3.5 Transfer of *SlAIM1* to Potato Plants by *Agrobacterium*-mediated Gene Transformation Method

3.5.1 Propagation of potato plants in tissue culture conditions

In order to propagate Agria potato cultivar, MS-0 nutrient medium was used to plant growth boxes (Magenta boxes) are used to grow the plants. Nodes were used for propogation and 4 nodes were grown in each grow box. Plants were grown at 27 ± 2ºC with a day length of 16 h light, provided by fluorescent tubes in growth chamber. The content of MS nutrient medium is given in Table 3.9.

Table 3.9. Nutrients and amounts of MS (Murashige and Skoog (1962)) medium

Nutrients	**Ingredients and**	**Amount (mg/l)**
Macro nutrients	NH4NO3	1650,00
	KNO3	1900,00
	CaCl2.2H2O	440,00
	MgSO4.7H2O	370,00
	KH2PO4	170,00
Micro nutrients	KI	0,83
	H3BO$_3$	6,20
	MnSO$_4$.4H$_2$O	22,30
	ZnSO$_4$.7 H$_2$O	8,60
	Na2MoO4.2 H$_2$O	0,25
	CuSO4.5 H$_2$O	0,025
	CoCl2.6 H$_2$O	0,025
	FeSO4.7 H$_2$O	27,80
	Na2EDTA.2 H$_2$O	37,30
Vitamins	myo-Inositol	100,00
	Nicotinic Acid (free acid)	0,50
	Pyridoxine-HCl	0,50
	Thiamine-HCl	0,10
Amino Acid	Glycine	2,00

TOTAL (gms/l): 4,4

The component of MS-0 media for 1 L

Agar	8.0 g
MS-Salt	4.4 g
Sucrose	30.0 g
pH	5.7

3.5.2 Preparation of bacteria inoculation

Agrobacterium tumefaciens strain *AGL-1* harboring *SlAIM1* gene was used for genetic transformation of potato cultivars. Prior to co-cultivation with potato tissues, the Agrobacterium strain harboring *AIM1* gene were streaked on LB plates containing 50µg/mL of kanamycin and incubated for 24–48h at 28 ºC (Figure 3.5). After incubation period, single colony was picked up by using small tips and inoculated in 10 mL of LB broth containing 50mg/mL of kanamycin in a 50 mL falcon tube (Figure 3.6). Then, the bacteria were cultured overnight on a rotary shaker at 28 °C with shaking at 180 rpm.

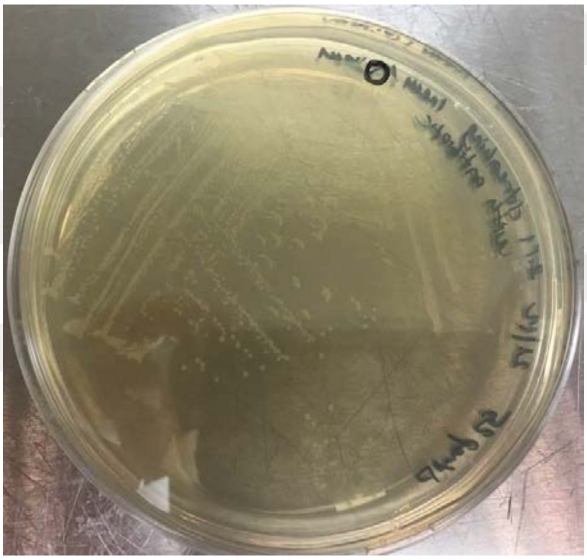


Figure 3.5. Streaking of *Agrobacterium* plasmid suspension overnight growth on LB agar plate with kanamycin

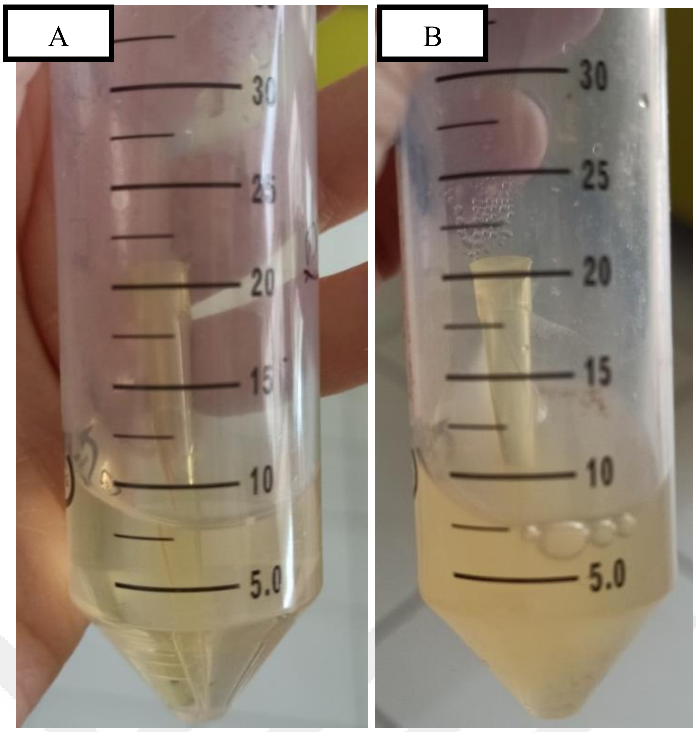


Figure 3.6. Overnight grown culture of *SlAIM1* gene plasmid suspension, (A) taking a single colony in liquid LB medium with kanamycin antibiotic, (B) overnight grown bacteria

3.5.3 Transfer of *SlAIM1* to plants

3.5.3.1 Inoculation of explant with *Agrobacterium* culture

Leaf, node, internode and microtuber explants of Agria potato cultivar were used for gene transfer when plants were uniformly sized 4-5 nodes. First of all, explants were transferred to petri dishes containing sterile liquid LB medium and 2 ml of *A. tumefaciens* culture containing *pEarleyGate 100-SlAIM1* vector was added and inoculated for 15 minutes with gentle shaking (Figure 3.7). The inoculated explants put on co-cultivation media [MS-0, Acetosyringone (100mM) 1ml/l] and kept in incubator chamber at 24 ° C, 16 hours light and 8 hours dark.

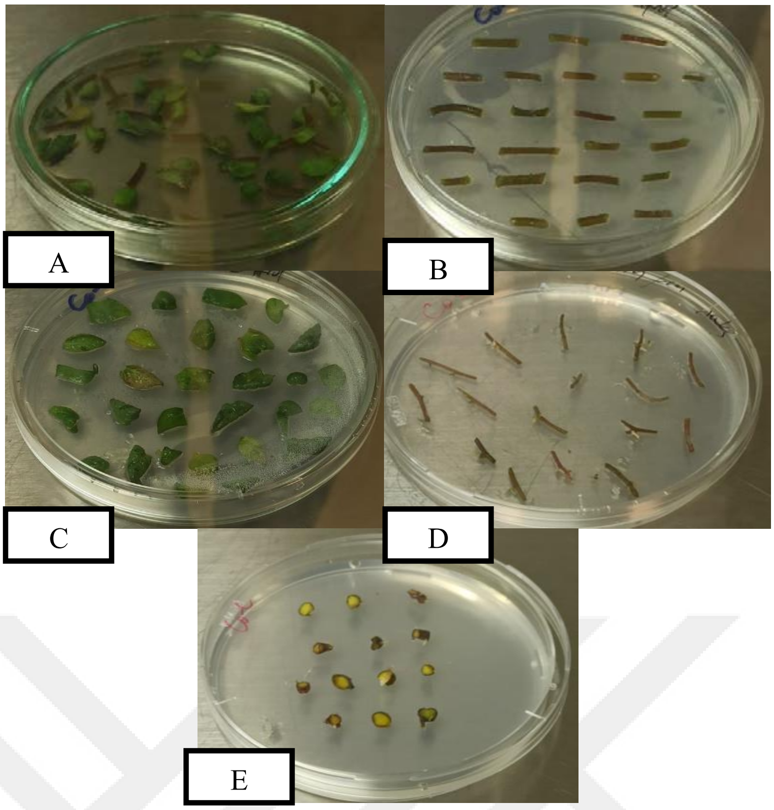


Figure 3.7. Transfer of *SlAIM1* to Agria cultivar by *Agrobacterium tumefaciens*; (a) cutting the explants in sterile environment and inoculating *Agrobacterium tumefaciens* in LB medium, (b) internodes on co-cultivation medium after inoculation, (c) leaves on co-cultivation medium after inoculation, (d) nodes on co-cultivation medium after inoculation, (e) micro-tubers on co-cultivation medium after inoculation

After 2 days, explants were washed by sterile distilled water and sulcid for 15 min. The explants were then dried on sterile paper. Washing process was performed to remove all bacteria remaining on the explants. Then the explants were cultured in RSM selection medium, with 10 explants in each petri dish (Figure 3.8). The explants were kept in a growth chamber at 24 ° C, 16 hours light and 8 hours dark.

On the other hand, control vector (empty *pEarleyGate 100* in *AGL-1* strain) was transferred to the Agria cultivar. The control vector does not contain *SlAIM1* gene and it has controlled by CamMV 35S promoter which is useful as a negative control in experiment.

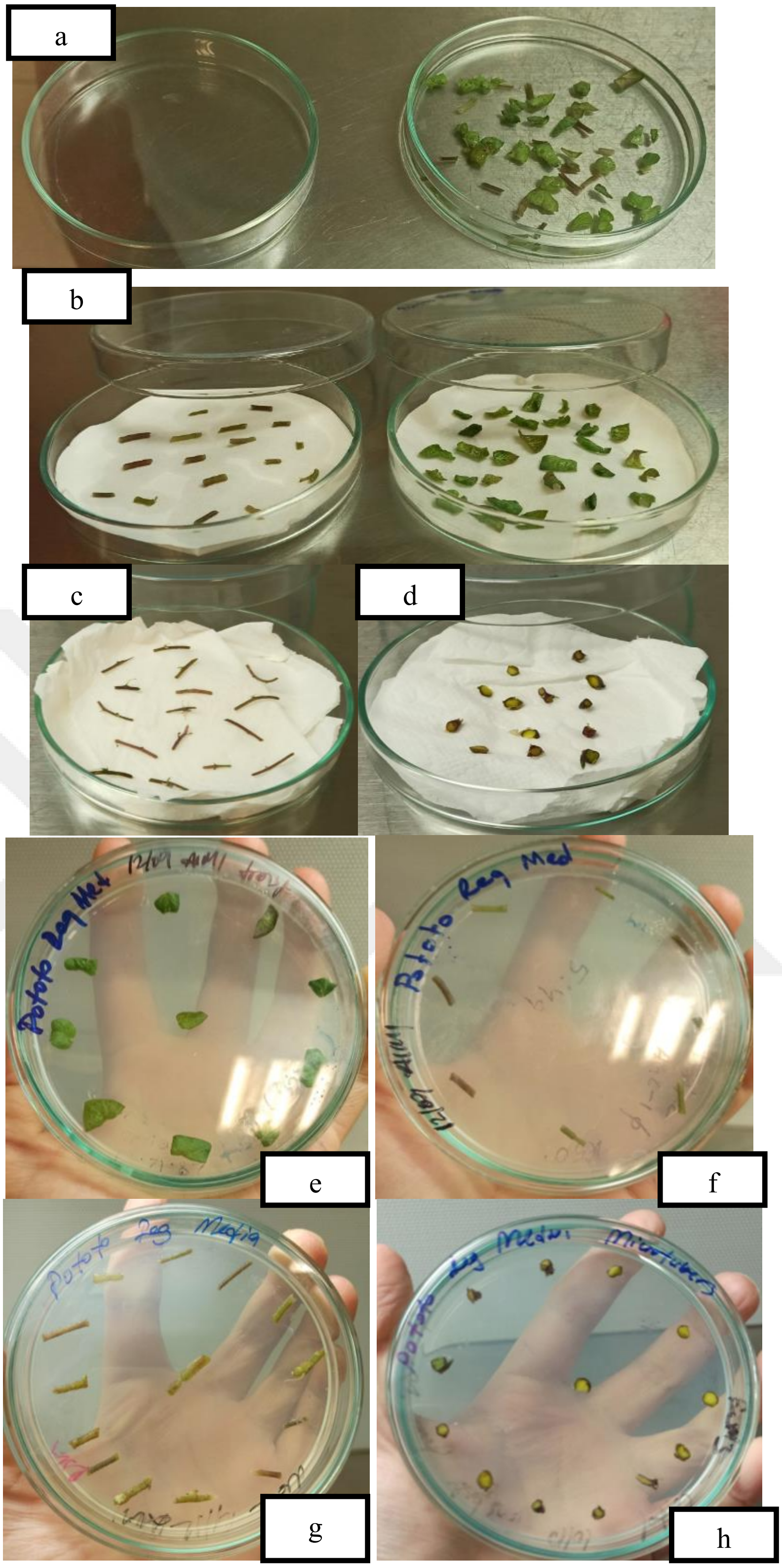


Figure 3.8. Transfer of inoculated explants from co-cultivation media to the RSM media; washing the explants with sterile water and antibiotic (sulcid) (a), drying the explants (b,c,d), placing leaves on RSM media (e), placing internodes on RSM media (f), placing nodes on RSM media (g), and placing microtubers on RSM media (h)

3.5.3.2 Regeneration selection medium (RSM)

In order to obtain callus formation and candidate transgenic plants, RSM media was used (Table 3.10). Sulcid was used to prevent the development of *A. tumefaciens* and phosphinothricin (PPT) was used for plant selection in gene transfer.

