The Role of *HvBWMK1* in Barley Tolerance to Abiotic Stress

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ABSTRACT

Barley (*Hordeum vulgare* L.) is an important crop grown throughout the world. Quality and quantity of barley is compromised by abiotic stresses, salinity in particular which is already widespread in many regions. Mitogen Activated Protein Kinase (MAPK) cascades are signal transduction pathways involved in biotic and abiotic plant defence mechanisms. Here, the function of the barley gene *HvBWMK1* has been investigated. Transgenic barley plants that overexpress *HvBWMK1*, or in which *HvBWMK1* has been down-regulated by antisens, were generated, as well as empty vector control plants. The transformation efficiency using *Agrobacterium* was 10.4% independent transgenic plant per embryo. Northern blot analysis of *HvBWMK1* primary overexpression lines showed up-regulation of mRNA level, conversely the antisense lines of *HvBWMK1* showed clear down-regulation. Real-time PCR analysis in the T_1 generation revealed that two over-expresser lines showed higher expression as compared with wild type (by 79% and 35%) while the *HvBWMK1* antisense construct reduced endogenous gene expression partially (by 14%, 23% and 39% compared with wild type). Barley seedlings were subjected to two weeks of salt stress (150 mM NaCl) then biochemical and physiological parameters were measured.

The overexpression lines showed an increase in tolerance to salinity stress compared to antisense lines and controls. Tolerance was accompanied with increasing endogenous proline and chlorophyll levels and a reduction in hydrogen peroxide content after salinity exposure. Overall these results suggest that the barley MAPK *HvBWMK1* acts as a positive regulator in barley tolerance to salinity stress.

DEDICATION

With my pleasure, this thesis is dedicated to my lovely family:

wife:

Salemah Saleh Saud Al-awjely

sons:

Ahmed

Saleh

Mohamed

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May God bless all of you.

DECLARATION

I, Salem M. A. Rajab, hereby declare that I am the author of this thesis. All the work described in this thesis is my own, except were stated in the text. The work presented here has not been accepted in any previous applications for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of reference.

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ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
%	percent
µg	Microgram
µl	Microliter
µm	Micromole
µM	Micromolar
6BAP	6-benzylaminopurine
A_{xnm}	Absorption at wavelength of x nm
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
bar	phosphinothricin N-acetyltransferase gene
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BWMK1	Blast and Wound-induced MAP Kinase
CaMV 35S	Cauliflower Mosaic Virus 35S promoter
CAT	Catalases
cDNA	Complementary DNA
CIM	Callus induction medium
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
dH_2O	Distilled water
Dicamba	3,6-dichloro-2-methoxybenzoic acid
DIG	Digoxygenin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	dioxyribonuclease
dNTP	Deoxyribonucleoside 5´-triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate

E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
et al.	and others
etc.	et cetera
eV	electron volt
Fig.	Figure
FW	fresh weight
g	Gram or gravitational force
GC-MS	Gas chromatography-mass spectrometry
H_2O_2	Hydrogen peroxide
hpt	hygromycin phosphotransferase gene
i.e.	Latin words: id est = that is
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kbp	Kilo base pair
kDa	Kilo Dalton
l	litre
LB	Luria Bertani broth
LB	left border
M	Molar
MS	Murashige and Skoog basal salt mixture
MAPK	Mitogen activated protein kinase
MES	4-morpholinoethanesulphonic acid
mg	miligram
ml	mililiter
mM	mili Molar
mm	milimeter
MP	Megapixel
mRNA	messenger-RNA
NaCl	Sodium chloride
N:P:K	ratio of nitrogen (N), phosphorus (P) and potassium (K) in fertilizer
NBT	nitrotetrazolium blue chloride
ng	nanogram
nm	nanomolar

OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PCA	Principle component analyses
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
pH	a measure of the acidity or alkalinity of a solution
pmol	picomolar
POX	Peroxidase
RB	right border
RNA	ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutases
T_0	regenerated transgenic plant
T_1	progeny derived from self-pollination of T_0
Taq	*Thermus aquaticus*
T-DNA	transfer-DNA
TDY	Thr-Asp-Tyr
TEY	Thr-Glu-Tyr
Ti	Tumor inducing
Tris	2-Amino-2-hydroxymethylpropane-1.3-diol
u	unit
UV	Ultraviolet
V	Volt
v/v	volume to volume
w/v	weight to volume
WT	wild-type plant
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactoside

CHAPTER 1 INTRODUCTION

1.1 Barley

Barley (*Hordeum vulgare* L.) is one of the important annual cereal crops with a short season, which is widespread and grown nearly all over the cultivated area of whole regions of the world (Table 1.1). Generally, barley is cultivated in two cultivations seasons, the autumn season and the spring season. It can be sown in the autumn season in September or early October, whereas the spring varieties can be sown from January to April depending on the latitude (Newton *et al*. 2011). Archaeological remains of barley grains found at different regions in the Fertile Crescent confirmed that barley was domesticated about 8000 - 6500 B. C. (Badr *et al*. 2000, Newman and Newman 2008). Many researchers consider the origin of barley is the Fertile Crescent (Palestine, Jordan, Syria, Iraq and Iran) where its wild relative *Hordeum spontaneum* is still existence (Badr *et al*. 2000, Newman and Newman 2008). Depending on certain traits, barley is classified to spring varieties or winter varieties, two rows or six rows, hulled or hulless by presence or absence of hull tightly adhering to the grain, and malting or feed by end-use type (Baik and Ullrich 2008).

1.1.1 Taxonomy and botanical description

Barley was one of the first domesticated cereal crops, belonging to the *Tritiaceae* tribe, grass family *Poaceae*, which is consider the largest family in the monocotyledons included the important domesticated crops wheat, maize, rice and sorghum. The genus *Hordeum* compromise 32 species (Newman and Newman 2008).

Barley is an annual crop, diploid with 14 chromosomes (2n=14) and self-pollinating, thus each flower containing both the male and female organs, anthers and ovary. Barley has a strong fibrous root system, comprise two types, seminal roots: about 5 to 7 roots initiate from coleorhiza tissues in the embryo and many nodal or adventitious roots develop from the lower basal nodes of the crown as tiller develop. The barley stem is a cylindrical culm divided to several hollow internodes by solid nodes, the stem reach about 60 to 120 cm high. Barley has several tillers arising from the axils of the basal leaves with the same structure of the main stem. Each leaf of barley consists of flattened blade and tubular sheath wraps around the stem completely, one leaf arises on each node alternately on opposite side of each internode. The barley spike consists of a solid, flat, zigzag rachis carrying a triplet of spikelets which alternate on opposite sides of each node, on the six-rows all spikelet are fertile whereas on the two-rows only the

central spikelet is fertile. The caryopses (grain) comprises of two main parts enveloped by the seed coat, the bigger part: the endosperm and the smaller part: the embryo which lies at the bottom of dorsal side, the embryo consists of the scutellum and axis of growth, comprise the shoot and the root initials (Briggs 1998, Morris and Bryce 2000, Newman and Newman 2008, Horsley *et al*. 2009).

1.1.2 Economical importance

Barley is fourth in rank of total cereal production area in the world with 560,000 square kilometres cultivated, whereas the average of the annual rate of the world barley production during 2009-2013 was 137 million metric tons approximately (Table 1.1) (USDA 2013, IGC 2013). Barley has three important major uses: feed grain, malting and seeds (to sell to farmers) where about 60% is used as an animal feed grain and stock feed manufacture, about 30% for malting, 7% for seeds with only a small amount of about 3% is used for a human feedstuff (Newman and Newman 2008). In addition the straw is used as forage for grazing, hay or silage.

In term of the plant genetics, barley is considered as a good genetic model to study the *Tritiaceae* tribe. In this respect, barley has important traits such as the low number of chromosomes (2n = 14) with large size, is naturally diploid with a high degree of self-pollination, cross-breeding easily and is easily cultivated in different climate conditions. These traits contribute to the broad utilization of genetic studies of the cereal crops (Saisho and Takeda 2011, Nalawade *et al*. 2012). It also provides an excellent and useful model system representing a number of small grain cereal species, especially wheat, which is more important crop and has a larger and more complex genome (Goedeke *et al*. 2007, Bartlett *et al*. 2008). Barley was suggested as a model for wheat due to its highly efficient transformation rates and smaller, less complex genome (Harwood 2012).

Table 1.1: Regional barley production and consumption.*

Region	Production (Thousand Metric Tons)				
	2009/10	2010/11	2011/12	2012/13	Aug 2013
North America	14,996	12,224	11,771	13,369	13,786
South America	2,443	3,869	5,535	6,304	5,440
European Union - 28	62,393	53,691	52,026	54,572	57,117
Other Europe	1,163	1,112	1,163	1,181	1,212
Former Soviet Union	35,988	21,682	32,245	25,977	30,030
Middle East	11,320	11,100	11,670	10,275	12,619
North Africa	7,340	4,589	4,448	3,408	4,983
Sub-Saharan Africa	1,933	1,865	2,049	2,058	2,047
East Asia	2,646	2,215	2,602	2,652	2,615
South Asia	2,330	1,957	2,192	2,327	2,447
Oceania	8,300	8,303	8,589	7,197	7,800
Total	**150,852**	**122,607**	**134,290**	**129,320**	**140,096**
Consumption					
North America	14,021	12,701	11,808	12,118	12,706
South America	2,933	2,937	3,072	3,490	3,370
European Union	56,770	56,610	51,230	51,820	52,220
Other Europe	1,282	1,309	1,336	1,370	1,399
Former Soviet Union	28,088	20,677	26,323	22,285	23,660
Middle East	20,403	19,694	21,786	21,996	22,936
North Africa	5,847	5,925	6,199	5,262	6,350
Sub-Saharan Africa	1,963	1,961	1,998	1,937	2,160
East Asia	6,276	5,771	6,375	6,250	6,355
South Asia	2,163	1,967	2,122	2,237	2,407
Oceania	4,925	4,550	3,700	3,525	3,700
Total	**144,671**	**134,102**	**135,949**	**132,290**	**137,263**

* USDA (United States Department of Agriculture) 2013.

1.2 Genetic modification techniques of cereal crops

1.2.1 Historical review

For long time, classical plant breeding by crossing was the main technique to produce new varieties with desirable traits despite this taking a long time, for example 10-12 years for cereals (Horsley *et al*. 2009). In the early 1980s four groups of plant researchers working independently succeeded in creating transgenic plants. Three groups, headed by Jeff Schell (Belgium), Mary-Dell Chilton (USA) and Rob Horsch (USA), announced in January 1983 that they had inserted bacterial genes into plants whereas the fourth group headed by John Kemp (USA) announced in April 1983 that they had inserted a plant gene from one species into another species (Vasil 2005, Newell-McGloughlin and Re 2006).

Compared with the dicotyledonous plants, success in cereal crops transformation was achieved in the late 1980s. Rice (*Oryza Sativa*) (Zhang and Wu 1988, Zhang *et al*. 1988, Toriyama *et al*. 1988, Shimamoto *et al*. 1989), maize (*Zea mays*) (Rhodes *et al*. 1988, Gordon-kamm *et al*. 1990), orchard grass (*Dactylis glomerata*) (Horn *et al*. 1988), then 1990s followed by wheat (*Triticum aestivum*) (Vasil *et al*. 1992, Vasil *et al*. 1993, Weeks *et al*.1993), oat (*Avena sativa*) (Somers *et al*. 1992), and barley (*Hordeum vulgare*) (Ritala *et al*. 1993, 1994, Wan and Lemaux 1994).

In order to deliver gene into cereal plants, several gene transformation technologies have been attempted by scientists and researchers in laboratories around the world. In their reviews, Danilova (2007), Darbani *et al*. (2008), Rao *et al*. (2009) and Rivera *et al*. (2012) describe most of these technologies which comprise electroporation of cells and tissues, chemical (PEG) treatment of protoplasts, silicon carbide fibers, biolistic transformation (particles bombardment), ultrasound-mediated transformation, vacuum infiltration, liposome-mediated transformation, shockwave-mediated transformation, microinjection, macroinjection, laser microbeams, transformation via the pollen-tube pathway, desiccation based transformation, *Agrobacterium*-mediated transformation and electrophoresis of embryos. Three methods to date predominate: *Agrobacterium*-mediated transformation, electroporation and biolistic transformation (Rivera *et al*. 2012). Each method has advantages and disadvantages (Table 1.2) which make them suitable for this plant or procedure and unsuitable for the other. The successful methods have ability to integrate foreign genes (transgenes) not only from other non-related plants but also from fungi, viruses, bacteria and animals (Rivera *et al*. 2012).

Table 1.2: Comparsion of the most popular methods for genetic transformation of plants*.

Method	Procedure	Advantages	Disadvantages
Agrobacterium	Plant pathogenic bacteria able to transfer DNA fragment and integrate it into the plant cell genome.	Facilitates the precise integration of fewer copies of gene. High degree of stability, inheritance, and consistent gene expression over the generations. Suitable for different cell types. Reliable, reproducible and efficient protocols. Suitable for many dicots and monocots crops. High efficiency.	Various parameters not easy to handle affect transformation efficiency and plant regeneration. Time-consuming and slow process requires sterile materials. Introduction of unnecessary partner vectors that produce unknown genetic expressions into the plant.
Electroporation	Electric pulses form transient micropores in the cell membrane allowing DNA to pass into the cell.	Low cost, simple, and quick. It can be applied to any plant protoplasts. Suitable for different cell types.	Labour-intensive protocols. Often requires protoplast formation. Depends on the electrophysiological properties of the cells. Low transformation efficiency.
Biolistics	Small fragments of the target DNA coated with heavy-metal micro-particles accelerate by gun to penetrate the cell wall.	Easy and simple. It has a short processing time. No pre-treatment of the cell wall required. Does not depend on the physiological properties of the cell. Transformation with multiple transgenes is possible.	Expensive. Requires continuous supply of con-sumables. DNA can be damaged. Impossible to control the copies number of inserted genes in one cell. Limitation on the size of introduced genes. Low transformation efficiency.

* Rivera *et al*. 2012 with modifications

Efforts are currently in progress to modify the old transformation methods described above or develop new methods. Ohadi *et al*. (2012) in their review have pointed to some of these methods which employ the nanotechnology such as nanoparticle, carbon nano-tube, quantum dots, nanoemulsion, etc. The combination between nanotechnology based biotransformation and other commonly applied transformation methods might improve the efficiency, productivity and minimize the transgene silencing.

Rivera *et al*. (2012) in their review concluded several requirements must be taken into account to evaluate any transformation method, low cost, safe to operator, easy procedures, simple technically, lead to large number of transformants, low copy number of genes introduced into each cell, ability to generate transgenic plants from single transformed cells and capability to introduce in a stable way only the desired DNA without vector sequences which are not required for gene integration or expression.

1.2.2 Biolistic transformation (particles bombardment)

Biolistic transformation is a process by which the fragments of the target DNA coated onto heavy-metal microparticles (tungsten, gold or platinum) are introduced into the cells using a device often referred to as a gene gun. It was invented and developed by John Sanford with Edward Wolf between 1983 and 1987 (Sanford 2000). The particle mixture accelerates at hundreds of meters per second under high pressure of gas such as helium, nitrogen or carbon dioxide to penetrate the cell wall and nuclear membrane to incorporate the DNA within the genome.

The target tissues capable of regeneration and used for cereal biolistic transformation include the embryogenic cells suspension cultures from maize (Gordon-kamm *et al*. 1990), barley (Ritala *et al*. 1993); embryogenic callus cultures from oat (Somers *et al*. 1992), wheat (Vasil *et al*. 1992, Weeks *et al*.1993), barley (Ritala *et al*. 1994, Wan and Lemaux 1994, Manoharan and Dahleen 2002, Abdallah 2007), sugarcane (Xiong *et al*. 2013); apical meristem from oat and barley (Zhang *et al*. 1999); microspore from barley (Carlson *et al*. 2001, Obert *et al*. 2008), wheat (Folling and Olesen 2001); immature embryos callus from barley (Cho *et al*. 1998, Harwood *et al*. 2000, Travella *et al*. 2005, El-Assal *et al*. 2011,Yadav *et al*. 2012); immature scutella and inflorescences from wheat (Cui *et al*. 2011); mature embryos callus from barley (Um *et al*. 2007) (Table 1.3).

1.2.3 Electroporation

Electroporation is a process where electric pulses lead to a temporar pore formation in the cell membrane. Small amount of plant material, such as leaf fragments, buds, pollen, microspores, seeds, embryos, callus, cell suspension or protoplast with the desired DNA are placed in a suitable cuvette which is then placed between electrodes (anode and cathode). High-voltage (200 to 1500 V/cm) electrical pulses in short duration (dozens of microseconds to dozens of milliseconds) disturb the phospholipid bilayer of the membrane and facilitate the formation of transient aqueous micropores in the cell membrane and allow the insertion of molecular compounds into the cell and then the nucleus (Danilova 2007, Rao *et al*. 2009, Husaini *et al*. 2010).

For electroporation, the first successful attempts were reported in the nineteen eighties for maize (Rhodes *et al*. 1988) and rice (Toriyama *et al*. 1988, Zhang *et al*. 1988, Shimamoto *et al*. 1989). The protocol is suitable for many cereal species (Table 1.3) using different plant tissues such as immature embryos protoplast; maize (Rhodes et al. 1988), rice (Shimamoto *et al*. 1989, Toriyama *et al*. 1988); leaf base callus protoplast for rice (Zhang *et al*. 1988); immature embryos for wheat (He and Lazzeri 1998, Sorokin *et al*. 2000), maize (D'Halluin *et al*. 1992); mature embryo for barley (Gürel and Gözükirmizi 2000); anther derived embryos for wheat (Haliloglu *et al*. 2004); immature inflorescences for tritordeum (He and Lazzeri 1998, He *et al*. 2001); embryogenic microspore protoplast for barley (Salmenkallio-Marttila *et al*. 1995); shoot apices for rice (Muniz de Padua *et al*. 2001).

1.2.4 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a rod shaped, non-sporing, aerobically growing, motile (surrounded by a small number of peritrichous flagella), soil-borne, gram-negative, plant pathogenic bacterium (Kado 2002, Pitzschke and Hirt 2010). It belongs to the *Agrobacterium* genus, family *Rhizobiaceae*, which contains saprophytic and pathogenic species. It invades a wide range of dicotyledonous plants in more than 60 different plant families, especially member of the rose family (Rosaceae) such as plum, apple, peach, cherry, apricot, pear, almond, raspberry and roses (Kado 2002, Opabode 2006) and it is ranked third in a list of the most important bacterial plant pathogens (Mansfield *et al*. 2012). *Agrobacterium* causes a plant disease called crown gall, forming a big tumour in the region between the root and the stem of plant (Kado 2002). The bacterium

encourages the gall to synthesize opines (unusual amino acids) which are used by these bacteria as an important source of carbon and nitrogen needed for energy and growth (Hellens *et al*. 2000, Pitzschke and Hirt 2010, Pacurar *et al*. 2011).

Agrobacterium contains a plasmid called the Ti (tumour inducing) plasmid, approximately 200 kbp in size (Kado 2002), which contains: (1) T-DNA region (transfer DNA) fragment flanked by two 25-bp border repeats called the left border (LB) and right border (RB), any DNA fragment between these borders is suitable for transformation into the plant cell. (2) Approximately 35 virulence genes (not located on the T-DNA region) encode genes producing proteins responsible for the transformation process: excision, transfer and integration the T-DNA fragment into the plant host genome. Virulence genes are divided in at least eight groups (*virA, virB, virC, virD, virE, virF, virG* and *virH*) (Pacurar *et al*. 2011). (3) Opine catabolism genes, which allow *Agrobacterium* to consume and catabolizes the opines (amino acids and sugars) produced by crown gall cells. The bacteria invade the plant tissues through the open wound after being attracted by phenolic compound (acetosyringone, hydroxy-acetosyringone) which are excreted from wounded tissues, and the phenolic compounds induce the expression of the *vir* genes, which transfer a single stranded copy of the T-DNA fragment and integrate it into the plant cell genome, and so consequently alter the metabolism and development process of the invaded cell resulting in abnormal cell division and synthesis of nutritive compounds (opines), which are used by *Agrobacterium tumefaciens* (Pacurar *et al*. 2011).

The process of T-DNA transformation starts when phenolic and sugars compounds are released from wounded plant cells which act as chemotactic attractants of *Agrobacterium* to move toward plant tissue and attach to wounded cells, in addition the phenolic compounds induce *vir* gene expression by binding to the constitutively expressed *virA* gene product which acts as a receptor (Pitzschke and Hirt 2010). The VirA and VirG proteins stimulate the expression of the other *vir* genes. VirD1 and VirD2 recognise the 25 bp border sequences, and nick the T-DNA at the right border to form a T-strand, VirD2 caps the 5′ end of the single-strand DNA (T-strand) to form a T-strand (complex). In addition VirE2 coats the T-strand to protect it from nuclease degradation in the plant cell. The VirB protein creates a VirB complex comprising of at least 12 vir proteins and then enables the T-strand complex to pass through the inner and outer bacterial membranes as well as the plant host cell wall and membrane. Once

the complex enters into the plant cell, it is transported directly to the nucleus, because the proteins associated with the T-DNA complex contain nuclear localisation signals (Gelvin 2003, Lee and Gelvin 2008, Pitzschke and Hirt 2010, Pacurar *et al*. 2011).

In order to employ *Agrobacterium tumefaciens* in genetic engineering of plants, binary vector plasmids system were developed. The system consists of two independent and complementing plasmids: T-DNA binary vector plasmid and the vir helper vector plasmid located in the same *Agrobacterium* cell (Gelvin 2003, Lee and Gelvin 2008). The T-DNA binary vector plasmid generally comprises: firstly, T-DNA region containing multiple cloning sites in addition to a plant selectable marker gene, both are placed between right and left border of the T-DNA. Secondly, within backbone sequences there are origin of replication suitable for both *E. coli* and *A. tumefaciens* and antibiotic resistance gene for selection in both *E. coli* and *A. tumefaciens* as well. The vir helper vector plasmid contains the vir genes required for T-DNA mobilisation (Lee and Gelvin 2008). The addition of more virulence genes leads to the development of super-binary vectors which enhance the capacity of *Agrobacterium* to infect several types of recalcitrant plants such as important cereals (Komori *et al*. 2007).

Many different *Agrobacterium* strains exist naturally in the soil but most of them are only suitable for a specific host plant (Danilova 2007). To overcome this issue, artificial hypervirulent strains were developed such as AGL0, AGL1, EH101, EH105, C58 (ABI), GV2260 and LBA4404, which have been used to transform cereal crops (Table 1.3). In addition to the type of *Agrobacterium* strain many factors play a pivotal role in transformation success such as the binary plasmid vector, the host plant species, the type of explant, antibiotics and the transformation conditions (Opabode 2006).

In 1983 tobacco became the first plant transformed using the *Agrobacterium*-mediated transformation method (Barton *et al*.1983, Zambryski *et al*. 1983), since that time this method has become the dominant transformation method for dicotyledonous plants whereas monocotyledonous plants including cereal crops were thought to be recalcitrant plants because monocots are not natural host for *A. tumefaciens* (Newell 2000, Goedeke *et al*. 2007, Abdul *et al*. 2004). It took another ten years to develop highly efficient transformation method suitable for monocots using *A. tumefaciens*, despite the considerable efforts that were made by many researchers to obtain transgenic monocot plants such as maize (Graves and Goldman 1986, Gould 1991), and rice (Chan *et al*. 1993). Finally the first *Agrobacterium*-transformed monocot plant, rice, was achieved

(Hiei *et al*. 1994). After two years this was followed by successful efforts in some important cereal crops such as maize (Ishida *et al*. 1996), subsequently, wheat (Cheng *et al*. 1997), barley (Tingay *et al*. 1997), sugarcane (Arencibia *et al*. 1998) and sorghum (Zhao *et al*. 2000). Later on, the range of transformable species was extended to rye (Popelka and Altpeter 2003), triticale (Nadolska-Orczyk *et al*. 2005, Hensel *et al*. 2009) and oat (Gasparis *et al*. 2008).

Agrobacterium-mediated transformation has succeeded in transforming several species of cereal crops using various types of tissues, however the immature embryos still remain the most desirable tissue (Table 1.3).

1.2.5 Modifications and combinations of *Agrobacterium*-mediated transformation

In order to obtain high transformation efficiencies, researchers attempted to combine more than one method. Most of these combinatorial methods employ *A. tumefaciens* for gene transfer together with different methods employed to injure the plant tissue to facilitate agroinfection (Danilova 2007). Hansen and Chilton (1996) developed a technique called Agrolistic, a combination of the *Agrobacterium* and biolistic methods to get the advantages of *Agrobacterium* in respect of low copy number of the target gene integrated, with no vector sequence as compared to the disadvantages of biolistic such as multiple copy number including plasmid vector sequence which can lead to instability of expression and interactions that silence transgene expression. In other experiments, barley immature embryos were injured by shooting the scutellum surfaces with gold particles then inoculated with *Agrobacterium* suspension (Fang *et al*. 2002). Vortexing silicon carbide fibres with *Agrobacterium* suspension in order to injure explants leads to 33.3% transformation efficiency compared to 2.4% in case of transformation with *Agrobacterium* without silicon carbide fibres (Singh and Chawla 1999). For simplification of *Agrobacterium* penetration into the explants, biolistic shooting was performed on maize embryogenic callus (Danilova and Dolgikh 2005).

A number of different modifications were developed to make the method more simple and of higher efficiency. For example Ziemienowicz *et al*. (2012) instead of using *Agrobacterium* use an in vitro-prepared nano-complex consisting of transferred DNA, virulence protein D2, and recombination protein A delivered to triticale microspores with the help of a Tat_2 cell-penetrating peptide. The process led to

integration of intact single copy genes and prevented degradation, and in addition the transferred gene was expressed in all transgenic plants regenerated from microspores.

In attempts to save time in the tissue culture process, Zale *et al*. (2009) used a floral dip method to transform wheat, which depends on dipping the spikes in *Agrobacterium* suspension and then screening the produced seeds. Alternatively, Supartana *et al*. (2006), Razzaq *et al*. (2011) carried out an *in planta* transformation protocol to transform wheat. In this protocol, dry seeds of wheat were soaked with distilled water, sterilized, and then allowed to germinate till the emergence of coleoptiles which were incised through the apical meristem, after that by using a syringe, the apical meristem was inoculated with *Agrobacterium*, and the seedlings were regenerated to producing seeds. 26% of treated coleoptiles were confirmed successfully transformed.

1.3 Barley varieties amenable to transformation

Despite the large number of barley varieties, only relatively few of these varieties were successfully subjected to *Agrobacterium*-mediated transformation experiments such as Golden Promise, Chebec, Schooner, Sloop, Helium, Optic, Femina, Salome, Corniche, Alexis, Giza 123, Giza 130, Haruna, Nijo, Dissa and Scarlett (Table 1.3). Golden Promise is a gamma-ray induced semi-dwarf mutant of the cultivar Maythorpe (Forster 2001). This spring two-row barley variety is still considered the most readily transformed and widely used cultivar; it was the first transgenic barley transformed by biolistics (Wan and Lemaux 1994) and the first *Agrobacterium*-mediated transformed barley as well (Tingay *et al*. 1997). Since that time Golden Promise has become a standard genotype for barley transformation with high responsiveness for various sources in tissue culture (anther, ovule, microspore, immature embryos etc.) which can be transformed and then manipulated to produce high numbers of green transgenic fertile plants (Forster 2001) and also it shows good susceptibility to *Agrobacterium* infection (Harwood 2012) and is amenable to high successfully transformation with several genetic transformation methods.

Table 1.3: Cereal crops and varieties amenable to transformation by different methods.

Crop and variety	Transformation method	Target tissue	Reference
Barley Golden Promise	*A. tumefaciens* (AGL1)	Immature embryos	Tingay *et al*. 1997 Patel *et al*. 2000 Bartlett *et al*. 2008
Barley Golden Promise	*A. tumefaciens* (AGL0)	Embryos	Trifonova *et al*. 2001
Barley (Mentor, Baronesse, Pongo Cecilia, Filippa,)	*A. tumefaciens* (AGL1) Particle bombardment	Immature embryos	Roussy *et al*, 2001
Barley Golden Promise	*A. tumefaciens* (AGL0) + Biolistic	Immature embryos	Fang *et al*. 2002
Barley (Chebec, Golden Promise, Schooner, Sloop)	*A. tumefaciens* (AGL0)	Immature embryos	Murray *et al*. 2004
Barley Golden Promise	*A. tumefaciens* (LBA4404)	Immature embryos	Coronado *et al*. 2005 Shrawat *et al*. 2007
Barley Golden Promise	*A. tumefaciens* (AGL1) Particle bombardment	Immature embryos callus	Travella *et al*. 2005
Barley Golden Promise	*A. tumefaciens* (AGL0)	Ovule	Holme *et al*. 2006
Barley Golden Promise	*A. tumefaciens* (AGL0)	Immature embryos	Lange *et al*. 2006
Barley Golden Promise, Helium, Optic	*A. tumefaciens* (LBA4404) (AGL1)	Immature embryos	Hensel *et al*. 2008
Barley Golden Promise, Femina, Salome, Corniche, Alexis	*A. tumefaciens* (AGL0)	Ovule	Holme *et al*. 2008
Barley Golden Promise	*A. tumefaciens* (LBA4404)	Immature embryos	Hensel *et al*. 2009
Barley (Giza 123 Giza 130)	*A. tumefaciens* (AGL1)	Immature embryos	Ibrahim *et al*. 2010
Barley Golden Promise	*A. tumefaciens* (AGL1)	Immature embryos	Soltész *et al*. 2012
Barley (Scarlett, Golden Promise)	*A. tumefaciens* (AGL1) Particle bombardment	Immature embryos	Zalewski *et al*. 2012
Barley Golden Promise	*A. tumefaciens* (AGL1, LBA4404)	Immature embryos	Kapusi *et al*. 2013
Barley (Pokko)	Particle bombardment	Embryogenic cells suspension cultures	Ritala *et al*. 1993
Barley (Kymppi)	Particle bombardment	Immature embryos	Ritala *et al*. 1994

Barley Golden Promise	Particle bombardment	Embryogenic callus. Immature embryos. Microspore-derived embryos (MDEs).	Wan and Lemaux 1994
Barley (Haruna, Nijo, Dissa Golden Promise	Particle bombardment	Immature embryos	Hagio *et al*. 1995
Barley (Galena, Harrington, Golden Promise	Particle bombardment	Immature embryos	Cho *et al*. 1998
Barley (DH10, Crystal, Morex, Harrington)	Particle bombardment	Shoot meristematic	Zhang *et al*. 1999
Barley Golden Promise	Particle bombardment	Immature embryos callus	Harwood *et al*. 2000, 2002
Barley (Igri)	Particle bombardment	Microspore	Carlson *et al*. 2001
Barley (Giza 123)	Particle bombardment	Scutellum derived calli	Abdallah 2007
Barley (EL-Taif, EL-Dwaser)	Particle bombardment	Immature embryos callus	El-Assal *et al*. 2011
Barley (Indian cultivars)	Particle bombardment	Immature embryos callus	Yadav *et al*. 2012
Barley Golden Promise	Polyethylene glycol	Immature embryos protoplast	Kihara *et al*. 1998
Barley (Igri)	Polyethylene glycol	Protoplast	Kihara *et al*. 2000
Barley (Clipper, Dissa, Derkado)	Polyethylene glycol	Scutellum protoplast	Nobre *et al*. 2000
Maize (A188, R91)	*A. tumefaciens* (LBA4404)	Embryogenic callus	Danilova and Dolgikh 2005
Maize (Hi II)	*A. tumefaciens* (EHA105)	Immature embryos	Hensel *et al*. 2009
Maize (inbred A188)	Electroporation	Protoplast (immature embryos callus suspension	Rhodes *et al*. 1988
Maize	Particle bombardment	Embryogenic cells suspension cultures	Gordon-kamm *et al*. 1990
Maize	Particle bombardment	Embryogenic calli	Nguyen and Sticklen 2013
Oat (Bajka, Slawko, Akt)	*A. tumefaciens* (AGL1, LBA4404, EH101)	Immature embryos leaf base segments	Gasparis *et al*. 2008
Oat	Particle bombardment	Embryogenic callus	Somers *et al*. 1992
Oat (Garry)	Particle bombardment	Shoot meristematic	Zhang *et al*. 1999
Rye inbred L22	*A. tumefaciens* (AGL0)	Immature embryos	Popelka and Altpeter 2003
Rice (Taipei 309)	Electroporation	Protoplast (leaf base callus suspension)	Zhang *et al*. 1988