Table 3.10. Ingredients of regeneration selection medium (RSM)

MS Salts with vitamins	4.4g /l
Sucrose	30g/l
Plant Agar	8g/l
BAP	2mg/l
NAA	0.2 mg/l
Kinetin	1 mg/l
Trans zeatin	1 mg/l
PPT (Phosphinothricin)	0.5 mg/l
Sulcid	500mg/l
pH	5.7

3.5.4 Transfer and selection on shoot and root induction medium

Approximately 6 weeks later, callus and shoot formed on the explants. Callus and developed candidate transgenic shoots were then grown in shoot-induction MS media [2mg / l BAP, 0,2mg / l NAA, + 30 g / l sucrose + Agar 8 g / l + MS Salt 4,4 g / l + 400 mg / l Sulcid + 0,5 mg/l PPT]. Developed shoots were propagated under tissue culture conditions on MS0 media.

3.6 Confirmation of Transgenic Plants by Molecular Analyses

3.6.1 Genomic DNA extraction from putative transgenic plants and PCR assays

TransDirect® Plant Tissue PCR Kit was used to extract genomic DNA from putative transgenic plant and direct pcr was done by the same kit.
The protocol is given below:

5 mg of plant tissues were cut from putative transgenic plants and put into the 40 µl of PD1 buffer. After homogenizing by vortex, samples were incubated at 95 ° C for 30

min. 40 µl of PD2 buffer was added to the samples and vortexed well. The lysates were directly used for PCR assay.

PCR components:

Nuclease free water	5.2 µl
Unpurified Lysate	4µl
2x TransDirect® PCR SuperMix (+dye)	10 µl
Primer BAR-F (10 µM)	0.4 µl
Primer BAR-R (10 µM)	0.4 µl
Total	20.0 µl

The PCR reaction conditions by BAR primers were followed at 94 ºC for 5 minutes for denaturation followed by annealing temperatures at 55 ºC for 30 seconds. Extension was carried out at 72 ºC for 30 seconds repeated 35 times. Final extension was carried out at 72 ºC for 10 minutes.

3.6.2 Calculation of transformation efficiency

Transformation efficiency was calculated for transgenic plants. Mean Percent Transformation Efficiency (%MTE) was calculated.

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

3.7 Drought Stress Application on Standart Agria Cultivar and Transgenic Plants

Rahamkulov and Bakhsh, 2020 protocol was used in this study and %20 PEG was applied to induce drought stress on transgenic plants. Polyethylene Glycol (PEG) 6000 (item number 8.07491.1000) from Merck-Millipore was used to apply drought stress on the Agria potato cultivar. 20% PEG was prepared and used for drought stress. First, 50 ml of MS0 nutrient medium was prepared in magenta (GA-7 autoclavable) containers and added 25 ml of sterile filtered 20% PEG and left for 1 day to absorb the nutrient medium. Candidate transgenic plants (*SlAIM1*-1 and *SlAIM1*-4), transformed plants by control vector and non-transgenic standart agria varity were subcultured as 2 nodes for root and shoot formation in normal MS-0 and MS0 / PEG nutrient media for ten days.

Table 3.11. Preparation of 20% PEG for drought stress

Content of mixture	**Amount**
PEG H(C2H4O)nOH=6000	20 g
Sterile water	80ml

3.8 Gene Expression Analysis in Control and Transgenic Plants

qRT-PCR analysis was performed to determine the expression of the *SlAIM1* gene in transgenic plants growing in MS-0 medium. The details of the method were explained below under subtitles.

3.8.1 Total RNA isolation and cdna synthesis of transgenic plants

First of all, total RNA isolation was performed from transgenic plants (*SlAIM1*-1 and *SlAIM1*-4), transformed plants by control vector and non-transgenic standart agria varity under drought and normal conditions. On the other hand; totally 5 candidate transgenic plants plants were subjected to RNA isolation as it described before. Trizol protocol was used for RNA isolation. After RNA isolation, genomic DNA was removed from RNA preparations. The quality of RNAs was checked by NanoDrop spectrophotometer. RNA integrity was also checked by electrophoresis.

The extracted RNAs were converted to cDNA by using Thermoscientific Revert Aid First Strand cDNA Synthesis Kit (Cat. No. K1621) as it described before.

3.8.2 qRT-PCR analysis

qRT-PCR analysis was performed to determine the expression of the *SlAIM1* gene. The *EF1α* gene, considered the most stable gene in the potato plant (Nicot et al., 2005), was used as a reference gene for normalization. All real time PCR samples were performed as duplicate. The protocol of qRT-PCR analysis was given in Table 3.13. Rotor-Gene Q (QIAGEN) device and instrument software were used for gene expression analysis. The proportional variation of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The protocol is given below:

Table 3.12. Primers used in qRT-PCR

EF1-F	5'-ATTGGAAACGGATATGCTCCA-3′
EF1-R	5'-TCCTTACCTGAACGCCTGTCA-3′
qRT_AIM1-F	5'-AGTTGTCCAGCCAGAGAG-3′
qRT_AIM1-R	5'-AAGCAAGTTGTCGGAATC-3′

Table 3.13. The content of qRT-PCR

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

Table 3.14. Temperature conditions of qRT-PCR

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

Gene expression results were analyzed with the JMP Statistical Package program.

RESULTS

CHAPTER IV

4.1 Amplification and Cloning of *SlAIM1* Gene

For amplification and cloning of *SlAIM1* gene, following steps were performed.

4.1.1 Amplification of *SlAIM1* gene

cDNA from Tiny Tim tomato cultivar was used as a template to amplify 652 bp fragment of *SlAIM1* gene. The gradient revealed best results at 56 ° C and 62° C (Figure 4.1 A). PCR amplified product was purified from agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific #K0691) (Figure 4.1 B). Eluted fragment was further used for cloning purpose.

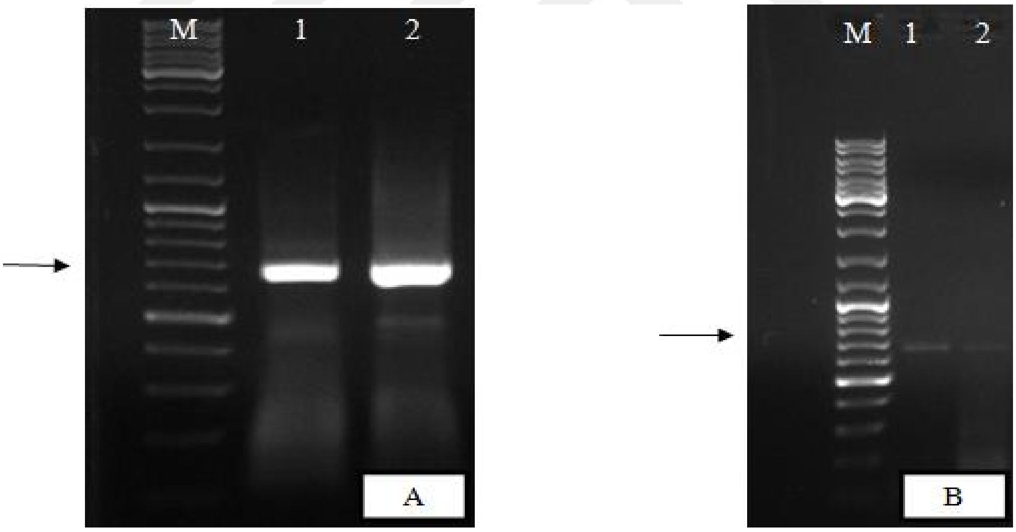


Figure 4.1. Amplification of *SlAIM1* gene by PCR, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1-2: *AIM1* gene amplified at 56° C and 62° C, respectively (A), Gel eluted fragment of the amplified gene (B)

4.1.2 Transformation of *SlAIM1 into the pDRIVE* plasmid in *E. coli* (*Top10*)

After ligation reaction in TA cloning, the ligated product was then transformed to *E.coli* component cell according to protocol of transformation. Colony PCR was performed to confirm positive clones by using M13 (M13 F and M13 R) primers and specific gene primer (*SlAIM1*-F and *SlAIM1*-R).

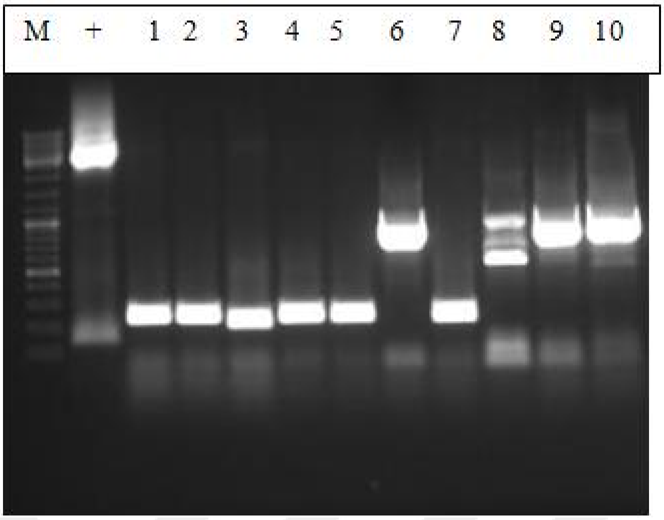


Figure 4.2. Colony PCR assay for the confirmation of TA cloning by using M13 primers, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: positive control, pDRIVE plasmid, Lane 1-10 DNA results with M13 primers

Lane 6, 9 and 10 were selected as a positive as they give expected amplicon size (900 bp). Positive clones were tested by specific gene primer (*SlAIM1*-F and *SlAIM1*-R).

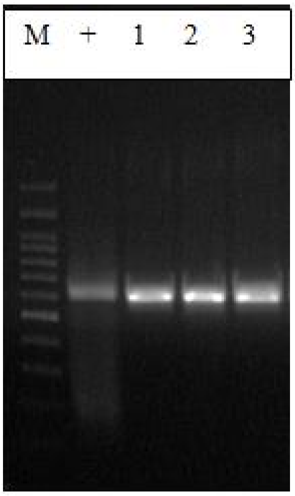


Figure 4.3. Colony PCR assay for the confirmation of TA cloning by using gene specific primers, (*SlAIM1*-F and *SlAIM*-R), M: 100 bp DNA Ladder (Solis BioDyne), Lane +: positive control, gel eluted fragment of *SlAIM1* gene, Lane 1-3: DNA results with gene specific primers, (*SlAIM1*-F and *SlAIM1*-R)

4.1.3 Confirmation of clones by restriction digestion

In Figure 4.2, Lane 6 and Lane 9 were selected and they were confirmed again. The positive clones (Lane 6 and Lane 9) were further confirmed by restriction digestion analysis with *BamHI* and *SacI* restriction enzymes (Figure 4.4). As seen in Figure 4.4, the expected band sizes (672 bp) were observed after restriction digestion.

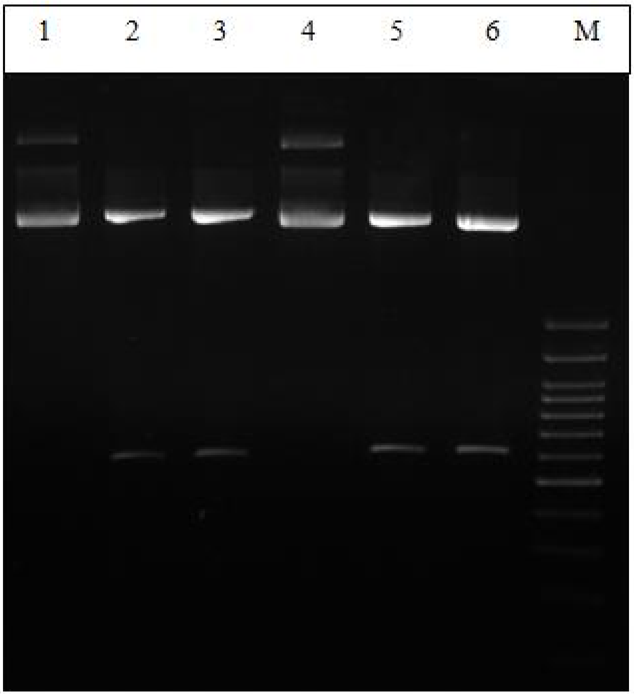


Figure 4.4. Restriction Digestion Analysis of *AIM1-pDRIVE* plasmid, M: 100 bp DNA Ladder (Solis BioDyne), Lane 1: Undigested plasmid (sixth clone), Lane 2, 3: digested plasmid by *BamhI* and *SacI,* Lane 4: Undigested plasmid (ninth clone), Lane 5, 6: digested plasmid by *BamhI* and *SacI*

4.2 Amplification of *SlAIM1* Gene by Gateway Primers

cDNAs from tomato varieties were used as a template to amplify *SlAIM1* gene by *SlAIM1*-GTF and *SlAIM1*-GTR primers. The gradient was arranged at 62° C (Figure 4.5).