Rice	Electroporation	protoplast	Toriyama *et al*. 1988, Shimamoto *et al*. 1989
Rice (Pi-4, Taipei309)	Polyethylene glycol	Protoplast (mature scutella callus suspension)	Zhang and Wu 1988
Sugarcane	*A. tumefaciens* (EH101, LBA4404)	Meristematic tissues callus	Arencibia *et al*. 1998
Sugarcane	Particle bombardment	Embryogenic callus	Xiong *et al*. 2013
Sorghum (Tx430, P898012, 296B, C401)	*A. tumefaciens* LBA4404, EH101	Immature embryos	Gurel *et al*. 2009
Triticale (Wanad)	*A. tumefaciens* (AGL1, LBA4404, EH101)	Immature embryos	Nadolska-Orczyk *et al*. 2005
Triticale (Bogo)	*A. tumefaciens* (LBA4404)	Immature embryos	Hensel *et al*. 2009
Triticale (Bogo)	*A. tumefaciens* (AGL1)	Immature embryos	Hensel *et al*. 2012
Triticale (Ultima)	T-DNA / VirD2, RecA protein complex	Microspores	Ziemienowicz *et al*. 2012
Wheat (Bobwhite)	*A. tumefaciens* C58 (ABI)	Embryogenic callus	Cheng *et al*. 1997
Wheat (Yan361, Yan2801)	*A. tumefaciens* (EH105)	Coleoptile	Zhao *et al*. 2006
Wheat (Certo)	*A. tumefaciens* (LBA4404)	Immature embryos	Hensel *et al*. 2009
Wheat (Crocus)	*A. tumefaciens* (C58C1, AGL1)	Floral dip	Zale *et al*. 2009
Wheat (GA-2002)	*A. tumefaciens* (LBA4404)	Imbibed seed apical meristem	Razzaq *et al*. 2011
Wheat (HD2329, PDW215	*A. tumefaciens* (LBA4404, GV2260)	Dry mature seeds	Chugh *et al*. 2012
Wheat (Giza 164)	Laser micropuncture	Immature embryos	Badr *et al*. 2005
Wheat	Particle bombardment.	Embryogenic callus	Vasil *et al*. 1992
Wheat	Particle bombardment	Immature embryos	Vasil *et al*. 1993
Wheat (Bobwhite)	Particle bombardment	Embryo-derived calli.	Weeks et al.1993
Wheat (BCDH18 BCDH46, CWDH239)	Particle bombardment	Microspore	Folling and Olesen 2001
Wheat (Oasis)	Particle bombardment	Embryogenic callus	Melchiorre *et al*. 2002
Wheat (Bobwhite)	Particle bombardment	Immature scutella and inflorescences	Cui *et al*. 2011

1.4 Antibiotic and selectable marker genes

Selectable marker genes are several types of genes encoding enzymes that play a crucial role in transformation techniques, conferring a high ability for successfully transformed cells to survive and grow in the presence of a specific substrate that is toxic to the untransformed cells. Because of the very low efficiency of transformation where only small number of the total cell population are transformed (only one cell out of several thousand or million cells will take up the DNA), using selectable marker genes facilitates the ability to distinguish between the very few true transformed cells and the bulk of untransformed cells (Penna *et al*. 2002, Abdul *et al*, 2004, Miki and McHugh 2004, Sharma *et al*. 2005).

In the binary vector system, plant selectable marker genes are placed between the right and left border of the T-DNA and in addition there are antibiotic resistance genes for selection of both *E. coli* and *A. tumefaciens* within the plasmid backbone sequences.

Selectable marker genes sometimes are combined with a very important partner, reporter genes system, to monitor transgenic events, and to confirm and improve transformation efficiency. Reporter genes allow the visual detection and the manual selection of transformed tissues with the help of laboratory equipment or special staining to visualise enzyme activity. Furthermore, in some cases reporter genes can be employed as selectable marker genes. The most widespread reporter genes are *GFP* (green fluorescent protein) isolated from the jellyfish (*Aequorea victoria*), *LUC* (luciferase) isolated from firefly (*Photinus pyralis*), *GUS* (β-glucuronidase) derived from *E. coli* and *CAT* (chloramphenical acetyltransferase) obtained from *E. coli* (Abdul *et al*, 2004, Miki and McHugh 2004, Wei *et al*. 2012).

To date there are a large numbers of selectable marker genes that exist for plants (Miki and McHugh 2004, Sundar and Sakthivel 2008, Penna and Ganapathi 2010, Rosellini 2011, Tuteja *et al*. 2012, Wei *et al*. 2012), but only few are widely used for barley transformation by *Agrobacterium*, most of these confer resistance to antibiotics or herbicides. The majority of the published scientific papers relating to transgenic cereal crops revealed the antibiotics kanamycin or hygromycin and a herbicide (phosphinothricin, PPT) were widely utilised as selection agents (Sundar and Sakthivel 2008). One of the most common selectable marker genes is *nptII* (neomycin phosphotransferase II) gene that confers the resistance to the antibiotic kanamycin (Miki

and McHugh 2004). The other most commonly used gene is the *bar* gene (phosphinothricin N-acetyltransferase) which confers tolerance to some herbicide active ingredients such as bialaphos, phosphinothricin and glufosinate ammonium (Miki and McHugh 2004), it was successfully used with barley transformation experiments carried out by Tingay *et al*. (1997), Patel *et al*. (2000), Trifonova *et al*. (2001), Travella *et al*. (2005), Shrawat *et al*. (2007), Zalewski *et al*. (2012). Also, hygromycin phosphotransferase (*hpt*) gene is widely used as a selectable marker gene, it encodes the enzyme hygromycin phosphotransferase (HPT) and confers tolerance to the antibiotic hygromycin B (Miki and McHugh 2004, Sundar and Sakthivel 2008). The antibiotic hygromycin B is produced originally by the bacteria *Streptomyces hygroscopicus* and also isolated from *Escherichia coli* (Zheng *et al*. 1991). Hygromycin comes second after kanamycin as an antibiotic selection marker and has been successfully used to generate transgenic plants in many cases (Hagio *et al*. 1995, Coronado *et al*. 2005, Shrawat *et al*. 2007, Bartlett *et al*. 2008, Holme *et al*. 2008, Hensel *et al*. 2008, 2009, Ibrahim *et al*. 2010, Soltész *et al*. 2012).

It is valuable to mention here that all commonly used antibiotic and herbicide resistance genes have been isolated from bacteria which exist in nature, therefore the humans, animals and plants are naturally exposed to these genes and enzyme (Ramessar *et al*. 2007). In spite of that, the consumers' societies and environmental groups raise concerns about biosafety and risks of using selectable marker genes in commercialized transgenic crops and its products (Afolabi 2007, Ramessar *et al*. 2007, Tuteja *et al*. 2012, Mehrotra and Goyal 2013). To win the consumers acceptance of transgenic crops and limiting their concerns, researchers and scientists are developing non-antibiotic based selectable markers based upon metabolic selection (Rosellini 2011, Wei *et al*. 2012) or so called clean gene technology or selectable marker-free technology such as co-transformation, site-specific recombination, negative selection, multi-autotransformation vector, transposition system and homologous recombination (Darbani *et al*. 2007, Penna and Ganapathi 2010, Tuteja *et al*. 2012).

1.5 Abiotic stress

Plants are exposed to many types of stresses during their life cycle, including biotic ones such as pathogens, weeds, mites, nematodes, and insects, and abiotic ones such as drought, salinity, pollution, UV light and high or low temperature. Drought and salinity in particular are already widespread in many regions, and it is expected that serious

salinization of more than 50% of all cultivated lands by the year 2050 will occur (Vinocur and Altman, 2005). Therefore, several control systems used to increase the plant tolerance to these stresses must be implemented. This includes agricultural control, chemical control, biological control, physical control, plant breeding and genetic engineering. In the last couple of decades, genetic engineering plays an important role in the development of plant protection.

1.5.1 Salinity

Salinity refers to the concentrations of mineral salts that are found in soil or dissolved in irrigation water which cause harmful effects to plants. Soil salinity problems and using saline water for irrigation affect approximately about one-third of the entire world's irrigated lands in humid as well as in arid and semi-arid regions (Yaron 1981). The United Nations Environment Program estimates that approximately 20% of arable lands and 50% of cropland in the world is exposed to salt stress (Yokoi *et al*. 2002). The total quantity of dissolved salts in water or soil solution is measuring by electrical conductivity (EC). The EC values of the majority of irrigation water are between in the range of 0.15 to 1.5 mmhos/cm, equivalent to 96-960 mg/l salt (Table 1.4). According to the USDA salinity laboratory, soil is considered as a saline soil when the electrical conductivity of the saturated paste extract (EC) is 4dS/m (4 dS/m = 40 mM NaCl) or more (Chinnusamy *et al*. 2005).

Table 1.4: Classification of saline waters*.

Water class	Electrical conductivity dS/m	Salt concentration (mg/L)	Type of water
Non-saline	< 0.7	< 500	Drinking, irrigation
Slightly saline	0.7-2	500-1500	Irrigation
Moderately saline	2-10	1500-7000	Primary drainage and groundwater
Highly saline	10-25	7000-15,000	Secondary drainage and groundwater
Very highly saline	25-45	15,000-35,000	Very saline groundwater
Brine	> 45	> 45,000	Seawater

dS/m = deciSiemens per metre. 1 dS/m = 10 $mmol_c/l$ = 700 mg/l

*Source: Rhodes *et al*. 1992.

Many crops are sensitive to salinity even when the soil EC is < 3 dS/m, therefore the increase in the rate of soil salinity reflects on the plant as a decrease in yield (Table 1.5) (Chinnusamy *et al*. 2005). Cereal crops differ greatly in its tolerance to salinity, rice is the most sensitive, and on the other way around barley is the most tolerant whereas bread wheat is moderately tolerant (Munns and Tester 2008). Soils and water naturally contain different concentrations and elements that make up salt. The major ions in water that may be responsible for salinization are the anions of carbonate (CO_3^{-2}), bicarbonate (HCO_3^-), chloride (Cl^-) sulphate (SO_4^{-2}) and nitrate (NO_3^-), and the cations of sodium (Na^+), calcium (Ca^{+2}), magnesium (Mg^{+2}) and potassium (K^+). In some cases other components could be found such as aluminum (Al^{+3}), iron (Fe^{+2}), manganese (Mn^{+2}) and molybdenum (Mo^{+3}). Soil salinity occurs as a result of irrigation with saline water, poor drainage and human activity. The consequences of salinity are effects on plant growth and yield, damage to irrigation pipes, reduction of water quality for users and soil erosion as a result of reduction of plant growth.

Table 1.5: The relationship between salinity and the yield of some important crops*.

Crop	Threshold salinity dS/m	Decrease in yield slope % per dS/m
Bean (*Phaseolus vulgaris* L.)	1.0	19.0
Eggplant (*Solanum melongena* L.)	1.1	6.9
Onion (*Allium cepa* L.)	1.2	16.0
Pepper (*Capsicum annum* L.)	1.5	14.0
Corn (*Zea mays* L.)	1.7	12.0
Sugarcane (*Saccharum officinarum* L.)	1.7	5.9
Potato (*Solanum tuberosum* L.)	1.7	12.0
Cabbage (*Brassica oleracea* var *capitata* L.)	1.8	9.7
Tomato (*Lycopersicon esculentum* Mill.)	2.5	9.9
Rice, paddy (*Oryza sativa* L.)	3.0	12.0
Peanut (*Arachis hypogaea* L.)	3.2	29.0
Soybean (*Glycine max* (L.) Merr.)	5.0	20.0
Wheat (*Triticum aestivum* L.)	6.0	7.1
Sugar beet (*Beta vulgaris* L.)	7.0	5.9
Cotton (*Gossypium hirsutum* L.)	7.7	5.2
Barley (*Hordeum vulgare* L.)	8.0	5.0

*Source: Chinnusamy *et al*. 2005.

1.5.2 Salinity symptoms in plants

In agriculture, salts accumulate in the root zone and cause effects that lead to crop yield reduction. The symptoms differ according to the type of plant, the stage of growth and the plant organs. But, in general the most common symptoms are similar to those of drought: older leaves become yellowish, there is interveinal leaf yellowing (chlorosis), brown edges of the leaf, die-back from the top to bottom, poor germination percentage and poor growth, stunting and susceptibility to pathogens.

Soil salinity affects plants by two ways, First, osmotic, where the presence of high concentrations of salts in the soil solution make it harder for roots to extract water and reduce the ability of the plant to take up water, this leads to slower growth (Munns and Tester 2008). Osmotic stress arrests the growth of plant and affects cell division and elongation. The division of cells is a crucial process which determines the meristem activity and the entire plant growth rate (Bartels and Sunkar 2005). Secondly, toxicity, where the presence of high concentrations of salt in the plant (intracellular and intercellular) can be toxic and leads to cellular damage (Munns and Tester 2008).

1.5.3 Salinity control

Salinity control is a water and soil salinity management regime which uses more than one technique together. These techniques include water desalination, drainage, crop rotation, leaching soluble salts out of soil with excess clean irrigation water, plant breeding and genetic engineering (modified plants) to withstand salinity.

Barley is one of the most tolerant cereal crops, and dies after exposure to extended periods at salt concentrations higher than 250 mM NaCl (equivalent to 50% sea water) conditions under which only species of halophytes (plants adapted to saline habitats) can survive and continue to grow, for example sea barley grass *Hordeum marinum* and tall wheatgrass *Thinopyrum ponticum*. Despite the relative tolerance of barley to slat, it is important to develop new barley lines with enhanced tolerance in view of the increasing problems of soil salinity. Some plants have mechanisms to be salt tolerant by using specific features of salt tolerance (Table 1.6).

Table 1.6: Mechanisms of salinity tolerance, organized by plant processes and their relevance to the three components of salinity tolerance.*

Process involved	Candidate genes	Osmotic stress	Ionic stress	
		Osmotic tolerance	Na^+ exclusion	Tissue tolerance
Sensing and signaling in roots	SOS3, SnRKs	Modification of long-distance signaling	Control of net ion transport to shoot	Control of vacuolar loading
Shoot growth	?	Decreased inhibition of cell expansion and lateral bud development	Not applicable	Delay in premature senescence of old (carbon source) leaves
Photosynthesis	ERA1, PP2C, AAPK, PKS3	Decreased stomatal closure	Avoidance of ion toxicity in chloroplasts	Delay in ion toxicity in chloroplasts
Accumulation of Na^+ in shoots	HKT, SOS1	Increased osmotic adjustment	Reduced long distance transport of Na^+	Reduced energy spent on Na^+ exclusion
Accumulation of Na^+ in vacuoles	NHX, AVP	Increased osmotic adjustment	Increased sequestration of Na^+ intro root vacuoles	Increased sequestration of Na^+ into leaf vacuoles
Accumulation of organic solutes	P5CS, OTS MT1D, M6PR S6PDH, IMT1	Increased osmotic adjustment	Alteration of transport processes to reduce Na^+ accumulation	Accumulation of high concentrations of compatible solutes in cytoplasm

*Munns and Tester 2008.

There are two main types of plant tolerance mechanisms, first, salt exclusion which mean minimizing the entry of salt into the roots of plant, for example the barley genotype (clipper) is able to exclude 94% of Na^+ and 91% of Cl^- when grown at 50 mM

NaCl (Munns 2005). Second, tissue tolerance which means minimizing the concentration of salt in the cytoplasm. Halophytes have both types of mechanisms, they exclude salt well and the cell can compartmentalize the salt in vacuoles, these mechanisms allow them to grow for long periods of time in saline soil. Roots of plant must exclude most of the salt in the soil solution or in time the salt will gradually build up in the shoot and become so high that it kill the leaves. To prevent salt building up in the shoot, roots should exclude 98% of the salt in the soil solution, allowing only 2% to be moved through the xylem to the shoot (Munns *et al*. 2006).

The salt arriving in leaves gradually builds up with time especially in older leaves. The salt concentration eventually becomes high enough to damage the cells, unless they can compartmentalize the salt in vacuoles, thereby the cytoplasm can be protected from the ions' toxicity (Munns *et al*. 2006, Munns and Tester 2008).

1.6 Mitogen activated protein kinase (MAPK) cascades

1.6.1 Background and historical review

Mitogen activated protein kinases (MAPK) are found in yeast, mammals and plants, in fact, all higher eukaryotes share about 60% of their genes with each other, including components of MAPKs (Nakagami *et al*. 2005). The first MAPK protein, called serine/ threonine kinase (MAP-2) was isolated from insulin treated 3T3-L1 mammalian cells by Sturgill and Ray in (1986), who later carried out several experiments to investigate its properties. They demonstrate this protein had the ability to phosphorylate microtubule associated protein-2 (MAP-2) and ribosomal protein S6 kinase II in vitro (Sturgill and Ray 1986, Ray and Sturgill 1987, 1988). Instead of the acronym "MAP" from "microtubule-associated protein" the term "MAP Kinase" from "mitogen-activated protein kinase" as it is now known was proposed by Rossomando *et al*. (1989).

Plant MAPKs came to light at the beginning of 1990s when Duerr *et al*. (1993) reported the recovery of a full-length cDNA clone encoding a MAP kinase from alfalfa (*Medicago sativa*) which has been named *MsERK1*, followed by isolation of *MsK7* from alfalfa as well, which shows 52% identity to animal MAP kinases (Jonak *et al*. 1993), then *D5* from pea (*Brassica napus*) which is thought to be a MAP kinase homologue, it shares approximately 48 to 51% amino acid identity with MAP kinase genes from mammals, amphibians, and yeasts (Stafstrom *et al*. 1993). Both the Jonak group and the Stafstrom group mention their report as the first demonstration of MAP kinase genes

from plants despite the previous published report by Lawton *et al*. (1989), who reported the isolation and characterization of cDNAs from the dicot bean (*Phaseolus vulgaris* L.) and the monocot rice (*Oryzae sativa* L.) that encode protein-serine/threonine kinase homologs. That might be due to the weakness of communications prior the internet and the misnomer prior MAP kinase nomenclature system which has been suggested by Ichimura *et al*. (2002). Then later, the isolation of plants MAPKs increased rapidly to include several plant species, for example Arabidopsis, *Arabidopsis thaliana* (Nobuaki *et al*. 1993, Mizoguchi *et al*. 1993a, b), tobacco, *Nicotiana tabacum* (Wilson *et al*. 1993), oat, *Avena sativa* (Huttly and Phillips 1995), maize, *Zea mays* (Hardin and Wolniak 1998), rice, *Oryza sativa* (He *et al*. 1999) wheat, *Triticum aestivum* (Takezawa 1999).

1.6.2 The components and classification of plant MAPK cascades

The MAP kinases are components of a linear cascade comprising a triple kinase module: MAPKK kinases (MAPKKKs), MAPK kinases (MAPKKs also known as MEKs), and MAPKs, those are linked in several ways to upstream receptors and downstream targets. In all plant species, MAPKs carry either a Thr-Glu-Tyr (TEY) or Thr-Asp-Tyr (TDY) phosphorylation motif at the activation site (Nakagami *et al*. 2005, Mishra *et al*. 2006).MAP kinase kinase kinase (MAPKKK) phosphorylates and activates MAP kinase kinase (MAPKK), which in turn activates the terminal MAP. The activation of MAPKK is regulated by phosphorylation on two conserved serine or threonine residues by MAPKKK (Mishra *et al*. 2006). Fig. 1.1 showed the set of three functionally interlinked protein kinases forms the basic module of a MAPK signal transduction pathway.

Eukaryotic cells use MAP kinase signal transduction cascades to deliver extracellular messages to the cytosol and nucleus, this signalling is involved in cell division, cellular differentiation, metabolism, and both biotic and abiotic stress responses (Morris 2001, Hirt 1997). Agrawal *et al*. (2003a,c) conducted several experiments on rice MAPKs genes such as *OsBWMK1*, *OsMSRMK3* and *OsWJUMK1* in response to diverse environmental stresses and suggested that MAPKs regulate a wide range of plant cellular activities from growth and development to cell death.

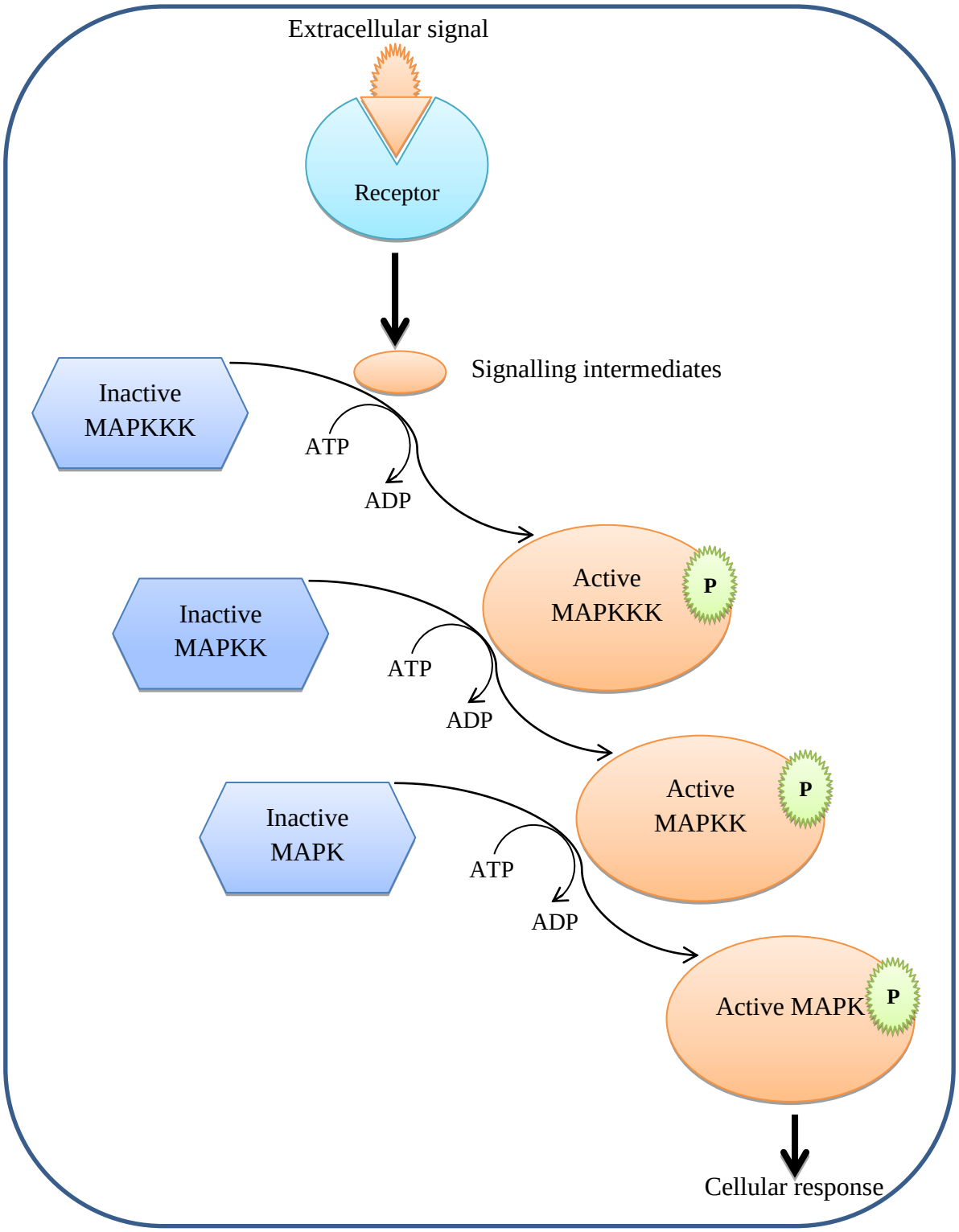


Figure 1.1: Illustrates MAP kinase signal transduction pathway.

An extracellular signal is received by a membrane located receptor. Several intermediate steps occure to activate the MAP kinase module (MAPKKK) which in turn allow the activation of other protein kinases, catalyse the phosphorylation of cytoskeletal components or the active MAP kinase translocated to the nucleus and activate transcription factors giving rise to the expression of target genes.

Since the first MAPKs isolated in 1986, researchers and scientists around the world identified and characterized many genes encoding MAP kinases and other upstream cascade components in plants (Morris 2001, Wei-Wei *et al*. 2012). For example, the Arabidopsis genome contains approximately 80 MAPKKKs, 10 MAPKKs and 20 MAPKs (Sinha *et al*. 2011), as yet, 16 (*SlMAPK*) genes from the tomato genome have been identified (Kong *et al*. 2012), in wheat a total of 15 MAPKs genes were identified (Wei-wei *et al*. 2012), and 17 MAPKs genes in rice have been identified and analyzed (Reyna and Yang 2006) in addition to 75 MAPKKK genes that have been identified in rice by Rao *et al*. (2010) using *in silico* analysis. Nicole *et al*. (2006) provide a complete survey of 10 MAPKK and 21 MAPK gene expression profiles in poplar (*Populus trichocarpa*), a large woody perennial tree. A total of 16 MAPK genes and 12 MAPKK genes were identified from purple false brome (*Brachypodium distachyon*) by database searches and they were classified according to their homology with known MAPK and MAPKK genes in Arabidopsis or rice, then classified into four groups A, B, C and D (Chen *et al*. 2012). 17 tobacco (*Nicotiana tabacum*) MAPKs were identified (Zhang *et al*. 2013).

Phylogenetic analysis based on amino acid sequence has attempted to classify plant MAP kinases into families. Thus Ichimura *et al*. (2002), Hamel *et al*. (2006) and Liu and Xue (2007) divided plant MAPK members into four distinct families (A, B, C and D) but in contrast, Cheong *et al*. (2003) grouped the plant MAPKs into five distinct families (I, II, III, IV and V) whereas Reyna and Yang (2006), Rohila and Yang (2007) divided the rice MAPK family into six major groups, A, B, C, D, E and F. Recently Wei-Wei *et al*. (2012) analysed 20 *AtMPKs*, 15 *TaMAPKs* and 17 *OsMPKs* and divided these into seven groups (A, B, C, D, E, F, and G). In a different approach, phylogenic analysis of the 16 *SlMAPKs* from tomato (*Solanum lycopersicum*) and 20 *AtMAPK*s from Arabidopsis indicated that the tomato *SlMAPK* genes were clustered into four major groups A, B, C and D (Kong *et al*. 2012). In order to divide 17 tobacco MAPKs to families, the amino acids sequences of MAPKs from four plants (Arabidopsis, tobacco, rice, poplar) were aligned using Clustal W program, resulted to six families A, B, C, D, E and F (Zhang *et al*. 2013).

1.6.3 The role of MAPKs in plant stresses response signalling

Activation of MAPKs has been observed in plants exposed to a variety of biotic and abiotic stresses such as pathogen attack, hormones, wounding, cold, salinity, drought,

oxidative stress and sugar starvation, etc. (He *et al*. 1999, Agrawal *et al*. 2003a, 2003c, Jeong *et al*. 2006, Ortiz-Masia *et al*. 2007, and Pan *et al*. 2012). There is a significant amount of evidence to show MAP kinase pathways in cereals are associated with abiotic stress resistance and that modulation of MAP kinase (or MAPK pathway) gene expression can led to enhanced resistance to abiotic stress.

For example, wild type maize seedlings were cultured in 250 mM NaCl, after 5 hour the expression level of *ZmSIMK* with treatment of NaCl was higher than untreated control (Wu *et al*. 2011). In transgenic rice plants, *OsMAPK5* overexpression lines exhibited increased specific kinase activity which was associated with increased tolerance to drought, salt, and cold stresses whereas the *OsMAPK5* suppression lines had significant reductions in drought, salt, and cold tolerance, interestingly these suppression lines significantly enhanced resistance to the pathogenicity of the fungus *Magnaporthe grisea* and the bacterium *Burkholderia glumae* (Xiong and Yang 2003). Under salinity stress of 250 mM NaCl for a week, overexpression of *OsMAPK44* (as a single copy) in transgenic rice plants showed less damage and greater ratio of potassium to sodium compared with *OsMAPK44* suppressed transgenic lines (Jeong *et al*. 2006). In the same way Lee *et al*. (2011) investigated the role of *OsMAPK33* in transgenic rice under drought and salinity stress, the suppressed lines did not display any significant difference in drought and salinity tolerance compared with wild type plants, whereas the overexpressing lines displayed greater reduction in biomass accumulation and higher sodium uptake into cells, compared with wild type and suppressed lines. Transgenic *Arabidopsis* carrying an overexpression construct for *ZmSIMK1* (*Zea mays* salt-induced mitogen-activated protein kinase 1) exhibited an increase in salt tolerance compared with wild type during the emergence of the coleoptile and demonstrated continued growth of the seedling when stressed by 200 mM NaCl (Gu *et al*. 2010). In respect of the drought stress, tobacco MAPKKK (*NPK1*) was over-expressed in transgenic maize leading to improved drought tolerance, under drought conditions transgenic maize plants maintained significantly higher photosynthesis rates and kernel weight than the non-transgenic plants (Shou *et al*. 2004). Furthermore, in similar experiments using the same gene as above, transgenic maize maintained a higher growth and showed increased tolerance to drought stress conditions as compared with the non-transgenic control under mannitol osmotic stress (Assem *et al*. 2009). When *DSM1* (drought-hypersensitive mutant1), a putative MAPK kinase kinase (MAPKKK) gene, was over-expressed in transgenic rice it resulted in increasing the tolerance of the seedlings to

dehydration stress (Ning *et al*. 2010). In a study on the maize *ZmMPK4* gene conducted by Zhou *et al*. (2012), it was reported that the overexpression of this gene in transgenic tobacco resulted in increased tolerance to low temperature stress. Overexpression of maize *ZmMKK3* gene conferred tolerance to osmotic and oxidative stresses and alleviated ABA sensitivity in transgenic tobacco plants, where the transgenic lines showed a significantly higher germination rate, accumulated a much lower level of H_2O_2 under osmotic stress condition and more chlorophyll accumulation under oxidative stress compared with wild type. Seed germination of these plants on MS medium supplemented with 10 μM abscisic acid (ABA) resulted in only 50% of the WT seeds germinating, but more than 78% of the transgenic plants seeds germinated (Zhang *et al*. 2012). Overexpression of the maize *ZmMPK17* gene in transgenic tobacco accumulated fewer reactive oxygen species under osmotic stress by affecting antioxidant defence systems. Furthermore these tobacco plants exhibited enhanced tolerance to cold with increased germination rates, proline and soluble sugar levels compared with wild type plants (Pan *et al*. 2012). In another study on one of the maize MAP kinase kinase genes, the researchers group reported that overexpression of *ZmMKK4* confers osmotic stress tolerance by scavenging reactive oxygen species (ROS) in transgenic tobacco plants. Also, the germination rates of *ZmMKK4*-overexpressing lines were significantly higher than that of the wild type, approximately 86 and 20% germination rates, respectively under osmotic stress (300 mM mannitol), the proline content was significantly higher in *ZmMKK4*-overexpressing plants, in addition the wild type plants showed more chlorosis and damage than the transgenic plants, furthermore the accumulation of reactive oxygen species (ROS) in *ZmMKK4*-overexpressing plants was much less than that of wild-type plants (Kong *et al*, 2011).

1.6.4 *BWMK1* gene

In rice (*Oryza sativa*), *OsBWMK1* (Accession No. AF177392) was the first MAPK to be discovered, it was isolated from rice Indica-type cultivar "IR36" after being treated either by infection with blast fungus *Magnaporthe grisea* or by mechanical wounding. Its name *OsBWMK1* is an acronym for Blast and Wound-induced MAP Kinase (He *et al*. 1999). *BWMK1* belongs to family D according to Ichimura *et al*. (2002), and Agrawal *et al*. (2003b) with a TDY motif at the activation site. Homologues are found among other plant species such as barley, goatgrass, maize, sorghum, wheat, alfalfa, soybean, and *Arabidopsis* (He *et al*. 1999). For example, in barley, Eckey *et al*. (2002)

found a gene in powdery mildew infected barley (Ingrid cultivar), they called it *PWMK1*, and it encodes a protein with 84% similarity to rice *OsBWMK1* with TDY in motif phosphorylation site and belongs to the group D of plant MAP kinases.

Many researchers from all over the world have made good progress in understanding the plant responses to abiotic stresses with respect to the role of *BWMK1* in different plants to detect its relationship to tolerance of biotic and abiotic stresses (Table 1.7). Hong *et al*. (2007) tested the response of *OsBWMK1* to several stresses, they fused the promoter of *BWMK1* with the coding region of the β-glucuronidase (*GUS*) reporter gene and transformed into rice, expression of *GUS* was induced in the transgenic rice by cold, drought, dark, and JA. Whereas light, SA, and BTH treatments suppressed *GUS* expression, which demonstrate that *BWMK1* is responsive to multiple abiotic stresses and environmental changes and may play a role in cross-talk between different signalling pathways. Agrawal *et al*. (2003c), investigated *OsBWMK1* gene expression in responce to diverse biotic and abiotic stresses and they found that *OsBWMK1* expression was strongly enhanced and enzyme activity was transiently regulated by wounding, gases produced by environmental stress (O_3 and SO_2), fungal elicitor, protein phosphatase inhibitors, heavy metals, high salt and sucrose, and drought. Cheong *et al*. (2003) reported that the expression of *BWMK1* was rapidly and transiently activated by several defense signals, including a fungal elicitor, hydrogen peroxide (H_2O_2), salicylic acid (SA), jasmonic acid (JA), and ethephon (which breaks down in the cell to release ethylene), in addition they reported that overexpression of *OsBWMK1* in transgenic tobacco plants enhanced resistance against fungal and bacterial infections, and finally concluded that overexpression of *BWMK1* enhances PR gene expression in the plant. Eckey *et al*. (2002) reported that the barley gene *PWMK1* (homologue to *OsBWMK1*) respond to inoculation with the biotrophic fungal pathogen *Blumeria graminis* f.sp. *hordei* and wounding but was not affected by cold stress of 5 °C, also they reported that the *PWMK1* knockdown resulted in higher fungal penetration efficiency.