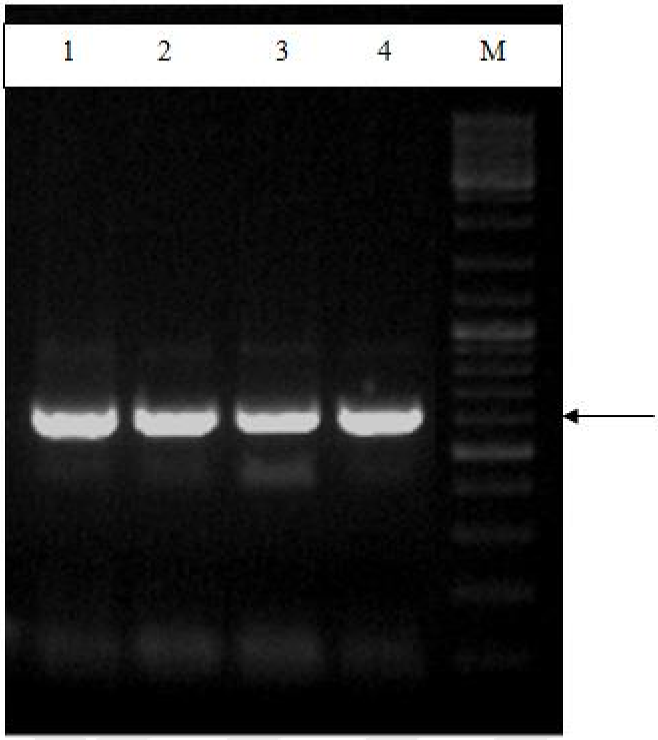


Figure 4.5. Amplification of *SlAIM1* gene by gateway primers, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1: *AIM1* gene amplified with cDNA from cherry at 62° C, Lane 2: *AIM1* gene amplified with cDNA from cherry- drought at 62° C, Lane 3: *AIM1* gene amplified with cDNA from Tiny Tim at 62° C, Lane 4: *AIM1* gene amplified with cDNA Tiny Tim- drought at 62° C. PCR assay showed amplification from all samples. Amplified *AIM1* gene from Tiny-Tim-drought cDNA was selected for further experiments

4.2.1 Transformation of *SlAIM1* into the *pDONR221* plasmid in *E. coli* (*Top10*)

After BP reaction, the product was then transformed to *E.coli* component cell according to protocol of transformation. Colony PCR was performed to confirm positive clones by using gateway gene specific gene primer (*SlAIM1*-GTF and *SlAIM1*-GTR) and M13 primers. As a positive control, gel eluted fragment of *SlAIM1* gene was used (Figure 4.6 and Figure 4.6).

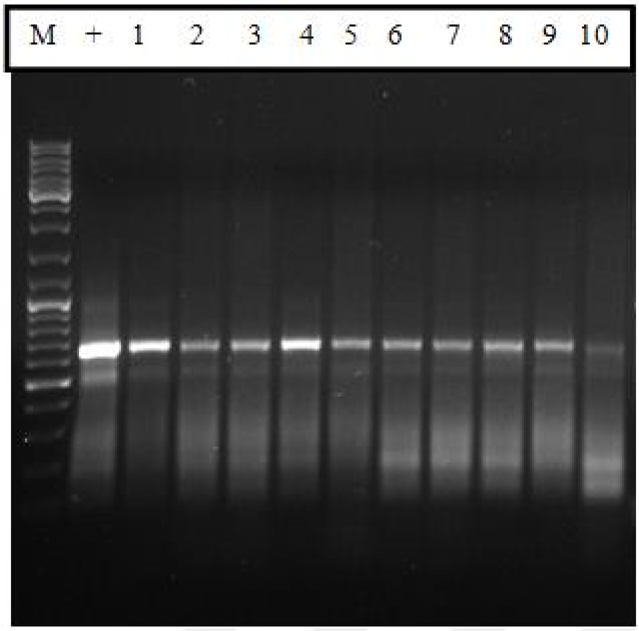


Figure 4.6. Colony PCR assay for the confirmation of BP reaction, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: positive control, gel eluted fragment of SlAIM gene, Lane 1-10 DNA results with gateway gene specific primers (*SlAIM1*-GTF and *SlAIM1*-GTR), Lane 1 was selected for furher experiment

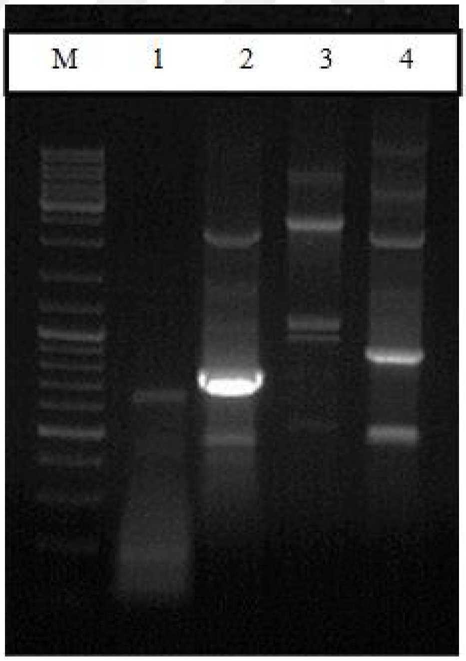


Figure 4.7. PCR assay for the confirmation of BP reaction, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1: BP plasmid (selected first clone) with gateway gene specific primers, Lane 2: BP plasmid with M13F and *SlAIM1*- GTR primers, Lane 3: *pDNOR221* plasmid with M13 primers, Lane 4: BP plasmid with M13 primers

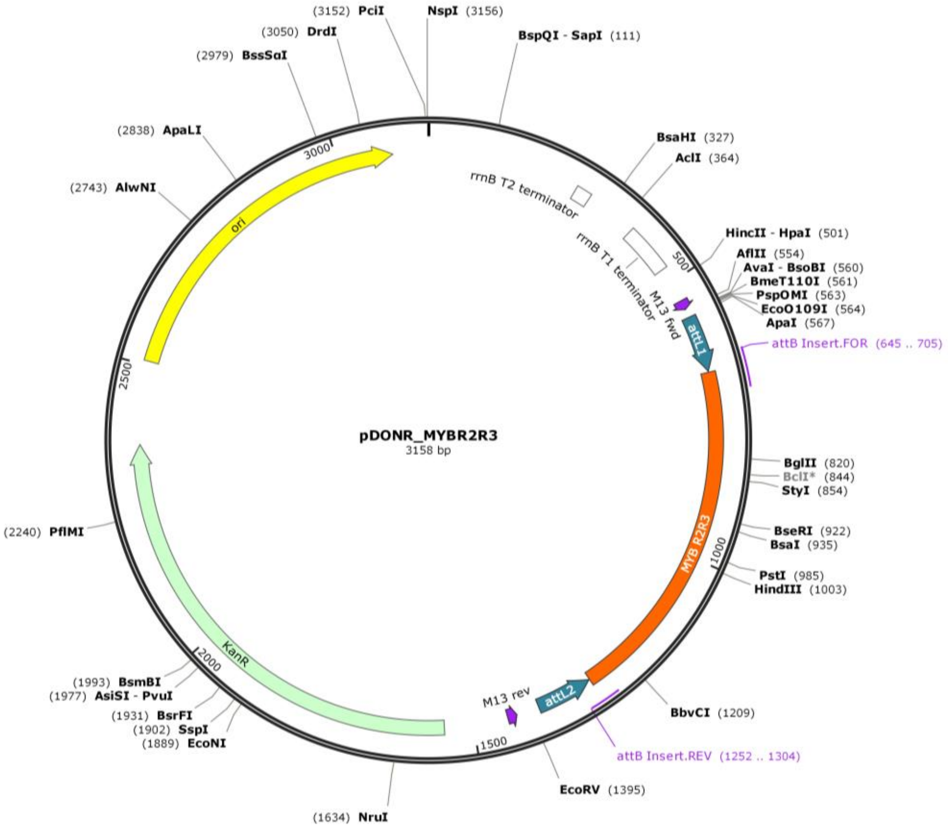


Figure 4.8. Generated entry clone, *pDONR221*-MYBR2R3 (*SlAIM1*), the map has been constructed using SnapGene

4.2.2 Transformation of *SlAIM1* into the *pEarleyGate 100* plasmid in *E. coli* (*Top10*)

After LR reaction, the product was then transformed to *E.coli* component cell according to protocol of transformation. Colony PCR was performed to confirm positive clones by using gateway specific gene primer (*SlAIM1*-GTF and *SlAIM1*-GTR) (Figure 4.9).

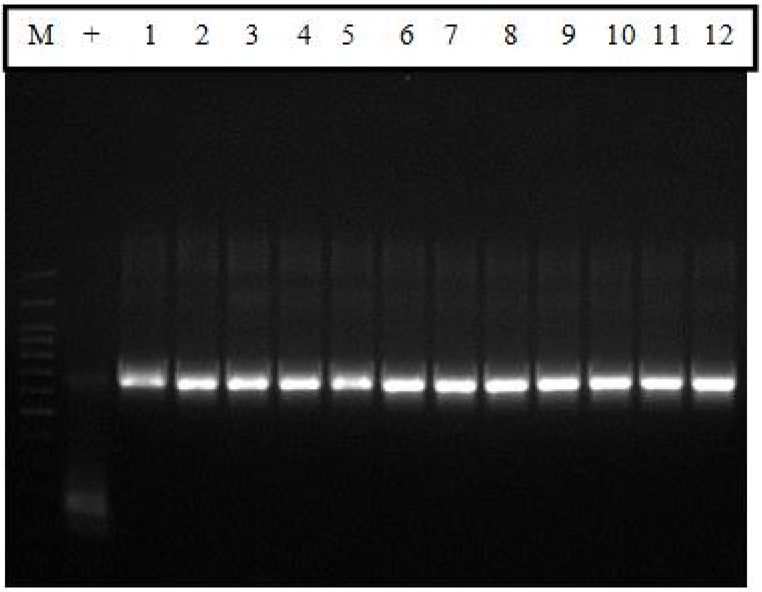


Figure 4.9. Colony PCR assay for the confirmation of LR reaction by gateway gene specific primers, Lane M: 100 bp DNA Ladder (Solis BioDyne), Lane +: positive control, gel eluted fragment of *SlAIM1* gene, Lane 1-12: DNA results with gateway gene specific primers, Lane 12 was selected as for further experiments

4.2.3 Transformation of *SlAIM1* into the *pEarleyGate 100* plasmid in *Agrobacterium* strain (*AGL-1*)

In order to confirm *SlAIM1-pEarleyGate 100* plasmid in *AGL-1* strain, colony pcr was performed by gateway gene specific primers (Figure 4.10), BAR and nptII primers (Figure 4.11 and Figure 4.12). The positive clones were grown in LB broth medium with kanamycin overnight.

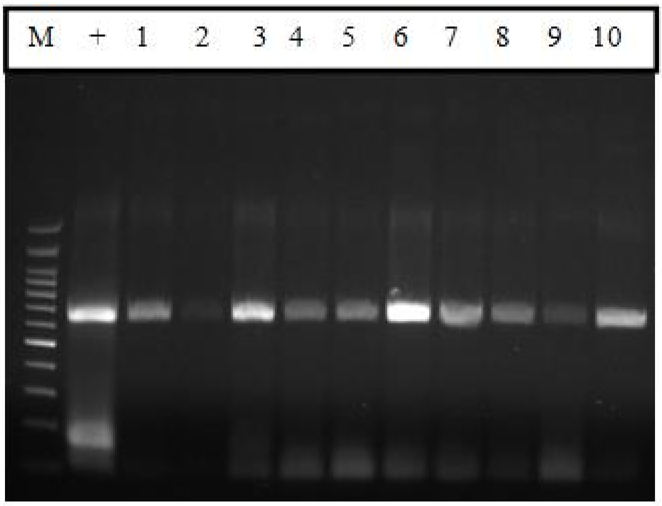


Figure 4.10. Colony PCR assay for the confirmation of LR reaction gateway gene specific primers, Lane M: 100 bp DNA Ladder (Solis BioDyne), Lane +: positive control, gel eluted fragment of *SlAIM1* gene, Lane 1-10: DNA results with gateway gene specific primers, Lane 1, 3,6,7,8 and 10 were selected and subjected to confirm by nptII and BAR primers

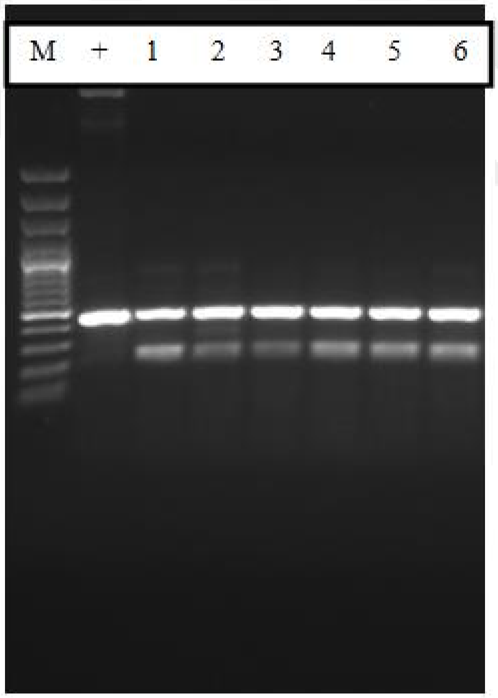


Figure 4.11. PCR assay for the confirmation of LR reaction by nptII primers, Lane M: GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific), Lane +: Empty *pEarleyGate 100* vector, Lane1-6: DNA results with nptII primers