Table 1.7: Stress-responsive plant *BWMK1* genes.

Gene	Plant	Stress	Reference
OsBWMK1	Rice	Fungal elicitor, Wounding	He *et al*. 1999
OsBWMK1	Rice	Wounding, JA, SO_2, H_2O_2, SA, Ethylene, sucrose, heavy metals Fungal elicitor, drought, UV, O_3 protein phosphatase, salt	Agrawal *et al*. 2003c
OsBWMK1	Rice, Tobacco	Fungal elicitor, SA, JA, H_2O_2 Ethephon	Cheong *et al*. 2003
promoter of *OsBWMK1*	Rice	Fungal elicitor, SA, BTH, JA Light and dark, Temperature Drought	Hong *et al*. 2007
OsBWMK1 splicing	Rice (CS)	Fungal elicitor from *M. grisea*, H_2O_2, JA, SA, NaCl.	Koo *et al*. 2007
HvBWMK1	barley	β-Glucosyl Yariv reagent, JA	Mashiguchi *et al*. 2008
OsBWMK1	Rice (CS) Tobacco (T) Arabidopsis (P)	SA, JA, H_2O_2	Koo *et al*. 2009

Cells suspension (CS), transgenic (T), protoplast (P), Jasmonic acid (JA), Salicylic acid (SA), Benzothiadiazole (BTH), Ozone (O_3) Hydrogen peroxide (H_2O_2), sulphur dioxide (SO_2), Ultraviolet irradiation (UV).

1.7 The criteria for assessing salinity tolerance of the transgenic plants

The harmful impacts of salinity on plants are one of the major challenges facing farmers around the globe. The effects of salinity on plant growth are caused by high osmotic potential of the soil solution (water stress), nutritional imbalance, specific ion effects (salt stress), or a combination of these factors (Ashraf and Harris 2004).

The criteria used to judge impact are grouped in several groups such as agronomic, physiological and biochemical. The agronomic criteria comprise the fresh weight, dry weight, the height of shoot, the length of root, survival percentage, leaf area, leaf injury, leaf expansion, relative growth rate, time of flowering and the weight of yield (Verslues *et al*. 2006). The physiological criteria included chlorophyll content, relative water content, photosynthesis, stomatal conductance, K^+/Na^+ and $Ca^{2+/}Na^+$ ratios and accumulation of solutes (Ashraf 2004, Verslues *et al*. 2006). The biochemical parameters include accumulation of soluble sugars, soluble proteins, amino acids, the quaternary ammonium compounds, polyamines, polyols, hydrogen peroxide (H_2O_2) and activity of ATPases (Ashraf and Harris 2004). These criteria have been used widely for long time to assess the new varieties in plants breeding or segregation between cultivated varieties. The following clarify some of that.

1.7.1 The agronomic criteria

The agronomic criteria which were used in this study comprise the fresh weight, dry weight, the height of shoot and the weight of 1000 seeds. The agronomic traits reflect the result of combination between genetic and environmental effects on plant growth. Campo *et al*. 2012 used some growth parameters such as mean height, seed production, number of spikes, and weight of 200 seeds to compare drought tolerance of wild type and transgenic rice carrying empty vector, or expressing the maize *ZmGF14-6* gene. In other experiment, 150 mM NaCl salt stress was imposed to evaluate and screen three wheat varieties ($186F_8$, Superhead#2 and Roshan) for salinity tolerance, resulted in a significant negative correlation between shoot dry matter and sodium concentration but in contrast, a significant positive correlations between shoot dry matter and K^+/Na^+ ratio (Azadi *et al*. 2011). It was also reported that two barley cultivars which differ in salinity tolerance, Clipper and Sahara, were grown hydroponically under conditions of high salinity (100 mM NaCl) for 6 weeks, and analysed by measuring dry weight, root length, and shoot height, Clipper was severely affected, having a growth reduction of up to 58% of dry weight compared with 14% in Sahara (Widodo *et al*. 2009). Similarly, the comparisons of two barley cultivars that differ in salt sensitivity resulted in that the salt stress of 100 mM NaCl lead to growth reduction by 40% in salt-sensitive cultivar (K305) and 15% in salt-tolerant cultivar (I743) (Ligaba and Katsuhara 2010). To compare the salt tolerance of two rice hybrid varieties (Shanyou 10 and Liangyoupeijiu), ten day old seedlings were treated with 200 mM NaCl, the length and

dry weight of shoots and roots were measured. The result revealed that the roots and shoots of Shanyou 10 were longer and heavier than those of Liangyoupeijiu (Ruan *et al*. 2011). In order to screening thirteen genotypes of rice, seedlings were grown on MS medium supplemented with 200 mM NaCl (salt stress) for 14 days. Growth parameters, including shoot height, root length, fresh weight, dry weight and leaf area in salt stressed plantlets of all genotypes were significantly inhibited (Cha-um *et al*. 2010). In another experiment to compare two wheat cultivars, a salt tolerant one and a salt sensitive one, showed that salt stress significantly reduced shoot and root dry mass of both wheat cultivars, however this decrease was more clear in sensitive cultivar (Ashraf *et al*. 2012). Also in another experiment to evaluate six cultivars of maize under 100 mM NaCl salt stress, the results indicate that salinity decreased shoot and dry weight in all cultivars compare with the controls (Carpici *et al*. 2010).

1.7.2 The physiological criteria

Visible plant characteristics and growth parameters are not enough for plant line screening, which make it necessary to combine this with physiological ones. Mostly, the physiological criteria display more reliable information than agronomic characters (Ashraf 2004). It was reported that chlorophyll content and relative water content (RWC) are good indexes for screening bread wheat genotypes for salinity tolerance (Azadi *et al*. 2011). Both relative water content and chlorophyll content were used in this study.

1.7.2.1 Relative water content (RWC)

RWC, in the mid 80s, was considered as the best parameter to measure the plant water status, and later was used widely instead of plant water potential. It is an accurate method of measuring the amount of absorbed water by plant which available in leaves and that consumed through transpiration (cited in Arjenaki *et al*. 2012).

El-Tayeb (2005) used RWC and several other physiological parameters such as the germination percentage, electrolyte leakage, lipid peroxidation, free amino acids and proline and the photosynthetic pigments, to investigate the response of barley (*Hordeum vulgare* cv Gerbel) to salinity (0, 50, 100, 150 and 200 mM NaCl) and found that RWC decreased with increasing NaCl concentration. In order to evaluate 6 wheat resistant varieties and 3 susceptible varieties, drought stress was applied by withholding water at anthesis stage, the results showed that RWC was different between the resistant and

susceptible varieties, being higher in the resistant varieties (Arjenaki *et al*. 2012). Similarly RWC with several parameters such as visual assessment, cell membrane stability, stomatal conductance, lipid peroxidation and lipoxygenase activity, water use efficiency, leaf chlorophyll content and antioxidant activity were able to evaluate and screen 174 landraces of oat under drought stress (Sanchez-Martin *et al*. 2012). It was also reported by El-Bassiouny and Bekheta (2005) that salt stress decreased the RWC of two Egyptian wheat cultivars, the effect increased with salinity level.

1.7.2.2 Chlorophyll

Chlorophyll is a green pigment found in high concentrations in chloroplasts of plant cells, that gives leaves their green colour. Four types of chlorophyll are described, chlorophyll (a), (b), (c), and (d). All green plants contain at least one of these forms. Chlorophyll is the main molecule responsible for absorbing sunlight that represents a critical step of photosynthesis. So the chlorophyll levels into plant leaves are considered a good indicator of the photosynthetic function under changing environmental circumstances. For example, Belkhodja *et al*. (1994) carried out an experiment on barley to successfully investigate the possibility of using chlorophyll fluorescence as a tool to screening barley genotype for salinity tolerance.

Salt stress generally causes chlorophyll levels to decrease in salt sensitive varieties and increase in salt tolerant varities. It was observed that chlorophyll content of 94 rice genotypes was differently affected by 7 days of salt stress (EC of 12 dSm^{-1}), where the mean total chlorophyll content decreased in 34 genotypes which were considered as a salt sensitive and increased in 60 genotypes which were considered as a salt tolerant (Kanawapee *et al*. 2012).

In a study to evaluate and screen the inbred wheat line (186F8) and its parents, Superhead#2 (salt sensitive variety) and Roshan (salt tolerance cultivar), chlorophyll content decreased in response to increasing concentrations of NaCl to 150 mM, but the chlorophyll was lower in the salt sensitive variety than the salt tolerant cultivar (Azadi *et al*. 2011). In another experiment to compare two wheat cultivars, a salt tolerant one and moderately salt sensitive, showed that higher levels of salts in the growth medium resulted in a marked decline in chlorophyll (a) and (b) at different growth stages in both wheat cultivars, chlorophyll (a) and (b) were lower in sensitive cultivar (Ashraf and Ashraf 2012). Also in an evaluation of six cultivars of maize under 100 mM NaCl salt

stress, salinity caused a decrease in total chlorophyll of all cultivars except two as compared with the controls, the decrease was more apparent in the salt sensitive genotypes than in tolerant ones (Carpici *et al*. 2010). In a similar way, salt stress (150 mM NaCl) was imposed on barley seedlings for 4 days, the chlorophyll (a) content in stressed seedlings was slightly increased whereas chlorophyll (b) and total chlorophyll were slightly decreased in stressed seedlings compared with controls (Fedina *et al*. 2002). In terms of increased plant tolerance to salinity by genetic transformation, the salt tolerance-related gene (*TaSTRG*) from wheat was transformed into rice, under salt stress the chlorophyll content was higher in leaves of the *TaSTRG* transgenic rice lines than in those of the control plants (Zhou *et al*. 2009).

1.7.3 The chemical criteria

1.7.3.1 Hydrogen peroxide H_2O_2

Abiotic stresses including salt-stress can increase the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). Low concentrations of endogenous H_2O_2 act as a stimulant of tolerance against several abiotic and biotic stresses (Fedina *et al*. 2009, Bhattacharjee 2012). It has been shown for barley that low concentration of exogenous H_2O_2 removed the inhibition of germination, the treatment of barley seeds with 30 mM H_2O_2 surpassed by the 2^{nd} day, values of the 7^{th} day final germination percentages of the control seeds (Çavusoglu and Kabar 2010). At high intracellular concentrations H_2O_2 causes oxidative stress which leads to programmed cell death (Fedina *et al*. 2009, Bhattacharjee 2012), this concentration differ from species to another, for example above 50 mM, exogenous H_2O_2 induces high necrotic cell death in tobacco BY-2 cells (Houot *et al*. 2001). Plants generally have the ability to alleviate the oxidative damage caused by ROS by using different antioxidants and ROS scavengers, which therefore enhance plant resistance to salinity stress (Ashraf and Harris 2004, Vinocur and Altman 2005).

It was reported that under normal conditions, H_2O_2 content of *OsCYP2*-transgenic rice was lower than in wild type, but after 24 h of treatment with 200 mM NaCl, H_2O_2 content decreased in both transgenic and wild type, but was still lower in transgenic compared with wild type, *OsCYP2* is a salt-induced rice cyclophilin gene (Ruan *et al*. 2011). In another experiment to compare two wheat cultivars, (a salt tolerance one and a salt sensitive one), this showed that 150 mM NaCl salt stress decrease accumulation

of H_2O_2 in both cultivars at the vegetative stage, the salt sensitive cultivar MH-97 had greater H_2O_2 accumulation than the salt tolerance cultivar S-24 (Ashraf *et al*. 2012).

1.7.3.2 Proline

Proline is one of the 20 known amino acids which make up proteins. It often found in higher plants accumulating in larger amounts than any other amino acids. Proline accumulation is a common physiological response, occuring in plants after various stresses such as salt, drought, high or low temperature, heavy metal, pathogen infection, anaerobiosis, nutrient deficiency, atmospheric pollution and UV irradiation (Verbruggen and Hermans 2008, Szabados and Savoure 2009). The amounts of proline in plants tissues are used as a physiological indicator to study the plant response to abiotic stresses.

The concentrations of proline in the leaves of 94 genotype of rice were measured after 7 days of salt stress (EC of 12 dSm^{-1}), the proline content in the stressed seedlings increased in all genotypes. Proline in non-stressed seedlings varied from 69 to 557 µg/gFW whereas it increased in stressed seedlings and ranged from 116 to 2006 µg/gFW (Kanawapee *et al*. 2012). In another experiment to compare two wheat cultivars, one salt tolerant and one salt sensitive, it was shown that 150 mM NaCl salt stress caused enhanced accumulation of proline in both wheat cultivars, this increase was considerably higher in salt tolerant cultivar S-24 than that in salt sensitive cultivar MH-97 (Ashraf *et al*. 2012). In work that evaluated and screened six cultivars of maize under 100 mM NaCl salt stress, salinity increased proline content in all cultivars except one compared with the controls, there were great differences in the increased proline contents of cultivars (Carpici *et al*. 2010). In term of increase plant tolerance to salinity by genetic transformation, a salt tolerance-related gene which increases plant tolerance to salt stress (*TaSTRG*) from wheat was transformed into rice; under salt stress (175 mM NaCl for 7 day) the three *TaSTRG*-transgenic lines accumulated approximately 1.8-2.2 times higher proline contents than the control, in addition the expression level of proline synthetase gene in the *TaSTRG* transgenic lines was 2-3 fold higher than that in the control plants (Zhou *et al*. 2009).

1.7.3.3 Metabolomics

Metabolomics refer to a new field of scientific study in particular the chemical processes involving metabolites. The terminology is derived from the new style of

"omics" terminology such as proteomics and genomics. Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" - specifically, the study of their small-molecule metabolite profiles (Tyagi, *et al*. 2010, Nanda *et al*. 2011). Metabolites are the small intermediates and end-products molecules (less than 1 kDa in size) of metabolic processes. It is estimated that plants produce more than 100,000 secondary metabolites (Bhalla *et al*. 2005).

Using GC-MS, the most popular metabolomics technique, makes it possible to determine the level of several hundred compounds belonging to diverse chemical classes including sugars, organic acids, amino acids, sugar alcohols, aromatic amines and fatty acids (Shulaev 2006, Saito and Matsuda 2010). GC-MS is able to identify at the same time around 50-150 of known metabolites from the same small amount of sample (Zuther et al. 2007). The main goal is getting unbiased identification and quantification for the most metabolites in a sample from an organism grown under particular conditions (Bhalla *et al*. 2005).

Metabolites play a key role in the evaluation of transgenic plants and response of plants to various biotic and abiotic stresses (Shulaev *et al*. 2008, Obata and Fernie 2012). Several metabolomics studies on plants response to abiotic stresses have been reported, salt stress, tomato (Johnson *et al*. 2003), *Arabidopsis thaliana* (Kim *et al*. 2007), *Thellungiella halophila* and *Arabidopsis thaliana* (Gong *et al*. 2005), grapevine (Cramer *et al*. 2007), Poplar: *Populus euphratica* (Brosché *et al*. 2005), halophytic grass: *Aeluropus lagopoides* (Sobhanian *et al*. 2010), water stress, Ryegrass: *Lolium perenne* (Foito *et al*. 2009).

In order to reveal the difference of metabolic profiles between XZ16 salt tolerant Tibetan wild barley (*Hordeum spontaneum*) and CM72 cultivated barley (*Hordeum vulgare*), an experiment was conducted under salt stress and normal conditions, profiles of 82 key metabolites were investigated in both barley cultivars. Under normal condition 16 and 10 metabolites showing significantly higher and lower contents in leaves of XZ16 than those in CM72, respectively, while under salt stress, 23 and 32 metabolites in leaves of CM72, and 20 and 34 metabolites in leaves of XZ16 showed significant up-accumulation and down-accumulation, respectively (Wu *et al*. 2013). Metabolomics analysis was carried out on two barley cultivars which differ in salinity tolerance, Clipper and Sahara and were grown hydroponically under conditions of high salinity (100 mM NaCl); the Clipper plants had elevated levels of amino acids,

including proline and γ-aminobutyric acid (GABA), and the polyamine putrescine whereas in the more tolerant Sahara plants, the levels of the hexose phosphates, TCA cycle intermediates, and metabolites involved in cellular protection increased in response to salt (Widodo *et al*. 2009).

1.8 Aims and objectives of the research

The main aim of the research reported here is to investigate the role of MAPK pathways to salt tolerance in barley. In particular, the barley homologue of the rice MAPK called *BWMK1* will be investigated as this MAPK has been demonstrated to be involved in mediating intracellular signalling in response to biotic and abiotic stress (He *et al*. 1999, Agrawal *et al*. 2003c, Cheong *et al*. 2003, Koo *et al*. 2007, 2009).

The objectives of this study were focused on identifying specific MAP kinase genes in barley, giving names to those genes according to MAP kinase nomenclature system which has been suggested by Ichimura *et al*. (2002) and the homology to the previously named and characterised rice MAP knases.