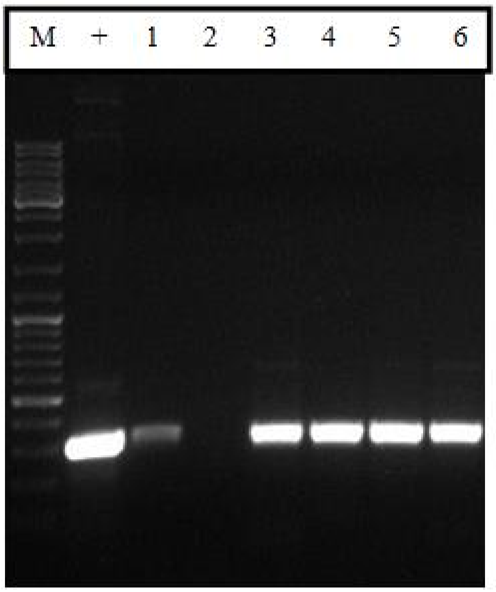


Figure 4.12. PCR assay for the confirmation of LR reaction by BAR primers, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: Empty *pEarleyGate 100* vector, Lane1-6: DNA results with BAR primers

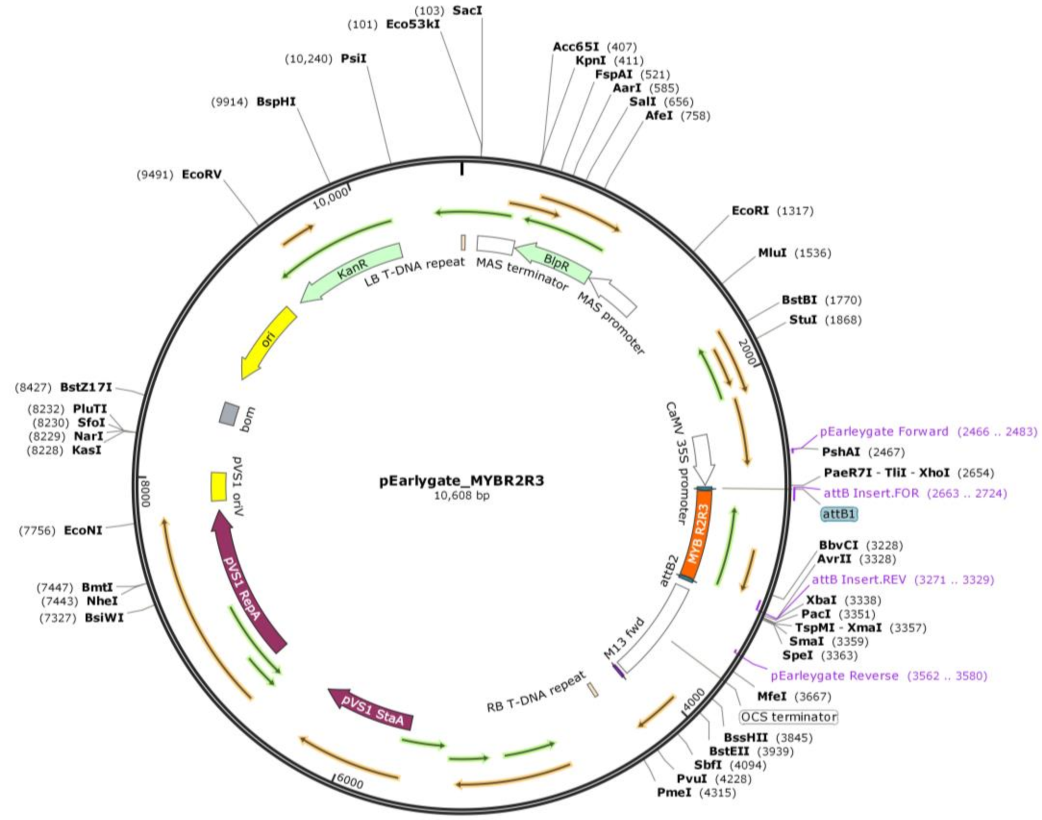


Figure 4.13. Generated expression clone, *pEarleyGate 100*-MYBR2R3 (*SlAIM1*), map has been constructed using SnapGene

4.3 Transformation of *pEarleyGate 100-SlAIM1* to Agria Cultivar by *Agrobacterium* Method

pEarleyGate 100-*SlAIM1* and control vector (Empty *pEarleyGate 100*) were transferred to the Agria cultivar. Studies have been carried out to form callus from internodes, nodes, leaves and microtubers in regeneration selection media. Callus was not obtained from explants for a long time in regeneration selection media and especially leaf explants died. In order to solve this problem, different regeneration selection media have been tested and optimized (Figure 4.14 and Figure 4.15).

4.3.1 Optimization of regeneration selection media for the transformation of Agria cultivar

In order to optimize and find a best regeneration media, different amount of hormones was added to the media. As seen in Figure 4.14, 5 different RSM media (RSMO, RSM1, RSM2, RSM3 and RSM4) with different ppt amount 0 mg/l, 1 mg/l, 1.5 mg/l, 2 mg/l, 2.5 mgl/l were tried in media, respectively.

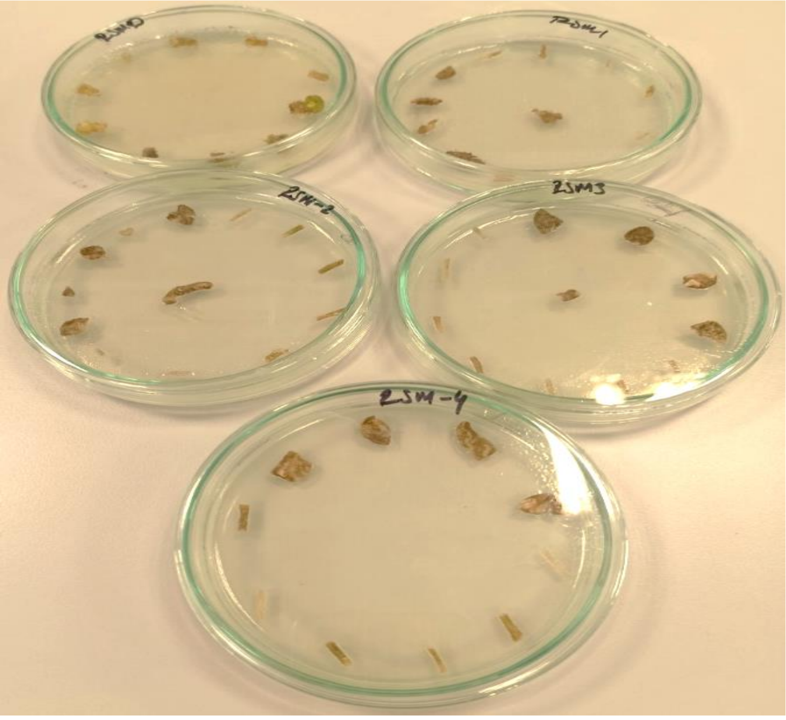


Figure 4.14. Different amount of ppt in RSM media

After 10 days, explants were healthy only RSM0 (0 mg/l ppt) amoung 5 media. All other explants were died. However, there should be ppt for the selection of plants in RSM. New media was prepared with 0.5 mg/L. The RSM media with 0.5 mg/L ppt amount was worked well and callus formation in internodes and nodes were observed in 20 days when the callus formation took 30 days in leaves and microtuber (Figure 4.16 and Figure 4.17).

On the other hand, two different RSM media was used to understand the effect of kinetin hormone. First RSM supplemented with kinetin 1 mg/l and second RSM was not supplemented with kinetin (Figure 4.15).

Finally, RSM media was optimized [2mg / l BAP, 0.2mg / l NAA, + 30 g / l sucrose + Agar 8 g / l + MS Salt 4.4 g / l + 500mg / l Sulcid + 1 mg/ L kinetin + 1 mg/ L transzeatin + 0.5 mg/l ppt].

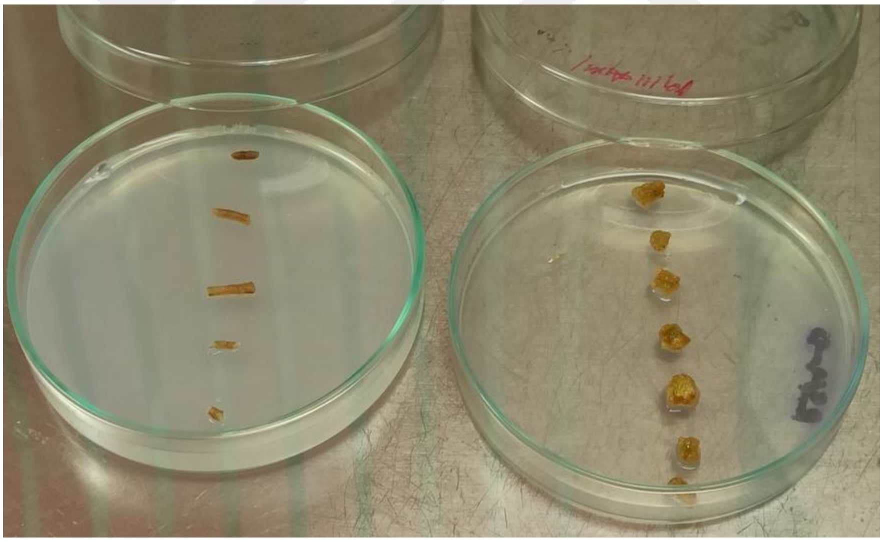


Figure 4.15. RSM media without kinetin hormone (left) and with kinetin hormone (right)

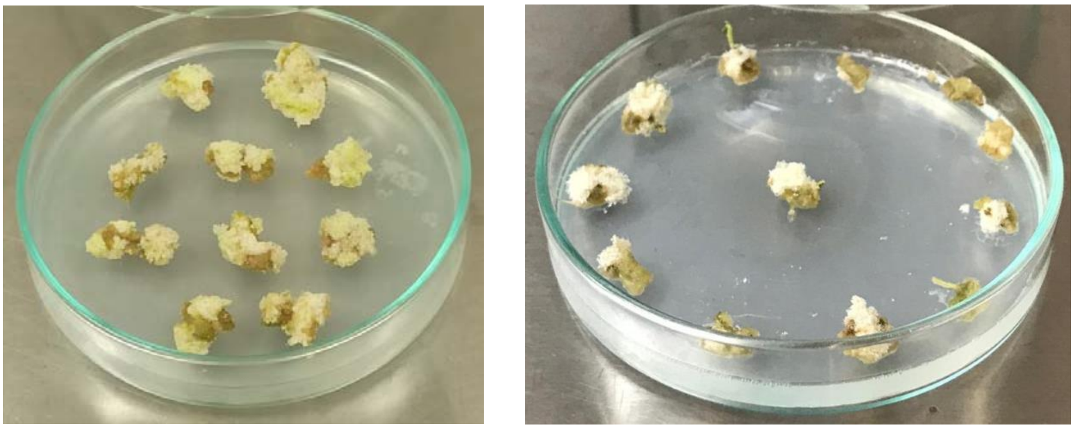


Figure 4.16. Callus formation from internodes (left) and callus formation from nodes (right)

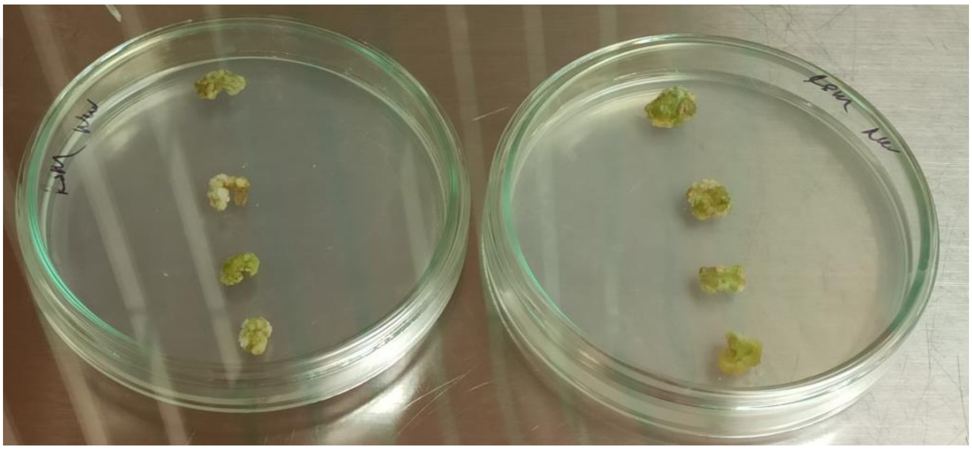


Figure 4.17. Callus formation from leaves

The highest callus induction was observed in node and internode explants. The percentage of callus induction efficiency was obtained %80 from node, %12 from internode, 4.66 from leaf and %2.08 from microtuber (Figure 4.18). Statistical analysis (by JMP Statistical Package program) showed that there is a significant difference between explants. (Table).

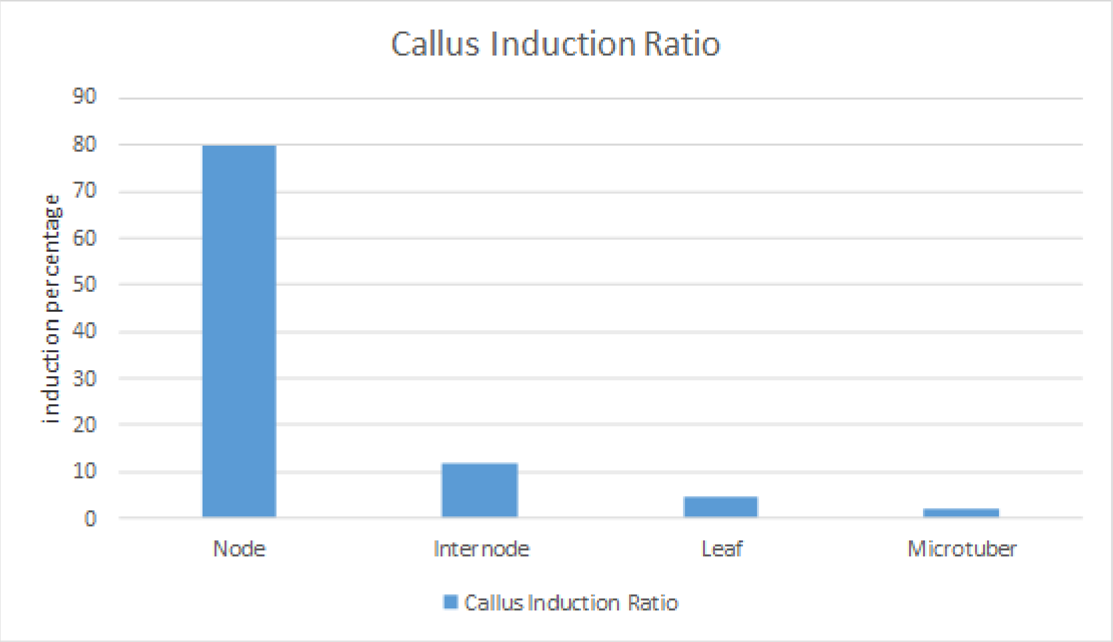


Figure 4.18. The callus induction percentage for different type of explants

Table 4.1. Variance analysis table of callus induction

Source	SS	MS Num	DF Num	F Ratio
Replication	7,57447	3,78723	2	1,1064
Explant	12509,6	4169,87	3	1218,189**
Replication*Explant&Random	20,5381	3,42301	6	1,008

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF))
**p≤ 0.01

4.3.2 Transfer and selection on shoot and root induction medium

After approximately 6 weeks, shoot formed on the explants. Callus and developed candidate transgenic shoots were then grown in shoot and root induction MS media (Figure 4.19 and Figure 4.20).

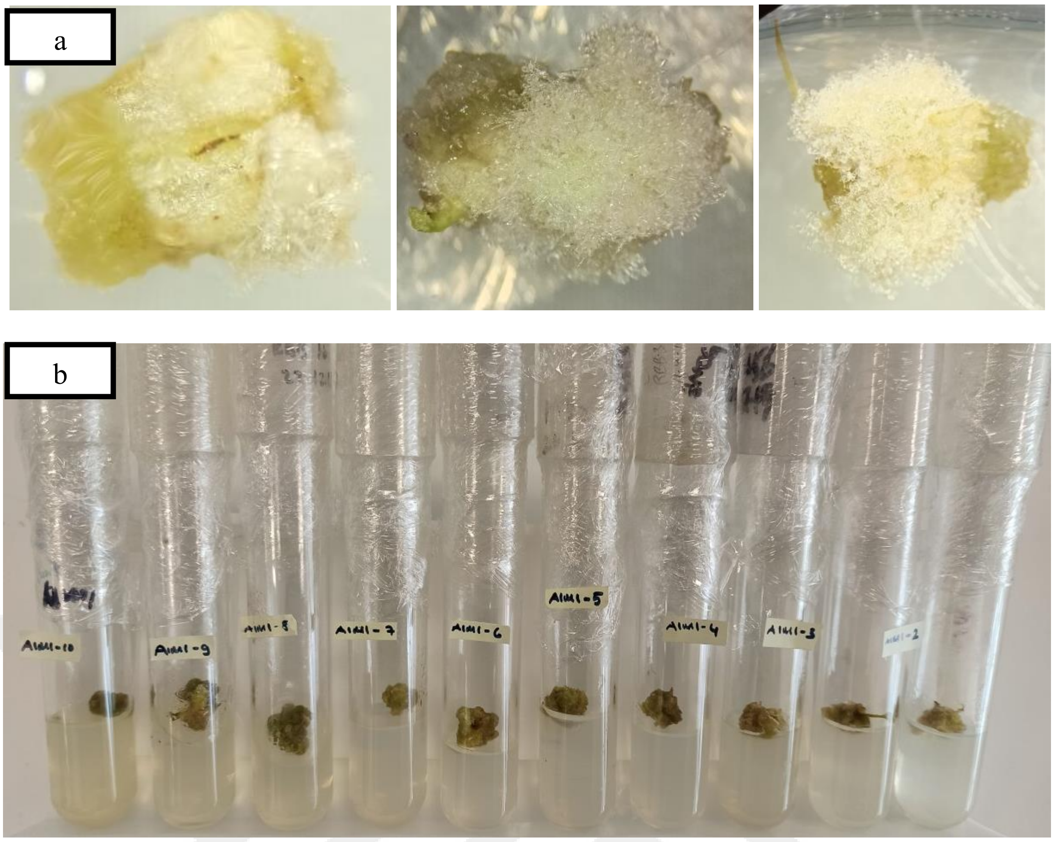


Figure 4.19. Transfering callus to the shoot and root induction medium, some callus examples from explants (a) and transferred callus to the shoot and root induction medium (b)

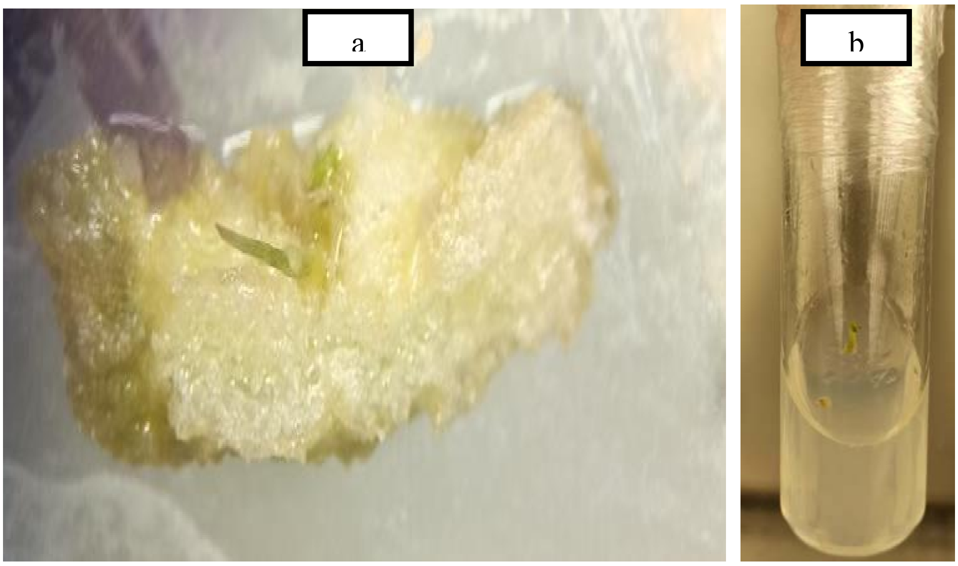


Figure 4.20. Transfer shoots from callus on shoot and root induction medium, shoot formation from callus (a) and transferring shoots to the shoot and root induction media (b)

The shoot induction rate from callus was calculated and statistical analysis (by JMP Statistical Package program) showed that there is a significant difference between explants (Table 4.2).

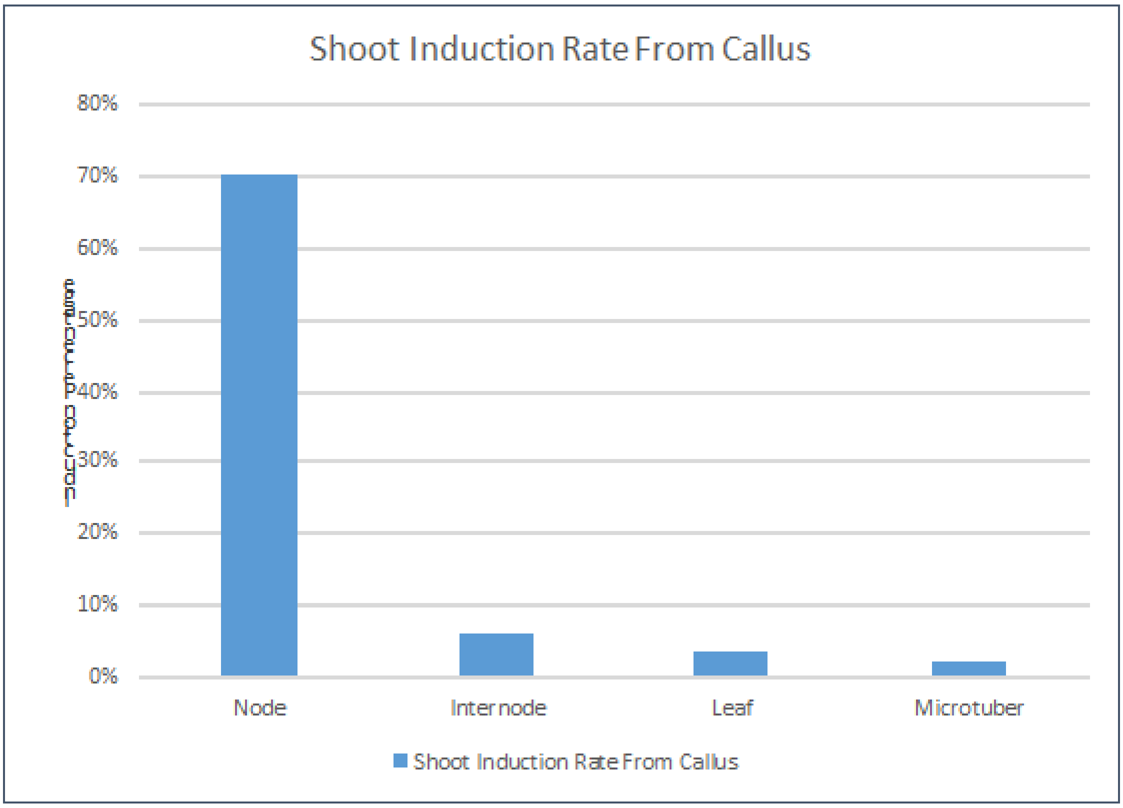


Figure 4.21. The shoot induction percentage from callus for different type of explants

Table 4.2. Variance analysis table of callus induction shoot induction from callus

Source	SS	MS Num	DF Num	F Ratio
Replication	253	1,26457	2	0,9892
Explant	9956,32	3318,77	3	2595,997**
Replication*Explant&Random	7,67052	1,27842	6	1,0009

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF))
**$p \leq 0.01$

After 5-6 weeks, the selected plants with well-developed shoots were subculture in MS normal media i.e. MS supplemented with vitamins, sucrose and agarose, with giberellic acid (Figure 4.22). Plants were continued to subculture on this media for 4-6 weeks.

Figure 4.22. *In vitro* growth of putative transgenic plants of Agria on shoot induction medium, transgenic shoot obtained from transformed leaf explant (a), transgenic shoot obtained from internode explant (B), transgenic shoot obtained from transformed node explant (c), transgenic shoot obtained from transformed microtuber explant (d)

Totally, 55 developed shoots from all explants were propagated under tissue culture conditions on MS0 media (Figure 4.23). 44 shoots from nodal explant, 8 shoots from internodal explant, 2 shoots from leaf and 1 shoot from microtuber were developed.

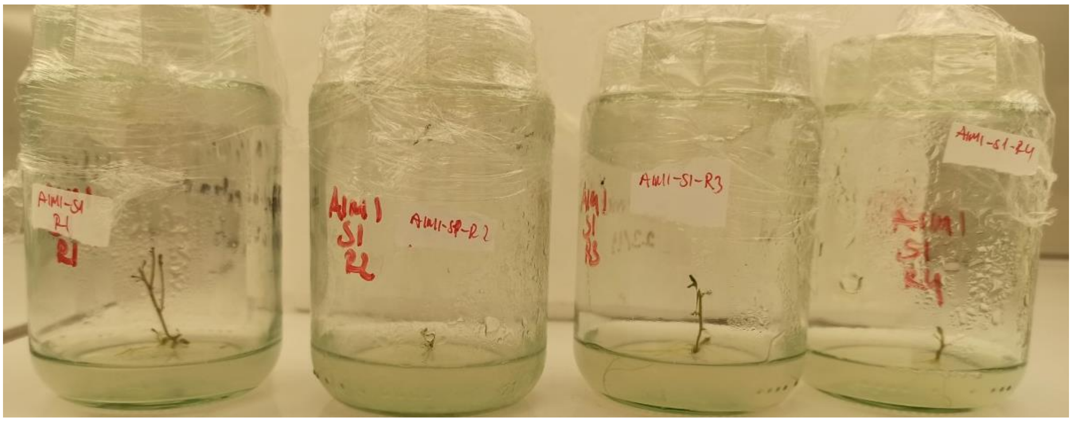


Figure 4.23. Propogation of developed candidate transgenic shoots under tissue culture conditions

4.4 Confirmation of Transgenic Plants by Molecular Analyses

4.4.1 PCR assays

Genomic DNA was taken from the plants and they were tested by PCR. PCR was performed by BAR primers to confirm transgenic plants (Figure 4.24).