To achieve the main aim, the barley gene *HvBWMK1* (Blast and Wound-induced MAP Kinase) was choosen to carry out this study in order to focus on its role in barley tolerance to salinity stress. Two constructions of T-DNA plasmid binary vector were designed to create transgenic barley alongside with empty binary vector (pWBVec.8) as a control, the first construction to overexpress the *HvBWMK1* gene to obtain high amount of *HvBWMK1* protein whereas the second construction was designed to silencing the endogenous *HvBWMK1*, therefore decrease the amount of *HvBWMK1* protein. Hygromycin resistance gene (hygromycin phosphotranferase, *hpt*) inserted in T-DNA region was used as a selectable marker for transgenic plants. The *Agrobacterium*-mediated transformation method was used to transinfect immature embryos of barley cultivar GoldenPromise to generate transgenic barley plants.

The generated transgenic barley plants (empty binary vector pWBVec.8 lines, *HvBWMK1* antisense and *HvBWMK1* overexpression) alongside wild type were exposed to salinity stress for particular time then the response of these plants was investigated. Several selection criteria comprise agronomic, physiological, biochemical and genetic criteria were measured and analysed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The suppliers of chemicals, reagents, enzymes, antibiotic, kits and other material used in this work are shown in Table 2.1.

Table 2.1: The suppliers of chemicals, enzymes, and other materials.

Suppliers	Chemical, reagents, enzymes and kits
GE Healthcare Life Sciences Little Chalfont, UK	Hyberdisation transfer nylon membrane
Eurofins MWG operon Ebersberg, Germany	PCR primers
Fisher scientific Leicestershire, UK	Filter papers, bulk chemicals
Fermentas Life Science	First strand cDNA synthesis kit, restriction endonucleases, T4 DNA ligase, λ *Hin*dIII DNA ladder and high range RNA ladder.
Melford Ipswich, UK	LB broth, Agar, Agarose, X-gal, Murashige and Skoog medium.
Primer Design	2X precision^{TM} Mastermix kit.
QIAGEN West Sussex, UK	Gel extraction kit for DNA
Roche Mannhein, Germany	DIG Northern starter kit, chemicals, Anti-Digoxigenin-AP, DIG RNA labelling mix, Nylon membranes positively charged.
Sigma-Aldrich Dorset, UK	Kodak X-ray film, Kodak developer and fixer solution, bulk chemicals.
Whatman Maidstone, UK	Sterile membrane filters, filter papers

Manufacturer and supplier's instructions and recommendations were followed for handled and stored all enzymes, antibiotics, chemicals and other materials.

2.2 Bio-informatic database searching

The amino acid sequences from seven publicly available barley MAP kinase clones (obtained from the Barley Germplasm Centre, Okayma University, Japan) were compared with the amino acid sequences of the 17 rice MAP kinases (retrieved from the NCBI website) by using the Clustal W program in order to identify the barley homolog of the rice MAP kinase *OsBWMK1*, which was subsequently named *HvBWMK1*.

The 7 full-length cDNA barley clones encoding MAP kinase homologues were donated by Dr. Kazuhiro Sato.

2.3 Bacterial strains

Escherichia coli strain XL1-blue MRF (Stratagene) was used as the host cell to carry modified plasmids during plasmid construction, whereas *Agrobacterium tumefaciens* strain EHA105 was used as the host to transform T-DNA from the binary vector to the plant tissue culture cells.

2.3.1 Bacterial growth conditions

LB liquid medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl, pH 7, autoclaved at 121°C for 20 minutes) was used to grow bacteria for plasmid preparation. *E.coli* was cultured at 37 °C on the shaker at 200 rpm overnight, whereas *Agrobacterium tumefaciens* was grown at 28 °C on the shaker at 200 rpm for 2-3 days. Likewise, LB solid medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 1.5% (w/v) agar, pH 7, autoclaved at 121°C for 20 minutes) was used to grow *E.coli* at 37 °C overnight or *Agrobacterium tumefaciens* at 28 °C for 2-3 days, from frozen stock or after transformation.

2.4 White-blue screening method

In order to prepare template for RNA probe, the target gene was ligated into the multiple cloning site (thus interrupting the *lacZ* gene) in the pBluescript II KS+ plasmid (Fig. 2.4) which contains the T3 and T7 polymerase promoters in addition to M13 foreward and reverse primers, then transformed to *E. coli* competent cells. Plates containing LB solid medium were prepared, these were supplemented with the appropriate antibiotic, followed by spreading 25 µl of X-gal (100 mg/ml in DMSO) and 100 µl of 0.1M of IPTG over the surface of the plate and left opened at least for 30 min

in a laminar flow hood to dry prior to plating cells. The plates were incubated at 37 °C overnight, next day after white and blue colonies had grown, some white colonies were picked and put in tubes containing liquid LB/antibiotic medium, only one colony for each tube. These were incubated on 37 °C overnight and were used for carrying out DNA plasmid minipreps.

2.5 Plasmids

Four types of plasmids were used in this study as shown in Table 2.2, together with their size and the source. Plasmids maps are provided in Figs. 2.1, 2.2, 2.3 and 2.4.

Table 2.2: The types of plasmids, which were used.

plasmid	Size bp	Function	source
pBluescript (pflcIII)-flbafl80a19	4698	Used to carry cDNA fragment of barley MAP kinase gene sent from Japan.	Dr. Kazuhiro Sato Okayama University, Japan
pTopoActin-OCS	5810	Contain Actin promoter and OCS terminator. Used to join *HvBWMK1* with promoter and terminator.	Dr. Peter Morris, School of Life Sciences, HWU
pWBVec.8	11280	Contain T-DNA region. Used to transfer and integrate T-DNA into barley genome.	Dr. Ming-Bo Wang Cooperative research centre for plant science, Canberra, Australia
pBluescript II KS+	2961	Used for white blue screening method and cDNA synthesis.	Stratagene

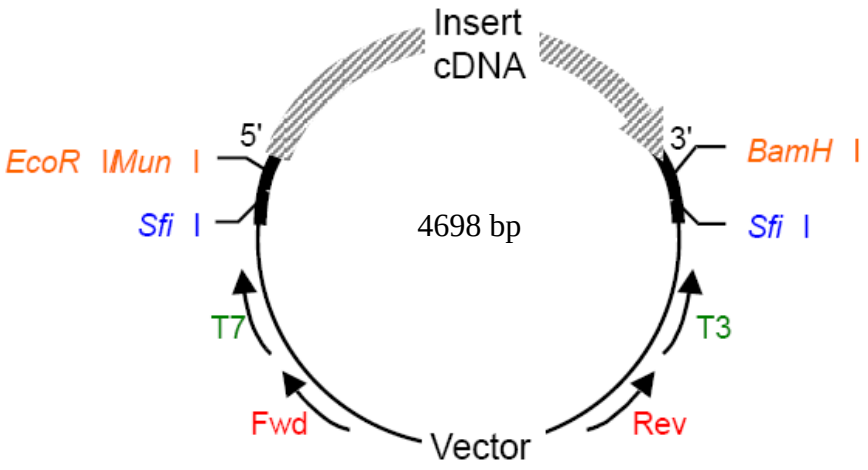


Figure 2.1: Modified pBluescript (pflc111)-flbafl80a19 plasmid map.

Insert cDNA: refers to one of 7 barley MAPkinases sent from japan.

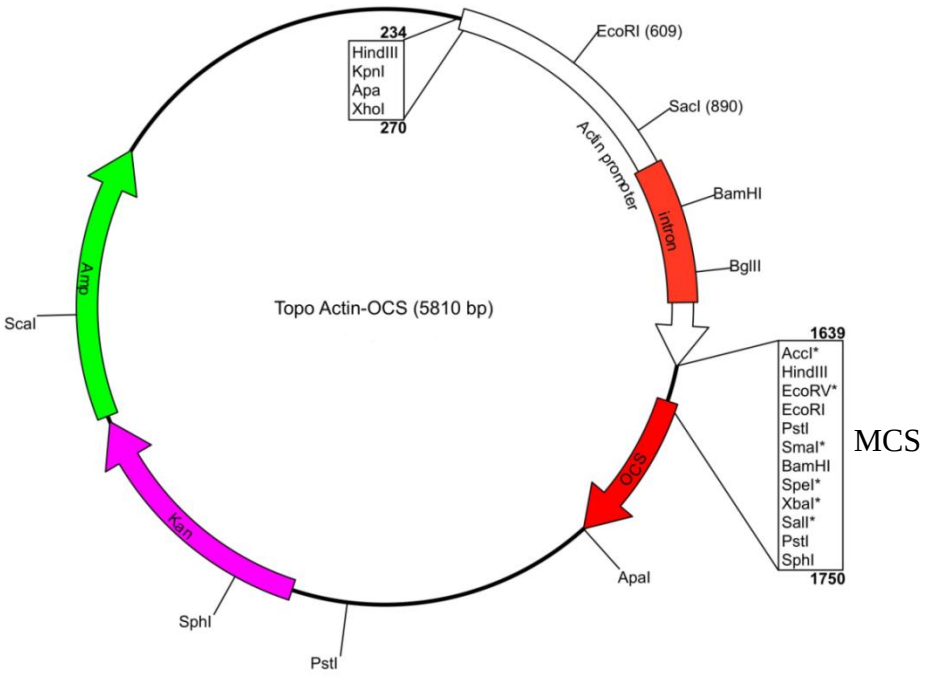


Figure 2.2: pTopo Actin-OCS plasmid map.

The plasmid contains:

Multiple cloning site (MCS) located between Actin promoter and OCS terminator.

*Apa*I restriction sites at the beginning of promoter and at the front of terminator.

Antibiotic genes: kanamycin (Kan) and ampicillin (Amp).

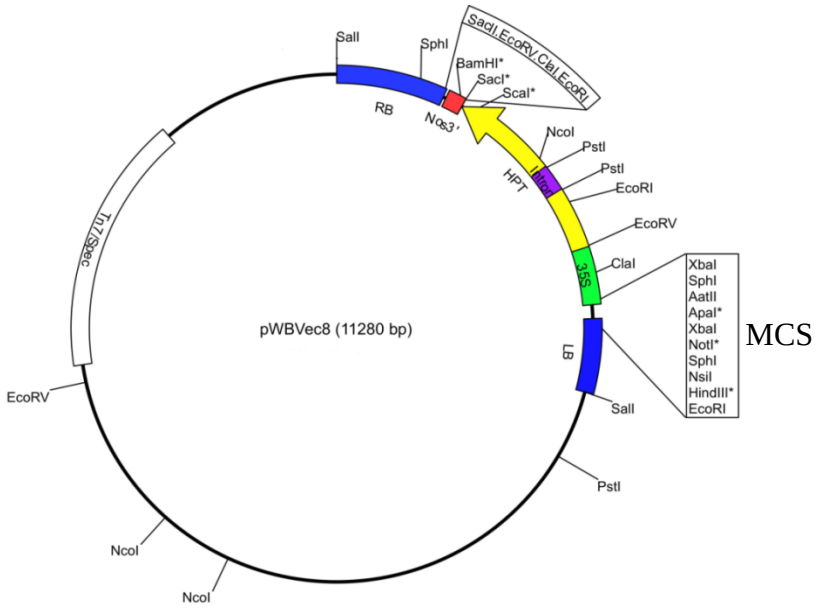


Figure 2.3: Binary vector pWBVec.8 plasmid map.

The plasmid contains:

T-DNA region flanked by right border (RB) and left border (LB) and contains multiple cloning site (MCS) and a hygromycin gene (HPT).

Antibiotic gene spectinomycin (Spec) is located on the plasmid backbone.

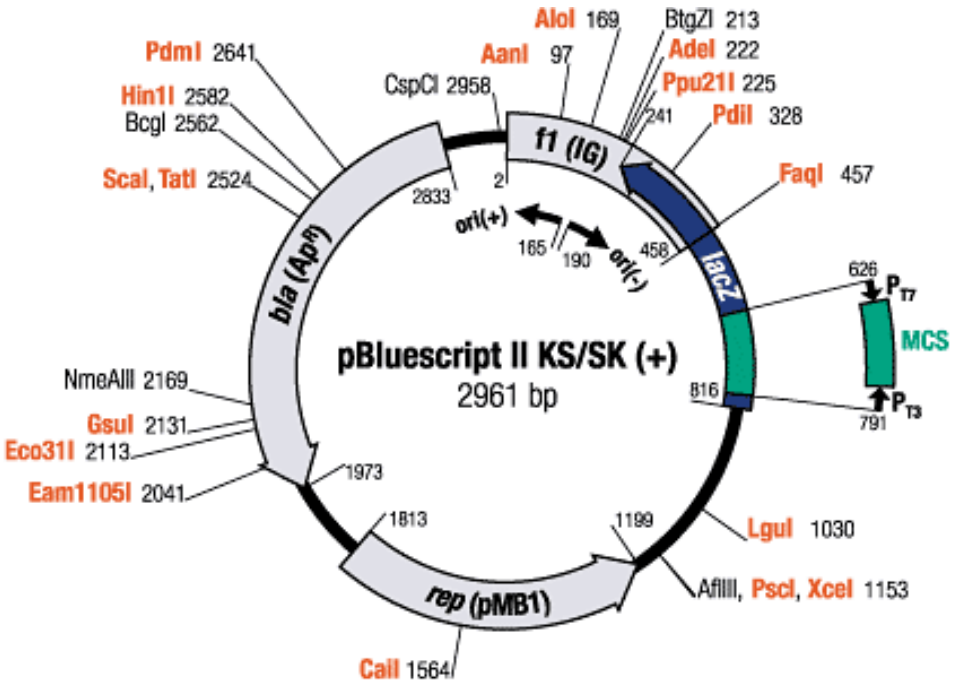


Figure 2.4: pBluescript II KS/SK+ plasmid map.

MCS: multiple cloning site flanked by forward and reverse M13 primers and reverse transcriptase primers T7 and T3 within the *lacZ* gene.

2.6 Antibiotics

Different types of antibiotic were used in this study for the selection of bacteria, transformed callus and the seeds of transformed barley plants. All antibiotics and their concentrations are detailed in Table 2.3.

Table 2.3: Antibiotics used in this work.

Antibiotic	Concentration mg/ l	Bacteria / plasmid
Ampicillin	50	pTopoActin-OCS pBluescript
Hygromycin	50 100	Transformed callus seeds of transformed barley
Kanamycin	50	pTopoActin-OCS
Rifampicin	25	*Agrobacterium tumefaciens*
Spectinomycin	50	pWBVec.8
Timentin	160	Transformed callus to remove *Agrobacterium*

2.7 Restriction enzyme digestion of DNA

In order to analyse genes from plant DNA, and to construct and analyse plasmid DNA, different endonuclease restriction enzymes were used in a total reaction volume of 10-20 µl depending on the concentration of undigested DNA, so that 5 units of enzyme was used to digest 1 µg of DNA with the appropriate 1 X buffer. The reactions were incubated at the appropriate incubation temperature for 1-4 hours. The restriction enzymes used in this study, their buffers, buffer composition, cleavage site, incubation temperature and thermal inactivation temperature are detailed in Table 2.4.

Table 2.4: Restriction enzymes, their cleavage sites, buffers, incubation temperature and thermal inactivation.

Enzyme	Fermentas buffer and composition	Cleavage site	Incubation temp. °C	Inactivation °C
*Apa*I	(Blue) 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.1mg/ml BSA	5´-GGGCC↓C-3´ 3´-C↑CCGGG-5´	30	65
*Eco*RV	(Red) 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/ml BSA	5´-GAT↓ATC-3´ 3´-CTA↑TAG-5´	37	80
*Pvu*II	(Green) 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 0.1 mg/ml BSA	5´-CAG↓CTG-3´ 3´-GTC↑GAC-5´	37	No
*Xba*I	(Tango) 33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66 mM K-acetate and 0.1 mg/ml BSA	5´-T↓CTAGA-3´ 3´-AGATC↑T-5´	37	85
*Xho*I	(Red) 10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/ml BSA	5´-C↓TCGAG-3´ 3´-GAGCT↑C-5´	37	80

2.8 Storage conditions

All DNA containing solutions (PCR products, plant genomic DNA, plasmid DNA and DIG-labelled DNA probes) were stored at -20 °C, whereas all the materials containing RNA or single strand DNA (RNA, DIG-labelled RNA probes and cDNA) were stored at -70 °C. The bacterial strains were also stored at -70 °C in liquid LB medium containing 15% (v/v) glycerol and the bacterial cultures on solid LB plate were stored at -4 °C.

2.9 Centrifugations

Small samples of solutions or bacterial cultures were centrifuged in 1.5 ml or 2 ml polypropylene microfuge tubes using Micro Centaur microfuge (MSE) at room temperature at the highest speed 13.000 rpm, unless otherwise stated. Larger volume samples were centrifuged in an Allegra™ X-12R, Beckman Coulter or Avanti® J-26 XP, Beckman Coulter centrifuges devices at the stated speeds and temperatures.