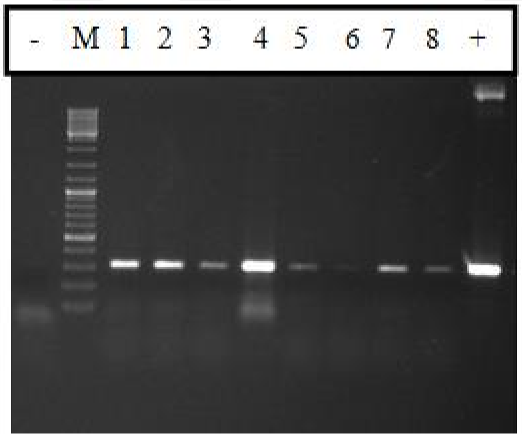


Figure 4.24. PCR assay for the confirmation of putative transgenic plants by BAR primers, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane -: Non-transgenic agria cultivar, Lane +: Empty *pEarleyGate 100* vector, Lane1: DNA of *SlAIM1*-1 plant, Lane2: DNA of *SlAIM1*-2 plant, Lane3: DNA of *SlAIM1*-3 plant, Lane4: DNA of *SlAIM1*-4 plant, Lane5: DNA of SlAIM1-5 plant, Lane6: DNA of *SlAIM1*-6 plant, Lane7: DNA of *SlAIM1*-7 plant, Lane8: DNA of *SlAIM1*-8 plant

4.5 Transformation Data and Calculation of Transformation Efficiency

The data of transformed explants of Agria cultivar is shown in Table 4.3. Calculation of transformation efficiency of transgenic plants was calculated results as 0.4% based on the PCR results.

Table 4.3. Regeneration data of gene transferred Agria cultivar

Type of Explant	**Total used explant**	**Callus induction Ratio**	**Number of shoots**	**Shoot Induction Ratio From Callus**	**Transgenic plants number based on PCR results**
Internode	1080	12%	8	6%	5 (*SlAIM1*-1, SlAIM1-2, *SlAIM1*-3, *SlAIM1*-4, *SlAIM1*-6)
Node	100	80%	56	70%	2 (*SlAIM1*-7, *SlAIM1*-8)
Leaf	900	4.66%	2	3.57%	0
Microtuber	48	2.08%	1	2.08%	1 (*SlAIM1*-5)

4.6 In vitro Drought Stress Application on Standart Agria Cultivar and Transgenic Plants

SlAIM1-1 and *SlAIM1*-4 transgenic plants were subjected to in vitro drought condition by 20% PEG treatment. For this purpose, transgenic plants, transformed plants by control vector and non-transgenic standart agria varity were subcultured as 2 nodes for root and shoot formation in normal MS-0 and MS0 / PEG nutrient media for ten days.

4.7 Gene Expression Analysis by qRT-PCR

Total RNA was isolated from all plants in drought stress experiment and *SlAIM1*-2, *SlAIM1*-3 and *SlAIM1*-8 transgenic plants in normal conditions. Contaminated DNA in RNA samples was removed.

The agarose gel analysis results of the pure RNA samples obtained are presented in Figure 4.25. RNA concentrations was also checked by nanodrop (Table 4.4).

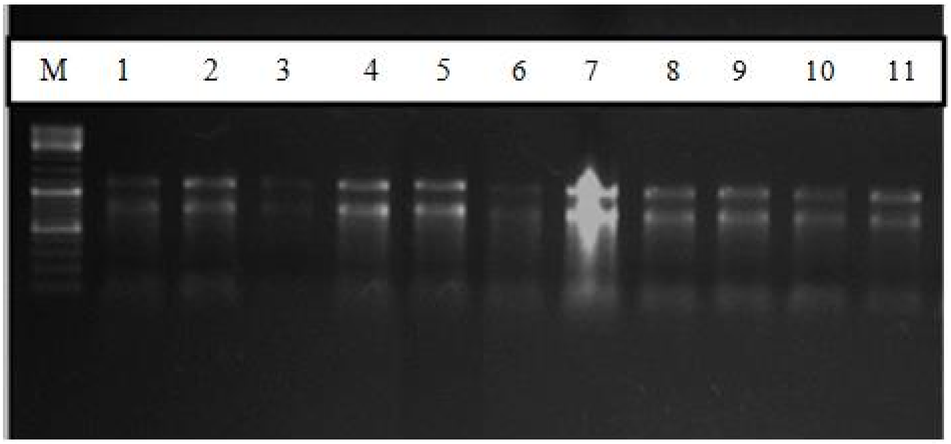


Figure 4.25. Agarose gel analysis of isolated total RNAs, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane1: RNA of *SlAIM1*-1 in normal condition, Lane2: RNA of *SlAIM1*-1 in drought condition, Lane3: RNA of non- transgenic plant in normal condition, Lane 4: RNA of non- transgenic plant in drought condition, Lane 5: RNA of *SlAIM1*-4 plant in normal condition, Lane 6: RNA of *SlAIM1*-4 in drought condition, Lane 7: RNA of transformed plants by control vector in normal condition, Lane 8: RNA of transformed plants by control vector in drought condition, Lane 9: RNA of *SlAIM1*-2 plant in normal condition, Lane 10: RNA of *SlAIM1*-3 plant in normal condition, Lane 8: RNA of *SlAIM1*-8 plant in normal condition

Table 4.4. Total RNA concentration in candidate transgenic potatoes on the nanodrop device

Sample Name	Check	Nucleic Acid Conc (ng/ µl)	OD 260/280	OD 260/230	OD260	OD280
SlAIM1-1 normal	✓	766,2	2,014	0,694	18,625	8,768
SlAIM1-1 drought	✓	266,44	2,03	0,161	2,394	1,179
SlAIM1-4 normal	✓	152,12	2,056	0,188	3,803	66,402
SlAIM1-4 drought	✓	239,12	2,063	0,192	5,978	2,898
Control vector normal	✓	338,4	2,028	0,541	6,302	3,014
Control vector drought	✓	834,3	2,103	0,79	12,006	5,709
Non-transgenic normal	✓	1075,24	2,086	0,462	26,881	12,882
Non-transgenic drought	✓	221,5	2,041	0,24	3,095	1,67
SlAIM1-2 normal	✓	217,96	1,981	0,354	5,449	2,75
SlAIM1-3 normal	✓	239,8	1,911	0,231	3,192	1,671
SlAIM1-8 normal	✓	483,6	2,089	0,707	12,09	5,789

Table 4.5. Analysis of transgenic plants

Sample No.	Detection by PCR (BAR primers)	qRT-pcr detection	Tested under PEG conditions
SlAIM1-1	+	+	+
SlAIM1-2	+	+	-
SlAIM1-3	+	+	-
SlAIM1-4	+	+	+
SlAIM1-5	+	-	-
SlAIM1-6	+	-	-
SlAIM1-7	+	-	-
SlAIM1-8	+	+	-

+; tested plants, -; nontested yet

For the purpose of the thesis, gene expressions of *SlAIM1* gene was examined under normal conditions and drought conditions. The gene expressions were analysed from transgenic plants under normal conditions (*SlAIM1*-1*,* *SlAIM1*-2, *SlAIM1*-3, *SlAIM1*-4 and *SlAIM1*-8) (Figure 4.28). On the other hand, the gene expressions were analysed from all plants in drought experiment (Figure 4.29). cDNAs synthesized from both conditions of standard Agria plant, transformed plants by control vector and transgenic plants were used as templates in gene expression analysis. In qRT-PCR analysis, the *ef1α* gene (Nicot et al., 2005) was used as a reference gene for normalization. It is recommended to use Elongation Factor 1α (*EF-1α*) as a reference gene during biotic and abiotic stress study (Nicot et al., 2005). The proportional change of gene expression was calculated according to the 2 − ΔΔCt method (Livak and Schmittgen, 2001).

Melting curve analysis has been performed to determine whether the PCR produces only a single product (Figure 4.26 and Figure 4.27).

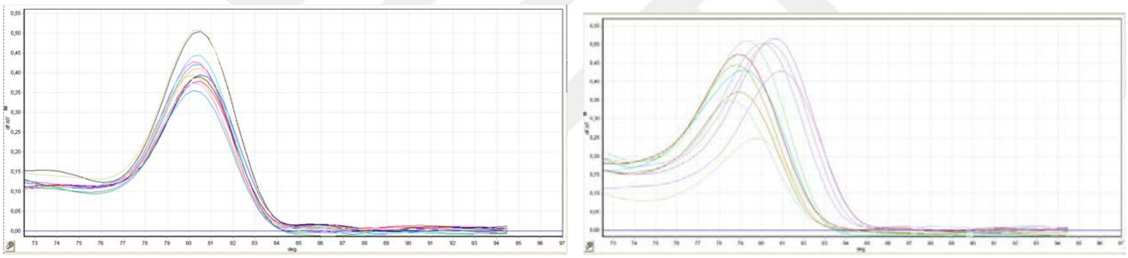


Figure 4.26. Melting temperature *of SlAIM1* gene (left) and *EF-1α* reference gene under drought conditions of transgenic plants

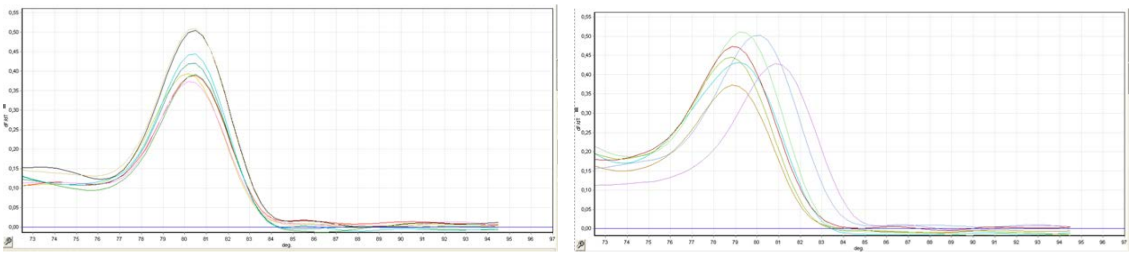


Figure 4.27. Melting temperature *of SlAIM1* gene (left) and *EF-1α* reference gene under normal conditions of transgenic plants

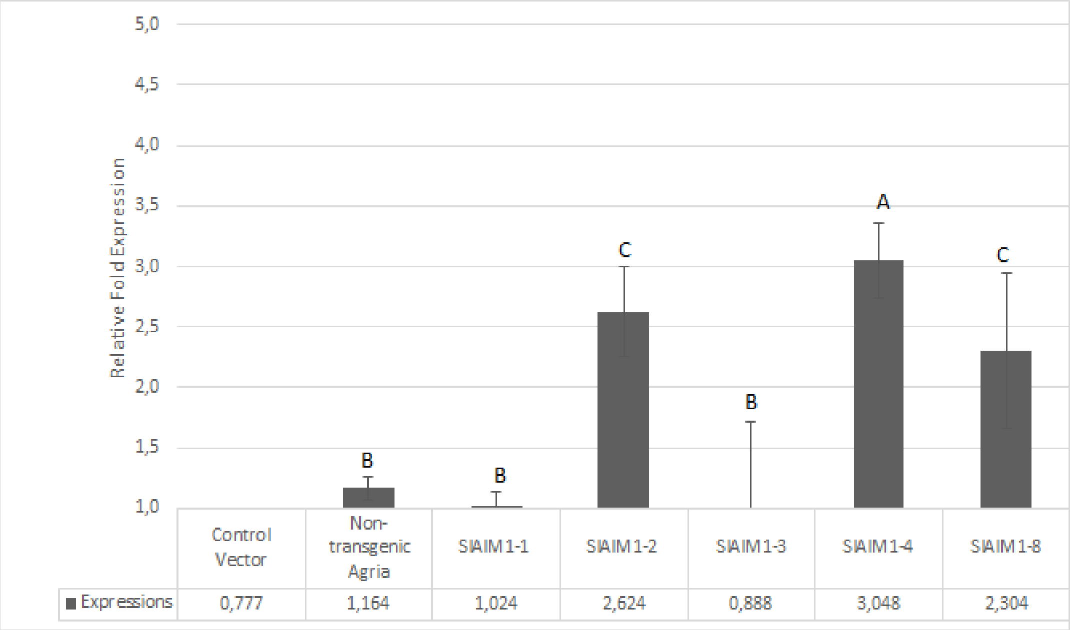


Figure 4.28. *SlAIM1* gene expression control vector (empty *pEarleyGate 100* vector) transformed plant, standard Agria and *SlAIM1*-1*, SlAIM1*-2, *SlAIM1*-3, *SlAIM1*-4 and *SlAIM1*-8 transgenic plants in normal conditions

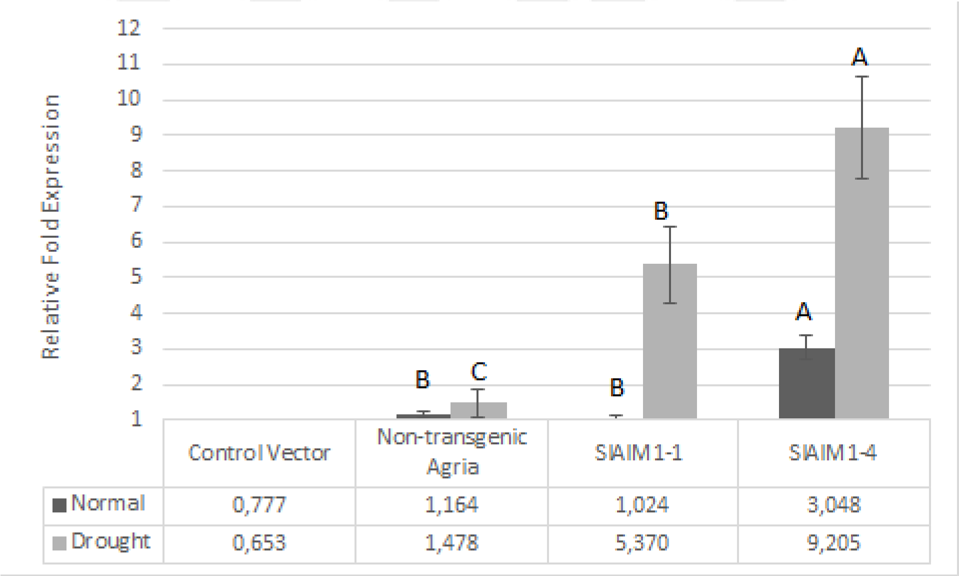


Figure 4.29. *SlAIM1* gene expression in control vector transformed plant, standard Agria and transgenic plants (*SlAIM1*-1 and *SlAIM1*-4) under normal and drought conditions

The results showed that *SlAIM*-4 transgenic plant has highest expression as compared to *SlAIM1*-1*, SlAIM1*-2, *SlAIM1*-3 and *SlAIM1*-8 plants (Figure 4.28). Variance analysis of transgenic plants (*SlAIM1*-1, *SlAIM1*-2, *SlAIM1*-3, *SlAIM*-4 and *SlAIM1*-8) was presented in Table 4.6. As seen on the the table, the value of genotype was statistically significant. Plant expression analysis were grouped by Tukey post-hoc test (Table 4.7).