2.10 Images

The pictures of agarose gel for nucleic acids RNA and DNA were taken using a UVP gel documentation system from Sony, whereas the digital camera (10 MP Samsung) was used to take the pictures of the whole plants, seeds and callus.

2.11 Techniques for recombinant DNA

2.11.1 *E. coli* plasmid DNA mini-prep

The mini-prep method for isolating plasmid DNA from *E. coli* was carried out by a rapid alkaline extraction method for isolating plasmid DNA as described by Birnboim (1983).

A single colony of *E. coli* previously grown overnight on solid LB medium at 37 °C was inoculated into 5 ml of liquid LB medium with appropriate antibiotic and incubated overnight at 37 °C on a shaker at 200 rpm. 1.5 ml from an overnight culture in an Eppendorf tube was centrifuged for 1 minute at 13.000 rpm. The supernatant was decanted while the pellet was resuspended by vortexing in 100 µl GTE (50 mM glucose, 25 mM Tris-HCl and10 mM EDTA, pH 8) with 1 µl of 10 mg/ml RNAse. 200 µl of NaOH/SDS solution (0.2 M NaOH and 1% (w/v) SDS) was added and the tube was inverted several times to mix followed by adding 150 µl 0f 5 M potassium acetate solution (pH 4.8) then inverted again several times and incubated on ice for 5 minutes. The mixture was spun down at 13.000 rpm for 1 minute then the supernatant containing the DNA (about 0.4 ml) was moved to a fresh tube in addition to 0.8 ml 95% ethanol, after mixing the sample was left for 2 minutes at room temperature followed by centrifugation for 1 minute at 13.000 rpm. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and another centrifugation was conducted at 13.000 rpm for 1 minute. Again the supernatant was discarded and the pellet dried at 70 °C for

5 minutes to remove the residual ethanol. The plasmid DNA was taken up in 30 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and stored at -20 °C.

2.11.2 *Agrobacterium tumefaciens* plasmid DNA mini-prep

The plasmid DNA of *Agrobacterium tumefaciens* was extracted by using the protocol that was provided by Birnboim and Doly 1979.

A single colony of *Agrobacterium tumefaciens* previously grown for 2 days on solid LB medium at 28 °C was inoculated into 5 ml of liquid LB medium supplemented with spectinomycin and rifampicin then incubated for 2 days at 28 °C on a shaker at 200 rpm. 1.5 ml of the culture was pipette into a 1.5 ml microfuge tube and spun down for 3 minutes at 13000 rpm. The supernatant was discarded and 3 cultures were combined then the pellet was resuspend in 100 µl ice cold GTE lysis buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8 and 2 mg/ml lysozyme) followed by vortexing for 30 seconds and incubation for 30 minutes at room temperature. 200 µl of freshly prepared NaOH/SDS buffer (0.2 M NaOH and 1% (w/v) SDS) was added and the mixture was mixed by gentle shaking and left to stand at room temperature for half an hour. 150 µl of ice cold 3 M NaOAc (pH 4.8) was added to the mixture then mixed and incubated on ice for 5 minutes. Afterward the mixture was centrifuged for 5 minutes at 13000 rpm. The supernatant was transferred to a fresh tube and an equal volume of phenol: chloroform (1:1) was added followed by mixing and centrifugation for 5 minutes at 13000 rpm. This resulted in a phase separation, the upper phase containing the DNA was transferred to fresh tube with addition 1 ml of 96% ethanol. After mixing, the mixture was spun down for 5 minutes at 13000 rpm and the pellet was washed with 1 ml of 70% ethanol then was spun down again for 3 minutes at 13000 rpm. The ethanol was discarded and the pellet was dried to remove the residual of ethanol. The pellet was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 1 µl of 10 mg/l RNase.

2.11.3 DNA amplification by Polymerase chain reaction (PCR)

A Gene Amp® PCR system 2700, (Applied Biosystem) thermal cycler was used to perform the DNA amplification for different purposes such as determination of gene orientation, to check the presence of manipulated genes in transformed plants, check the presence of a gene in synthesised cDNA to ensure that that gene was expressed, and for making probes. The PCR reaction mixture consist of 10-100 ng template DNA, 5 µl10x

(*Taq* or *Pfu* DNA polymerase) PCR buffer, 1 µl of 10 pmol/µl of each forward and reverse primers, 8 µl 1.25 mM dNTPs (dATP, dCTP, dGTP and dTTP) and 0.5-1 µl of *Taq* or *Pfu* DNA polymerase. The reaction mixture volume was brought up to 50 µl with sterilised distilled water in a 0.2 ml polypropylene tube. Primer stock solutions were prepared as recommended by the supplier by adding the particular amount of distilled water to get 100 pmol/µl as a final concentration. The different primers used in this study are shown in Table 2.5.

Table 2.5: Primers sequence and predicted size of PCR product.

Primer name	Primer sequence	Amplicon size
HvBWMK1-F *HvBWMK1*-R	ATA**TCTAGA**CGCATGGGGGGAGGGAACGG* ATA**TCTAGA**GCACTAGGCATGCATCTTGGAGACCT	1737 bp
M13-F *M13*-R	GTTTTCCCAGTCACGAC CAGGAAACAGCTATGAC	Dependent on cloned insert
Hyg-F *Hyg*-R	AAAAGTTCGACAGCGTCTCC ATTTGTGTACGCCCGACAGT	1068 bp
HvTub-F *HvTub*-R	TACCACCTCCCTGAGGTTTG CCATGCCTAGGGTCACACTT	217 bp
OCS-II-R	GAATGAACCGAAACCGGCGGTA	300 bp + cloned insert

*Bold letters refer to *Xba*I restriction site.

PCR reaction cycle conditions were 1 cycle of 94 °C as an initial denaturation stage for 5 minutes (*Taq* or *Pfu* DNA polymerase was added at the end of this stage), 30-35 cycles of 94 °C as a denaturation stage for 30 seconds, X °C as an annealing stage for 30 seconds (the annealing temperature depending on the primers used*) and 72 °C as an extension stage for X minute, that depends on the length of the target DNA fragment (1 minute per 1000 bp) and 1 cycle at 72 °C as a final extension step for 7 minutes followed by holding at 4 °C (Table 2.6).

*The annealing temperature 3-5 °C below the primers melting temperature.

The primers' melting temperature Tm = 2(A+T) + 4(C+G).

Where A, T, G and C are the number of occurrences of each nucleotide in the primers.

Table 2.6: Primers and PCR reaction cycle conditions.

Primer name	PCR reaction cycle conditions						Cycles
	1 cycle	30-35 cycles			1 cycle		
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**	**Holding**	
HvBWMK1	94 °C	94 °C	58 °C	72 °C	72 °C	4 °C	30
	5 min	30 sec	30 sec	2 min	7 min	∞	
M13	94 °C	94 °C	55 °C	72 °C	72 °C	4 °C	30
	5 min	30 sec	30 sec	2 min	7 min	∞	
Hygromycin	94 °C	94 °C	57 °C	72 °C	72 °C	4 °C	35
	5 min	30 sec	30 sec	1 min	7 min	∞	
HvTubulin	94 °C	94 °C	63.5 °C	72 °C	72 °C	4 °C	35
	5 min	30 sec	30 sec	30 sec	1 min	∞	

2.11.4 PEG Precipitation of PCR products

In order to remove unused primers and dNTPs from the PCR products, the PEG protocol was followed. 5 µl of PCR product was run onto a 1% agarose gel to ensure the PCR reaction had worked and the target gene amplified. The remaining 45 µl of PCR product was mixed by pipetting up and down with 20% PEG buffer (20% Poly Ethylene Glycol 8000 (MW 8000) and 2.5 M NaCl) followed by incubation at 37 °C for 15 minutes then centrifuged at 13000 rpm for 15 minutes. The supernatant was discarded and 125 µl of cold 80% ethanol was added, vortexed and spun for 2 minutes at 13000 rpm (this step was done twice). The supernatant was discarded and the pellet dried for 5-10 minutes on a low heat until there was no trace (visible) of ethanol. 25 µl of TE buffer (10 mM Tris and 0.1 mM EDTA) was added and vortexed. 2 µl of purified DNA was checked onto a 1% agarose gel by running for sufficient time to approximately quantify the recovery.

2.11.5 DNA dephosphorylation

This technique was employed to prevent the digested plasmid from re-circularising during the cloning process by removing completely the terminal phosphates from the 5´ end. One unit of shrimp alkaline phosphatase (SAP) was added to the linearised plasmid DNA fragments with 1x SAP buffer (50 mM Tris-HCl and 10 mM $MgCl_2$, pH 9). The mixture was mixed and incubated for 30 minutes at 37 °C. The phosphatase was then deactivated by heating at 70 °C for 10 minutes.

2.11.6 DNA fragment ligation

The linearised vector and insert were ligated to construct recombinant DNA molecules at a molar ratio of 1:3 vector: insert according to the following recipe: 2 µl 10x ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT and 5 mM ATP at pH 7.8), 0.5 µl (2U) T4 DNA ligase enzyme, 2 µl of 10x ATP and distilled water was added to complete the total volume to 10-20 µl. The mixture was incubated at 37 °C for 4 hours approximately followed by incubation on 65 °C for 15 minutes to inactivate the T4 ligase enzyme.

2.12 Competent cells

2.12.1 *E. coli* competent cells preparation

The competent cells of *E.coli* were prepared according to the protocol as described by Inoue *et al*. (1990). A single colony of *E.coli* (XL-1 Blue) strain was inoculated in 5 ml of LB medium containing 100 µl $MgSO_4$ and incubated overnight at 37 °C with vigorous shaking at 200 rpm. This culture was used to inoculate 250 ml of LB medium with 5 ml $MgSO_4$ in a 2 litre conical flask. The culture was grown at 37 °C on a shaker at 250 rpm with good aeration until the OD_{600} reached 0.4-0.6 by measuring aliquot with a spectrophotometer at 600 nm. The culture was then moved to a sterile bottle and was chilled on ice for 10 minutes followed by centrifugation at 3000 rpm for 10 minutes at 4°C. Afterward the supernatant was discarded and the pellet was gently dissolved in 80 ml ice cold transformation buffer (10 mM PIPES-HCl. pH 6.7, 15 mM $CaCl_2$, 0.25 M KCl and 55 mM $MnCl_2$) and was kept on ice for 10 minutes. The mixture was spun down at 3000 rpm for 10 minutes at 4 °C and the supernatant was removed. The pellet was gently resuspended in 20 ml cold transformation buffer with 1.5 ml DMSO. The

suspension was kept on ice for 10 minutes then divided into 200 µl aliquots in ice cold sterile microfuge tubes and stored at -70 °C.

2.12.2 *Agrobacterium tumefaciens* competent cells preparation

The competent cells of *Agrobacterium tumefaciens* were obtained by following the method as described by Höfgen and Willmitzer (1988) with simple modifications. A single colony of *Agrobacterium tumefaciens* strain EHA105 previously grown for 2 days on solid LB medium at 28 °C was inoculated into 5 ml of liquid LB medium supplemented with 25 mg/l rifampicin then incubated overnight at 28 °C on a shaker at 200 rpm. 2 ml of the overnight culture was added to 50 ml LB medium in 250 ml conical flask and incubated at 28 °C with vigorous shaking at 250 rpm till the OD_{600} reached approximately 0.5-1.0. The cell suspension was chilled on ice and spun down at 3000 g for 5 minutes at 4 °C. The supernatant was discarded and the pellet was suspended in 1 ml of cold 20 mM $CaCl_2$ solution. After that, the suspension was divided into 100 µl aliquots in precooled sterile microfuge tubes and stored at -70 °C.

2.13 Heat shock transformation of competent *E. coli* cells

An aliquot of stored competent cells was thawed on ice prior to transformation. Afterward, 10 µl of ligation mixture or plasmid DNA sample were added to the competent cells, mixed gently by inversion and incubated on ice for 30 minutes. The mixture was heat-shocked at 42 °C for 1 minute in a water bath then returned to the ice for 2 minutes. 1 ml of antibiotic free LB medium was added to the sample and incubated on heating block for 1 hour at 37 °C to allow bacterial cells to recover and to express the antibiotic selectable marker encoded by the plasmid. The cells were spun down at 6000 rpm for 3 minutes, most of the supernatant was removed, the cells were resuspended by tapping with a finger, and then up to 200 µl were spread on LB agar plate containing the appropriate selective antibiotic and grown overnight at 37 °C, with the plate inverted.

2.14 *Agrobacterium tumefaciens* transformation

The modified free-thaw method (Höfgen and Willmitzer 1988) was followed to perform the transformation process. The stored competent cells were taken from -70 °C and thawed on ice then mixed with 1 µg plasmid DNA. The mixture was incubated for 5 minutes at 37 °C followed by addition of 1 ml LB and incubation on a shaker for 4

hours at 28 °C. After centrifugation at 13000 rpm for 1 minute, the supernatant was discarded and the cells were resuspend in 100 µl LB medium, then plated on LB agar plate containing the appropriate antibiotic and incubated at 28 °C for 2-3 days until the growth of visible colonies for further propagation.

2.15 Cloning of *HvBWMK1* into a cereal transformation vector

The open reading frame (ORF) of *HvBWMK1* coding region was amplified from the cDNA cloned in pBluescript(pflc111)-flbaf180a19 plasmid by PCR using *HvBWMK1* primers with the *XbaI* restriction site on both the forward and reverse primers. The PCR products were digested with *XbaI* to make both ends sticky, and then gel-purified. After that, *HvBWMK1* was ligated into *XbaI* digested pTopoActin-OCS plasmid vector which containing *Actin* promoter and *OCS* terminator, then transformed into *E. coli*. The orientation of the insert (sense and antisense) was proved by digestion with different endonuclease enzymes. The resulting plasmid pTopoActin-BWMK1-OCS was digested with *Apa*I to cut out the fragment *Actin-BWMK1-OCS* which was then cloned into the *Apa*I site of the binary vector T-DNA plasmid pWBVec.8. The new recombinant plasmids pWBVec8-Actin-BWMK1-OCS constructions (in both orientations) alongside empty vector were transformed into *Agrobacterium tumefaciens*.

The *HvBWMK1* open reading frame of the overexpression construct was fully sequenced to confirm that no errors had been created during the process of PCR or cloning.

2.16 Preparation of DIG-labelled DNA probe

In order to prepare a labelled DNA probe for *HvBWMK1*, a standard 50 µl PCR reaction was conducted in presence of *HvBWMK1* forward and reverse primers, DNA of plasmid containing the ORF of *HvBWMK1* and 2 µl of DIG 11-dUTP (Roche) in addition to the usual level of dNTPs. The PCR product (probe) was checked on an agarose gel alongside with DNA from a non-labelled *HvBWMK1* PCR reaction to confirm the success of the labelling indicated by a size shift between the bands.

2.17 Filter-hybridisation of transformed *E. coli* colonies

In order to determine which bacterial colonies contain the target gene after the cloning step, a sample of target gene was labelled with Digoxigenin by PCR amplification (Section 2.16) and used as a DNA probe for hybridization as described by Roche. The

procedure of Buluwela *et al*. (1989) was used. Bacterial colonies were lifted from agar plated on to a nylon membrane (Amersham Hybond N, GE Healthcare Life Sciences) by placing the dry membrane onto the plate to contact the colonies. After marking the positions of the membrane, the filter was removed and the plate was re-incubated overnight at 37 °C to encourage regrowth of the bacterial colonies for later picking of positives. The nylon membrane was placed, colonies face-up on 3 MM Whatman filter paper pre-soaked in 2x SSC, 5% (w/v) SDS (300 mM NaCl, and 30 mM sodium citrate, pH7) and left for 2 minutes. After that, the membrane was placed in glass dish with a small amount of 2x SSC and transferred to a microwave oven and baked at 600 Watts for 2 minutes to lyse the bacterial cells, simultaneously denaturing and fixing the bacterial DNA to the membrane. The membrane was moved into a rotating roller bottle in presence of 10 ml of DIG Easy Hyb buffer (Roche) and subjected to prehybridisation at 65°C for 30 minutes. 250 ng of DIG-labelled DNA probe of *HvBWMK1* was denatured by boiling for 5 minutes in 0.5 ml hybridisation buffer then immediately cooled on ice. The prehybridisation solution was removed and another 10 ml of DIG Easy Hyb buffer (Roche) containing the denatured probe (25 ng/ml) was added and hybridised overnight at 65 °C. After the hybridisation was completed, the hybridization buffer was removed and the membrane was washed once with preheated low stringency 2x SSC, 1% (w/v) SDS buffer for 20 minutes at 65 °C in a rotating oven followed by another twice washing with pre-warmed high stringency 0.2x SSC, 0.1% (w/v) SDS buffer for 20 minutes at 65°C. The membrane was then washed with 10 ml autoclaved DIG1 buffer (100 mM Tris-HCl and 1 M NaCl at pH 8), the blot was then sealed in a plastic bag filled with 10 ml autoclaved blocking buffer (maleic buffer (0.1M maleic acid and 1M NaCl, pH adjusted to 8 by NaOH), blocking reagent (0.5% Hammerstein casein, 0.3% Tween and 0.1% DEPC)), blocked for 1 hour at room temperature with gentle shaking and followed by another incubation for 30 minutes at room temperature with 10 ml blocking buffer in presence of 1 µl (1:10.000) alkaline phosphatase coupled DIG-antibody (Roche). The membrane was then picked up out from the plastic bag and placed on a tray with 100 ml of washing buffer DIG1and kept shaking on an orbital shaker for 10 minutes twice followed by another washing with 100 ml of detection buffer DIG4 (100 mM Tris and 100 mM NaCl at pH 9.5**)** for 2 minutes.