Drought stress experiment results have indicated that the expression of the *SlAIM1*-4 transgenic plant increased 5-fold under in-vitro drought condition compared to normal condition. Similarly, expression of the *SlAIM1*-1 transgenic plant was increased 3-fold under in-vitro drought condition compared to normal conditions (Figure 4.29). The overall results indicated the higher expression levels of *SlAIMI* gene under in vitro drought condition. Variance analysis of drought stress application of transgenic plants (*SlAIM1*-1 and *SlAIM*-4) was presented in Table 4.8. As seen on the table, the values were was statistically significant in terms of Treatment and Treatment*Genotype. Plant expression analysis were grouped by Tukey post-hoc test (Table 4.9).

Table 4.6. Variance analysis table of transgenic plants under normal conditions

Source	SS	MS Num	DF Num	F Ratio
Replicate	0,78964	0,39482	2	4,8225
Genotype	34,2334	8,55835	4	104,5358**
Replicate*Genotype&Random	0,65496	0,08187	8	1,01

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF))
**$p \leq 0.01$

Table 4.7. Tukey post-hoc test of transgenic plants

Potato Plants	Group	Mean
***SlAIM1*-4 transgenic**	A	23,29
***SlAIM1*-1 transgenic**	B	21,47
Non-transgenic	B	21,32
Control Vector	B	21,04
***SlAIM1*-3 transgenic**	B	21,02
***SlAIM1*-2 transgenic**	C	18,42
***SlAIM1*-8 transgenic**	C	17,74

Table 4.8. Variance analysis table of in-vitro drought application

Source	SS	MS Num	DF Num	F Ratio
Replicate	0,00048	0,00024	2	0,0041
Genotype	8,67617	2,89206	3	1,7473
Treatment	16,0067	16,0067	1	9,1534*
Replicate*Genotype	0,34476	0,05746	6	0,3803
Treatment*Genotype	5,24617	1,74872	3	11,5755**

(Sum of Squares (SS), Mean Square (MS), Degrees of Freedom (DF))
**p≤ 0.01, *p≤ 0.05

Table 4.9. Tukey post-hoc test of in-vitro drought application

Treatment	Potato Plants	Group	Mean
Normal	*SlAIM1*-4 transgenic	A	23,29
Normal	*SlAIM1*-1 transgenic	B	21,47
Normal	Non-transgenic	B	21,32
Drought	*SlAIM1*-1 transgenic	B	21,04
Normal	Control Vector	B	21,04
Drought	*SlAIM1*-4 transgenic	A	20,25
Drought	Non-transgenic	C	19,96
Drought	Control Vector	D	19,33

CHAPTER V

DISCUSSION

The current research work was performed to obtain transgene potato lines expressing *SlAIM1* gene and see the effect the gene under in vitro drought conditions. *SlAIM1* gene which belongs to MYB family was isolated from tomato cultivar Cherry and Tiny Tim. TA cloning was studied for subcloning of *SlAIM1* gene into the *pDRIVE* cloning vector. After that, the studies were done for the transferring of *SlAIM1* gene into the expression vector via conventional cloning method. Cloning of *SlAIM1* gene to the expression vector by conventional techniques were failed.

The amplification of *SlAIM1* gene was good as seen in Figure 4.1. After that, digestion reaction was performed to the gene and vector. The digestions were worked both on gene and vector. The gel purification of plasmid and inserts were worked well but ligation process was not working. Optimization of all procedures were done for traditional cloning. Different ligases were tried, and also overnight ligation was done to ligate the product into the vector. Ligation PCR was done and as a result, ligation didn't work. On the other hand, transformation step was performed and colony PCR results showed that absolutely ligation reaction didn't work. Different vectors have been studied in conventional cloning (*pCAMBIA1301*, *pBIN61*, *pJIT61*). By using all vectors, same problem had been seen. The conventional cloning methods have been taken very long time (approximately 1 year), and then gateway cloning method was performed to clone target gene into the expression vector.

Firstly, in 1990s, gateway cloning system was commercialized by Invitrogen. First of all, Bolivar et al., 1977 found the first vector, *pBR322*, which has been designed for cloning purposes. *pBR322* vector was very small and it had antibiotic resistance genes for selection. Being founded on *pBR322* vector, Perron et al., 1985 was designed pUC plasmids that contains 'screening system' (blue/white screening). The puC plamids had multiple cloning site that helps scientists for screening bacterial colonies with DNA insert. After some time, ligase independent cloning methods were created such as Gateway cloning method.

In order to obtain abiotic stress tolerant plants, many studies have been done by gateway cloning method with the transformation of MYB transcription factors to the various plants. Zhao et al., 2017 was used gateway cloning technology to clone *GmMYBJ3*, and R2R3-MYB TFs, in soybean for isoflavonoids biosynthesis. Isoflavonoids plays and important role in plants for abiotic stress resistance. *pDONR221* was used to obtain entry clone and pCB35SR1R2-GFP was used for LR reaction. Embryonic tips of soybean was used and transformed to the *A.tumefaciens EHA105* strain. As a result, they found the enhance the individual and total isoflavone contents. Wei et al., 2017 was cloned *CiMYB5* and *CiMYB3* transcription factors from *Cichorium intybus* by using gateway cloning in order to regulate abiotic stress (drought and cold) *pDONR201* was used as an entry clone and then transcription factors were transferred to the *pART7* destination vector. It's known that anthocyanins are flavonoids pigments which has role in the protection of plants from abiotic stresses (Shi et al., 2019). Expression of anthocyanin biosynthesis genes can be regulated by MYB proteins. Jian et al., 2019 studied that overexpression of *SlMYB75* gene enhance the accumulation of anthocyanin and this led to abiotic stress tolerance. In the study, they used gateway cloning method and final vector was transformed to the *GV3101* strain of *A.tumefaciens.* On the other hand, proanthocyanidins are secondary metobilites and the biosyhthesis of proanthocyanidins can be regulated by MYB TFs. Wang et al., 2019 was studied sucrose-induced MYB (*SlMYB*) TF to enhance the accumulation of proanthInocyanidins in tea plants. *pDONR207* was used for BP reaction and *pCB2004* was used for LR reaction in this study. Agrobacterium-mediated transformation of Arabidopsis and Tobacco was done.

In present study, gateway cloning method was performed, and it was relatively simple compared to the conventional cloning. It is known that backbone system of gateway vectors contains *ccdB* lethal gene. *ccdB* replaces with the gene of interest in a successful insertion. *pEarleyGate* destination vectors are used in gateway cloning method for the expression of target genes/protein (Earley et al.,2006). In present study, *pEarleyGate 100* vector was used.

The vector contains a negative selectable marker, the *ccdB* gene, to select against non recombinant clones. *pEarleyGate 100-SlAIM1* was created under the control of constitutive promoter (35S).

CamMV 35S promoter is a strong and constitutive promoter. Beside this, 35S promoter is used to serve as a backbone of develop variety of transcription control systems for repressible or inducible gene expression in plants (Amack et al., 2020). CamMV 35S promoter is an important component of transgenic construct in more than %80 of genetically modified plants (Hull et al., 2000).

Potato cultivar Agria was transformed with *pEarleyGate 100–SlAIM1* and the empty vector (empty *pEarleyGate 100*). The empty vector also contains *bar* resistance gene and control by CamV 35S promoter. *A. tumefaciens* is insensitive to the toxic effect of ccdB. Therefore, empty *pEarleyGate 100* vector in *AGL-1* strain was used as a control vector.

Agrobacterium-mediated transformation provide us making changes at the molecular level on the plants to obtain a new plant by used in agriculture. *Agrobacterium*-mediated transformation is one of the widely used method and the initial studies have been started on 1970s and Wenzler et al., 1989 have worked on the development of efficient transformation method to obtain transgenic potato plant. They used Desiree, Russet Burbank and FL1607 potato line. Leaves, stem, and tuber disc were used as an explant and they found that the most effective regeneration was observed in leaf and stem explants. Regeneration media was containing 200 mg/l naphthaleneacetic acid (NAA), 2.24 mg/l 6- benzylaminopurine (BAP), 10 mg/l GA3 and 60 g/l Sucrose. In Agrobacterium transformation LBA4404 was used and plasmid pBI121 carrying the NptII and GUS marker genes with the CaMV 35S promoter. Amoung the potato varieties, the highest transgenic potatoes obtained from FL1607 line. As a conclusion, they specified the potato plant genotype and antibiotics in plant selection are critical factor for the obtaining of transgenic plants.

In present study, Donmez et al., 2019 protocol was modified for Agrobacterium transformation. Donmez et al., 2019 was used different potato varieties and the inoculation time of *Agrobacterium tumefaciens AGL-1* strain with Agria cultivar was performed for 15 minutes. The inoculated explants were washed by sulcid for 15 minutes as in protocol. Internodes, nodes, leaves and microtubers were used as an explant for *Agrobacterium*-mediated transformation.

Mainly, internodes and leaves are the explants used in potato gene transformation. However, there are some reports that nodes can be used as an explant for gene transformation of potato. Khan et al., 2016 were generated transgenic plants from Desiree potato cultivar with *A. solani* resistance from nodes obtained by *Agrobacterium*-mediated transformation. They have observed 91.9 % of regeneration from nodal explants and transformation efficiency was 21% by biochemical and molecular analyses. In another study, Ahmad et al., 2012 were used nodes as explant of Desiree and Sh-5 potato cultivars. They have optimized many factors that affects *Agrobacterium*-mediated transformation of chitinase gene. They have found that transformation efficiency was genotype dependent. The highest transformation efficiency was 3.38 and 3.10 in Desiree and Sh-5, respectively. On the other hand, studies have shown that microtuber dics can be used as an explant for *Agrobacterium*-mediated transformation (Snyder and Belknap, 1993). Plants regenerated from tubers have low rank of somaclonal variation (Ishida et al, 1989). However, there are very rare studies for the transformation of potato varieties using in vitro microtubers and those studies are very prior. Kumar et al., 1995 studied the transformation of five different *Solanum* species by using microtubers. Transformation effiencies were different from species and transgenic plants were successfully obtained from microtubers.

In present study, phosphinothricin (PPT) was used for plant selection. Glufosinate ammonium is the chemically synthesized form of PPT. Glufosinate inhibits the glutamine synthetase enzyme, which is the key enzyme responsible for ammonium assimilation in the plant. Therefore, as a result of the cessation of glutamine synthase activity, ammonium accumulates in plant cells and accumulated ammonium cause a fatal effect on the plants. Phosphinotricin acetyl transferase (PAT) enzyme is an enzyme that can detoxify PPT by acetylation and PAT is synthesized by the BAR gene (Rathore et al., 1993; Block et al., 1988).

It has been determined that PPT used in selective nutrient medium is a good selection system for regeneration of plants, enabling us to reach the target result even though the transformation rate is low. The present study showed the effect of various concentrantions of PPT. The best amount of PPT was optimized to 0.5 mg/l.

Block et al., 1988 and Barrell et al., 2002 have suggested that PPT is not as efficient as a selection marker for potato when compared to kanamycin (nptII) and hygromycin (hpt). Barrell et al., 2002 improved the protocol of potato transformation on identical binary vector except for the presence of different selectable marker genes. Virus-free potato plants of cultivar Iwa was used in the study. As they compared kanamycin, hygromycin and phosphinothricin resistance on transformed plants, the results showed that callus initiation, shoot regeneration, root initiation and overall transformation efficiency was lower when selecting for resistance to PPT. Block et al., 1988 compared the kanamycin and phosphitinotricin as selective agents. Leaves of the potato cultivars Desiree and Bintje were infected by agrobacterium strain. The transformation experiments performed with using kanamycin or phosphitinotricin as selective agents. The results showed that transformation efficiency 100% was obtained when kanamycin was used whereas a rate of transformation efficiency was only 20% obtained when phosphinotricin was used for selection. Block et al., 1988 concluded that PPT resistance is an inefficient selective marker can be explained by the high sensitivity of potato leaves to PPT. So, this high sensitivity of potato leaves to can be the reason of less regeneration ratio and less callus induction ratio (4.66%) in this study. The PPT selective agent can be one of the reason of low transformation efficiency in present study.

The concentration of PPT should be adjusted according to different explants and plant varieties. Ahmed et al., 2017 studied by *SN19* gene to improve the resistance of Colorado potato beetle and tomato leafminer on two potato cultivars (Marabel and Innovator). The have used pTF101.1 vector that contains BAR and PPT was used as a selection agent for transgenic plants. 1 mg/l PPT was used to in MS media. In another study, Han et al., 2015 found the efficient method to prevent low transformation rate of *Solanum tuberosum L.* var. Atlantic in the presence of 0.5 mg/l and 1 mg/l ppt for transformant selection. Amiri, 2018 studied on a potato cultivar Marabel transformed with insecticidal *(cry1Ac*) and herbicidal (*bar*) genes using Agrobacterium mediated transformation. 1 mg/l PPT was used as selectable marker and overall transformation efficiency obtained 0.6%. As a general, studies showed that PPT amount used for potato transformation was used up to 2 mg/l.

On the other hand, the importance of kinetin hormone was described in callus induction. It was showed that only RSM media supplement with 1 mg/l kinetin was worked well and gave good callus. Kinetin is an important cytokinin for cell division and plant growth (Yamaguchi et al., 2010). However, there is no report that the kinetin directly affects callus formation in potato regeneration. Hanur, 2016 have studied the effect of kinetin to produce callus in tomato. It was found that kinetin is a priori for callus induction in tomato regeneration. On the other hand, there are some studies about the combination of kinetin and 2,4D for callus induction in potato. Dhaka and Nailwal, 2015 have observed that the combination of 2,4D and kinetin enhance the callus induction and its maintenance in potato.

The callus induction efficiency is mainly dependent on hormones, Agrobacterium strain and explant type (Laboney et al., 2013). Donmez et al., 2019 developed transgenic potato varieties (Desiree and *S. chacoense* M6) by using five different strains (*GV2260*, *AGL1*, *LBA4404*, *EHA105* and *GV3101*). In Desiree, *AGL-1* strain showed the maximum callus induction rate from internode and the lowest callus induction rate from leaf. In *S. chacoense* M6, *AGL-1* strain showed the lowest callus induction rate from leaf and internodal explant. It is known that internodal explants have high regenerative potential and callus induction rate in vitro (Soto et al., 2007). Kumlay and Ercisli, 2015 was found that nodal explants showed better performance for callus induction compared to leaf segments of different potato varieties. Bakhsh, 2020 developed stable and efficient genetic transformation of different potato cultivars. The results showed that highest percentage of callus development was found in Desiree and the lowest percentage of callus development was found in Innovator. The transformation efficiency of potato cultivars were calculated as 10, 15, 18.6, 20 and 22% from the intermodal explant whereas 6, 8, 17, 12 and 15% from leaf explants in Innovator, Désirée, Agria, Granola and Lady Olympia. In present study, Agrobacterium strain *AGL1* harboring *pEarleyGate 100* vector containing *SlAIMI* gene under the control of constitutive promoter (35S) was used to infect leaf, nodal, micro tuber and internodal explants of potato cultivar Agria. Agria is commercially cultivated in Turkey which has high productivity but susceptible for drought stress conditions (Hassanpanah D., 2010).

In the study, leaves, internodes, and nodes gave good response on callus inducing and the highest callus induction was observed in node and internode explants. The percentage of callus induction efficiency was obtained %80 from node, %12 from internode, 4.66 from leaf and %2.08 from microtuber. The results supported that the percentage of callus induction from microtuber was lowest and only 1 resistant callus was obtained from microtuber transformation. The best callus induction media was observed on MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA and 1 mg/l Kinetin, 1 mg/l Transzeatin, 500mg/l sulcid and 0.5 mg/l ppt.

There are many different factors that affect the transformation efficiency, including the wounding response of plants, Agrobacterium strain, co-cultivation period, inoculation time, different concentration of hormones, plant cultivars and type of explant (Jin et al., 2005; Bakhsh et al. 2014; Donmez et al. 2019). Sumer, 2018 studied to transfer a target gene by using same gene transfer method to different potato cultivars and it was determined that the transformation efficiency was 0.3% for Lady Olympia and 0.7% for Desiree. In another study, Marabel potato cultivar was used to transfer a target gene and the transformation efficiency was obtained the 0.6% from internodes and 0% from the leaf (Amiri, 2018). Rahamkulov and Bakhsh, 2020 was used Lady Olympia potato cultivar in the study and transformation efficiency was calculated as 0.98%. The overall transformation efficiency in present study was recorded as that is 0.4% less when compared to earlier report of potato transformation. The reason of low transformation efficiency may be explained by the low efficiency in T-DNA integration into the plant genome. The transformation process in different varieties shows major differences which can cause important effect of plant defense response on *Agrobacterium*-mediated transformation. The genotypic differences can change transformation efficiency.

In vitro culture of plants can cause variation which can be problematic or useful for plant breeders. In vitro culture system creates genetic variability and helps to produce plants with novel characters (Laboney et al., 2013). Transformation is limited practicaly for some of the plant species as their low transformation frequency. Beside that, high frequency in plant transformation cause some genetic change and expression in transgene unpredictably. The problems create problems in screening transformation events (Birch, 1997). The reproducible and efficient transformation protocol is still missing in potato transformation.

The lower transformation frequency, genotype dependency or somaclonal variations are the main problems of genetic transformation in potato which causes lower yield of stable transgenic plants (Beaujean et al., 1998; Bakhsh, 2020). Studies are focused on to obtain transgenic potato plants to improve biotic and abiotic tolerance, yield and quality. In 1995, Monsanto has been found the first biotech potato in Russet Burbank cultivar against Colarado Potato Beetle (CPB) with *cryIIIA* gene. The CPB-resistant biotech potato named as NewLeaf TM. After that, resistance Russet Burbank cultivar was obtained against PLRV (potato leafroll virus) (Perlak et al., 1993; Halterman et al., 2015). In addition to Monsanto, scientists and universities were studied about transgenic potato plant and they did field testing of transgenic plants. There are many studies to obtain transgenic potato plants by manipulating in gene expression and using genes from potato or its relative plants (Halterman et al., 2015).

It is fact that drought stresses cause a yield loss in potato (Mackerron and Jefferies, 1988). Thus, many reports focused on drought tolerance by using biotechnology. Amoung the different methods, transcription factors are one of the good applicants for genetic engineering to obtain abiotic stress-tolerant potato crops (Shin et al., 2011). Liu et al., 2020 was screened the potato genome and they have identified 138 *StMYB* gene of TFs. They have found out the abiotic stress-responsive expression levels to determine the roles of identified TFs in abiotic stress. They identified *MYB_CC* genes, PG0008401 and PG0018426, were up-regulated in drought stress and PG0011980 was identified in drought-tolerant cultivar. On the other hand, reports have evinced that the genes in R2R3-MYB family play an important role in abiotic stresses. Qin et al., 2012 obtained drought tolerant plants by overexpression of *TaMY33* gene. Shin et al., 2011 overexpressed *StMYBR-1* in potato to obtain drought tolerance.

There are some reports that polyethylene glycol (PEG) is usable for water stress inducers in plant varieties, including potatoes (Sirait and Charloq, 2017). Polyethylene glycol (PEG) induces drought stress by reducing osmotic potential and plants are not able to metabolize it (Marssaro et al., 2017). Si et al., 2012 was studied on development of drought tolerant potato cultivar by expressing of *BADH* gene. In order to induce water stress, they used %15 PEG to transgenic plants for 10 days. In another study, transgenic plants were exposured to four days stress and two days recovery period by

10% and 20% PEG. The study resulted that both conditions were sufficient to accumulate glycine betaine (GB) by *codA* transgene (Cheng et al., 2013).

Rahamkulov and Bakhsh, 2020 was determined the efficiency of pRCA and pRD29A promoters to express *gusA* gene in transgenic potato as compared to constitutive promoter, 35S promoter. The qRT-PCR analysis was performed under normal and drought (20%PEG) conditions. It was observed that the stress sensitive pRD29A promoter was 10 times higher in drought than optimal plant growing conditions. In another study, *StnsLTP1* gene was overexpressed in potato against multiple abiotic stresses including drought stress. qRT-PCR analysis results showed that *StnsLTP1* gene have 3-4 fold higher under drought conditions in *StnsLTP1* transgenic lines compared control potato plants (Gangadhar et al., 2016). In another study, overexpressing of *StMYB1R-1* in potato showed drought tolerance by the *RD29A* promoter and qRT-PCR analysis showed the more expression in drought condition as 4 to 7 fold compared to normal conditions (Gazendam et al., 2016).

In present study, qRT-PCR analysis was performed to determine the expression of the *SlAIM1* gene in transgenic plants (*SlAIM1*-1, *SlAIM1*-2, *SlAIM1*-3, *SlAIM1*-4 and *SlAIM1*-8) transgenic plants in MS-0 media. Variance analysis of transgenic plants was showed that when genotypes were evaluated, a significant difference was found between genotypes. The results showed that *SlAIM1*-4 transgenic plant has the highest expression whereas *SlAIM1*-3 transgenic plant has the lowest expression.

In present study, Rahamkulov and Bakhsh, 2020 protocol was used and %20 PEG was applied to induce drought stress on transgenic plants (*SlAIM1*-1 and *SlAIM1*-4) for ten days. Since *SlAIM1*-1 and *SlAIM1*-4 were grown in in vitro drought condition (MS0 / PEG), qRT-PCR analysis of these transgenic plants performed in comparison with plants obtained by standard Agria and transformed plants by control vector, it was observed that the expression increased significantly in transgenic plants under PEG conditions. The results showed that *SlAIM1*-4 transgenic plant has the highest expression and under PEG condition, the expression of the *SlAIM1*-4 transgenic plant increased 5-fold compared to normal conditions. Similarly, expression of the *SlAIM1*-1 transgenic plant was increased 3-fold compared to normal conditions.

Statistical analysis (by JMP Statistical Package program) showed that when only genotypes were evaluated, although the *SlAIM1*-4 transgenic plant showed more expression than the *SlAIM1*-1 transgenic plant in normal conditions and drought conditions, it did not differ statistically in terms of genotypes. However, a significant difference was found between treatment and (Treatment*Genotype) interactions.

Although it was observed that the expression of the *SlAIM1* gene increased in the Agria cultivar under drought condition, more research is needed to determine whether drought tolerance has also increased. The overall study is important for preliminary works on *SlAIM1* gene for drought stress response.

This study will help to understand the main role of *SlAIM1* gene under drought stress of potato plants in the future. Thus, it is expected that by enlightening drought tolerance mechanisms, more drought tolerant plants can be developed.

CHAPTER VI

CONCLUSION

Biotic and abiotic stress conditions on plants create many problems in the agricultural sector throughout the world and these problems turn into a global problem with the increase of the world population. There are many studies using new technologies to prevent such problems.

The present study is important as new step has been taken against drought stress. This is the very first study of potato harboring with tomato *AIM1* gene which is encoded by R2R3 MYB transcription factor under drought stress.

The study was fulfilled to optimize cloning steps and *Agrobacterium tumefaciens* mediated transformation in Agria potato cultivar. It was investigated that the composition of different plant growth regulators and different type of explants are affecting callus induction. An expression vector harboring nptII gene for kanamycin resistance and BAR gene for plant selection was used to obtain transgenic potato plants.

In this thesis work, a construct was created by gateway cloning method to transfer the *SlAIM1* gene to potato plant and transgenic plants were obtained from the transformations from gene transfer. There has been no previous study to analyse the expression of the target gene in potato plants. This study is a preliminary study and have a great importance to determine expression of the *SlAIM1* gene in transformed potato plants. In the thesis study, 8 transgenic plants (*SlAIM1*-1, *SlAIM1*-2, *SlAIM1*-3, *SlAIM1*-4, *SlAIM1*-5, *SlAIM1*-6, *SlAIM1*-7 and *SlAIM1*-8) were obtained and 2 transgenic plants (*SlAIM1*-1 and *SlAIM1*-4) were growed under drought condition in comparison with normal condition. Totally, 5 transgenic plants (*SlAIM1*-1, *SlAIM1*-2, *SlAIM1*-3, *SlAIM1*-4 and *SlAIM1*-8) were examined by qRT-PCR. The expression of *SlAIM1* gene was examined under normal and drought conditions. It was observed that the expression of the *SlAIM1* gene increased in the transgenic Agria cultivar under drought but more research is needed to determine whether drought tolerance has also increased.

The overall study is important for preliminary results on *SlAIM1* gene for drought stress response and more studies are needed to investigate the detailed role of *SlAIM1* gene against drought and other abiotic stresses. In the future, obtained transgenic plants in tissue culture conditions should be tested in greenhouse conditions and then in field conditions under drought and other abiotic stresses. The obtained plants may be included in breeding programs.

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CURRICULUME VITAE

Irem Aycan Sırel was born …………………. Turkey. She completed her primary, secondary and high school education in Nigde. After high school, she completed her B.Sc. in Nigde Ömer Halisdemir University, the Faculty of Agricultural Sciences and Technologies, Department of Agricultural Genetic Engineering. During B.Sc., she went to Germany for her internship and she was successfully completed her internship in Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) within the scope of Erasmus program. After completing her undergraduate education in 2018, she started her master's degree in Nigde Ömer Halisdemir University, Institute of Science, Department of Agricultural Genetic Engineering.