The membrane was sealed in a plastic bag with 10 ml of detection buffer DIG4 as a substrate for alkaline phosphatase in addition to 35 µl of BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 45 µl of NBT (nitrotetrazolium blue chloride) then incubated in

the dark at 37 °C until the clear purple colour of positive colonies appeared, to stop reaction the membrane was washed with distilled water and dried.

2.18 DEPC-treatment of solutions

In order to remove any traces of RNase from solutions which were expected to be used with RNA, a DEPC-treatment process was carried out by adding 100 µl of DEPC (diethyl pyrocarbonate) to 1 litre of the solution and stirred for 1 hour at room temperature, followed by autoclaving to remove residual DEPC.

2.19 The donor barley cultivar and growth conditions

Hordeum vulgare L. Golden Promise (a spring barley cultivar) was used as a donor plant in this study. The plants were grown in 3:1 mixture of compost (Scotts Professional) and vermiculite (Sinclair) and in addition one gram of slow release fertiliser (Osmocote N: P: K =14-13-13) was spread onto the surface of the soil at the time of planting, the microelements fertiliser (Miracle-Gro) 1.25 g/l was dissolved in irrigation water and added every other week, the pot size was 15 cm in height and 14 cm in width. The plants were watered with tap water and kept in an environmentally controlled growth chamber under the followed conditions: 18 °C, 16 h light, 8 h dark photoperiods (light level of 450 $\mu Em^{-2}.s^{-1}$ at head light) and 60-80% relative humidity.

2.20 Barley transformation

All the steps of barley transformation which are described below were conducted according to the protocol of Harwood *et al.* (2009) and Tingay *et al.* (1997) with modifications.

2.20.1 Preparation of *Agrobacterium* growth

Transformed *Agrobacterium* with the appropriate plasmid construction was grown on plate of solid LB medium supplemented with rifampicin 25 mg/l and spectinomycin 50 mg/L and incubated at 28 °C for 2-3 days. A single colony was picked and inoculated in 5 ml liquid LB medium with the same antibiotic as above and incubated on a shaker at 28 °C for 1-2 days. This broth culture was used for barley transformation.

2.20.2 Callus induction media preparation

Four different types of media were prepared for different stages of callus growth (Table 2.7). Except the Dicamba (3,6-dichloro-2-methoxybenzoic acid), antibiotics and 6-benzylaminopurine (6BAP) all the components of media were mixed together, dissolved in water and adjusted to pH 5.8 then autoclaved at 121 °C for 20 min after that kept at room temperature. Filter-sterilised Dicamba, antibiotics and 6-benzylaminopurine (6BAP) were added to the media after melting prior to use.

2.20.3 Barley immature embryos preparation

Plants of barley cultivar Golden Promise were grown in the growth room, the spikes of barley were harvested at two weeks post anthesis. The seeds were collected and sterilized in 70% ethanol for 1 minute and 10% bleach (diluted sodium hypochlorite solution) for 20 minutes, then rinsed four times in sterile distilled water. The caryopsis were dissected longitudinally along the central at the dorsal side of the grains, immature embryos of about 1.5-2.5 mm size were released, then the embryonic axis was removed with a sharp scalpel blade under a dissecting microscope, the explants were placed on solid callus induction medium. All these steps were conducted in sterile condition under laminar flow clean air hood.

2.20.3.1 Embryos of barley transformation

25 embryos were placed with the scutellum side down (Bartlett *et al*. 2008) on callus induction medium (CIM) in Petri dishes without antibiotic (Table 2.7). 1-2 µl of *Agrobacterium* suspension was dropped on each embryo directly on the area of the removed axes on the same day of isolation and the plates were left open for 30-60 minutes in the laminar flow hood to drain off any excess inoculum. The embryos were kept in the dark at 24 °C for 2 days as a co-cultivation period. The plates were checked several times daily to notice any *Agrobacterium* overgrowth and in case of that, the infected embryo was washed with Timentin (160 mg/l) for 30 seconds then rinsed twice in sterile distilled water.

2.20.4 Selection and plant regeneration

After the co-cultivation phase, the embryos were then placed on selection medium, which was the same as callus induction medium but supplemented with 50 mg/l hygromycin as a selective agent, 160 mg/l Timentin to kill the remaining *Agrobacterium* and 1.25 mg/l $CuSO_4.5H_2O$. Explants were maintained on selection medium for 4 weeks at 24 ±2 °C in darkness, and the embryos moved to new plates every two weeks. The healthy embryo-derived callus quickly showed signs of green areas during 2-3 weeks, after which they were transferred to regeneration medium (selection medium with 0.1 mg/l 6BAP only). The plates were incubated for 2 weeks in full light at 24 ±2 °C. Shortly after, the shoots (about 1-2 cm in length) were excised and transferred into square culture containers which contain rooting medium (CIM with the antibiotics hygromycin and timentin and without any growth regulators. The small plantlets were incubated under the same conditions and once they formed strong roots and the shoots reached the top of the tubes, they were transferred to the soil under the same donor barley growth conditions in a controlled environment growth room. Wild type plants were grown alongside in order to observe any morphological differences during different growth phases.

2.20.5 Seeds collection

Mature dry spikes of transgenic plants of T_0 lines and wild type were collected from plants. Each transgenic line was spread on labelled tray and incubated on 37 °C in incubation room for 6 weeks in order to more drying and break the dormancy. After that, the seeds were threshed and cleanly separated from the other components of spikes. The pure seed were stored in freezer on -20 °C for further experiments.

The collected seeds of transgenic lines of barley and wild type were checked by eye and under dissecting microscope in order to assess any differences in seed shape, colour and size. Then the weight of 100 seeds was taken.

Table 2.7: The compositions of the tissues culture media (per litre).

CIM (co-cultivation) 2 days	Selection medium 4 weeks	Regeneration Medium 2 weeks full light	Rooting Medium
4.3 g MS	4.3 g MS	4.3 g MS	4.3 g MS
30 g Maltose	30 g Maltose	30 g Maltose	30 g Maltose
1 g Casein hydrolysate	1 g Casein hydrolysate	1 g Casein hydrolysate	1 g Casein hydrolysate
250 mg Myo-inositol	250 mg Myo-inositol	250 mg Myo-inositol	250 mg Myo-inositol
650 mg Proline	650 mg Proline	650 mg Proline	650 mg Proline
1 mg Thiamine	1 mg Thiamine	1 mg Thiamine	1 mg Thiamine
2.5 mg Dicamba	2.5 mg Dicamba		
3.9 g Phytagel	3.9 g Phytagel	3.9 g Phytagel	3.9 g Phytagel
	1.25 mg $CuSO_4$. $5H_2O$		
10 mM MES	10 mM MES	10 mM MES	10 mM MES
	50 mg Hygromycin	50 mg Hygromycin	50 mg Hygromycin
	160 mg Timentin	160 mg Timentin	160mg Timentin
		0.1 mg 6BAP	
pH 5.8	pH 5.8	pH 5.8	pH 5.8

2.21 Extraction of DNA from barley leaves

Plant DNA was isolated according to Dellaporta *et al*. (1983). Fresh and young leaves were collected from barley plants. 100 mg was ground in a 1.5 ml microcentrifuge tube with a plastic pestle until fully liquefied in presence of 750 µl of extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, pH 8 and 10 mM 2-mercaptoethanol, added immediately before the start of extraction). The suspension was

incubated at 65 °C for 10 minutes, afterward 200 µl of 5 M potassium acetate (pH 4.8) was added and incubate on ice for 20 minutes then the mixture was centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to new tube and an equal volume of isopropanol was added then centrifuged at 13000 rpm for 2 minutes. The supernatant was discarded, the pellet was washed with 500 µl of 70% ethanol and centrifuged at 13000 rpm for 10 minutes. The supernatant was removed, the pellet was dried and dissolved in 30 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5).

2.21.1 Agarose gel electrophoresis of DNA fragments

To separate the DNA fragments a 1% (w/v) agarose was prepared by heating agarose till dissolved in 0.5x TBE buffer (10x stock TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, and dH_2O up to 1 litre at pH 8). Once the agarose solution had cooled to ~50 °C, ethidium bromide was added to a final concentration of 0.5 µg/ml. Afterward the comb was placed for well formation, and then the solution was poured into a casting tray containing the comb and allowed to solidify at room temperature. When the agarose gel completely solidified, the comb was removed carefully and the gel moved into an electrophoresis tank, then 0.5x TBE buffer was poured into the tank to cover the gel completely. The samples containing DNA were mixed with 6x gel loading dye buffer (60% (v/v) glycerol, 60 mM EDTA, 0.09% (w/v) xylene cyanol and 0.09% (w/v) bromophenol blue) to a final concentration of 1x and pipetted into the gel wells. To detect the size of DNA fragments, 2 µl of λ *Hin*d III marker (1 µg) was loaded on the agarose gel in a well alongside the DNA samples. λ *Hin*d III marker (Fermentas) gives detectable fragments of: 23130, 9416, 6557, 4316, 2322, 2027, 564 and 125 bp (Appendix 5.11 A). The gel was electrophoresed at 100 V for approximately 15 to 30 minutes, after which the agarose gel was removed from the tank, viewed and photographed on a UV transilluminator at 280 nm.

2.21.1.1 Purified DNA fragments from the gel

The QIAquick gel extraction kit from Qiagen was used to extract and purified DNA fragments from the gel. The target DNA fragment was excised as an agarose block from the gel under UV light with a clean and sharp scalpel, then the excised gel weighed. 3 volumes of QG buffer were added to one volume of excised gel and incubated at 50 °C in water bath for10 minutes until the gel was totally dissolved. One gel volume of isopropanol was added to the sample and mixed by vortexing. The sample was put into

a QIAquick spin column which was placed in a 2 ml collection tube and centrifuged for 1 minute at 13000 rpm and then the flow-through discarded. 750 µl of PE buffer was added to the QIAquick spin column and centrifuged for 1 minute at 13000 rpm and the flow-through discarded. An additional spin was conducted for 1 minute at 13000 rpm. The QIAquick spin column was placed into a clean 1.5 ml microcentrifuge tube and 50 µl of warm EB buffer was added then centrifuged for 1 minute at 13000 rpm and the elute stored at -20 °C. 2 µl of purified DNA was checked onto a 1% agarose gel by running for enough time to roughly quantify recovery.

2.22 Extraction of total RNA from barley leaves

The total RNA extraction was carried out according to Chomczynski and Sacchi (1987) with some modification by using Trizol reagent. This is a monophasic solution of phenol and guanidine isothiocyanate which inhibits RNAse activity to protect RNA from degradation while disrupting cells and dissolving its components. In general, 400 mg of young leaves were ground by using a pestle and mortar until fully liquefied in presence of 5 ml of Trizol buffer and 50 µl 2-mercaptoethanol. The mixture was transferred to five 2 ml microcentrifuge tubes and put on the bench at room temperature for 5 minutes then 200 µl of chloroform were added and mixed by vortexing. Afterward the mixture was centrifuged at 13000 rpm for 10 minutes. The upper aqueous layer was transferred to new tube followed by 1 ml isopropanol. After mixing, the solution was centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded and 500 µl 70% ethanol was added immediately and vortexed well. After 5 minutes centrifugation at 13000 rpm, the supernatant was removed, the pellet dried and dissolved in 40 µl sterile distilled water.

The concentration and purity of RNA was quantified by using a spectrophotometer to determine the optical density. 1 µl RNA was dissolved in 400 µl of TE buffer in microfuge tube, vortexed briefly then transferred to quartz cuvette with 10 mm path length. The measuring of UV light absorbance at 260 nm and 280 nm were recorded. An A_{260}/A_{280} ratio of between 1.8 and 2.0 determines the purity of the RNA whereas multiplying the A_{260} reading by the conversion factor of RNA quantitation (1 absorbance unit at 260 nm = 40 µg/ml) was used to calculate the quantity of RNA.

RNA concentration (µg/ml) = (OD_{260}) × (dilution) × (40 µg/ml).

2.22.1 RNA quality quick check

To test the presence of RNA and its quality quickly, a 1% (w/v) TBE-agarose gel was prepared in a tray and the comb cleaned with RNAse Zap solution (1% SDS, 2 mM $CuSO_4.5H_2O$ and dH_2O), the RNA sample was mixed with 2x RNA loading dye and immediately loaded into the gel wells and then submerged in adequate 0.5x TBE buffer in an RNAse Zap cleaned electrophoresis tank. The electrophoresis was conducted at 100 V for approximately 10 minutes, after which the gel was removed and checked on UV trans- illuminator at 280 nm and the image was taken.

2.22.2 Agarose gel electrophoresis of RNA fragments

1% Agarose Mops-formaldehyde gel was used to separate RNA fragments. A 1% (w/v) agarose was dissolved by heating in distilled H_2O and 1x MOPS buffer (pH 7.0, autoclaved 10x MOPS buffer: 200 mM MOPS 3-(N-morpholino) propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA) then cooled and 1% (v/v) of 37% formaldehyde was added and immediately poured into a casting tray containing a comb and allowed to solidify at room temperature. The samples containing RNA were mixed with two volume of RNA loading buffer (50% (v/v) formamide, 16% (v/v) formaldehyde, 10% (v/v) of 10x MOPS buffer, 0.1 mg/ml ethidium bromide and 0.01% (w/v) bromophenol blue), then denatured at 70 °C for 10 minutes. After cooling on ice, the sample was immediately pipetted into the gel wells. The electrophoresis chamber was filled with adequate 1x Mops buffer and electrophoresis set on 80 volts and monitored until the dye front migrated about $2/3^{rd}$ of the way down the gel, after which the gel was removed and visualized under UV trans-illuminator at 280 nm and the image was taken.

2.23 Phenol-chloroform extraction

This procedure was carried out to remove protein and cell debris from the nucleic acid containing solutions, one volume of the nucleic acid (DNA or RNA) solution was mixed with 1 volume of 50:50 Tris-HCl (pH 8) saturated phenol:chloroform for DNA or with water saturated phenol:chloroform for RNA in 1.5 ml microcentrifuge tube. The mixture was vortexed and then centrifuged for 10 minutes at 13000 rpm. The upper aqueous phase which containing the nucleic acid (DNA or RNA) was removed to a fresh microcentrifuge tube and mixed with an equal volume of chloroform to remove

any residual phenol, after a further 10 minutes centrifugation at 13000 rpm, the upper phase was transferred into a fresh tube and used for ethanol precipitation.

2.24 Ethanol precipitation of nucleic acids

The nucleic acids (DNA or RNA) were precipitated from the solutions by mixing with 0.1 volume of 3 M sodium acetate at pH 5.2 and 2.5 volumes of 96% ethanol. The mixture was mixed and incubated for 30 minutes at -20 °C followed by centrifugation for 15 minutes at 13000 rpm, the supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol. After further 10 minutes centrifugation at 13000 rpm, the supernatant was removed, the pellet dried by heating at 70 °C for 2-5 minutes and dissolved in TE buffer for DNA or DEPC treated water for RNA at the required volume.

2.25 Preparation of DIG-labelled RNA probe

The target DNA was cloned in pBluescript II KS+ plasmid (Stratagene). The cloned orientation was tested by digestion with different endonuclease enzymes. The target DNA fragment was amplified from a cloning vector through a standard PCR reaction with M13 forward and reverse primers. Depending on the insert orientation, one of the T3, T7 or SP6 RNA polymerases (Fermentas) was used to synthesis a digoxigenin labelled antisense single strand RNA. The probe synthesis reaction components contained 100-200 ng of purified PCR product template DNA, 4 µl 5x transcription buffer, 1 µl RNAse inhibitor (Fermentas), 4 µl 5x DIG RNA labelling mix (Roche), 2 µl RNA polymerase (T7) and DEPC-treated water (Fermentas) brought up to the final volume of 20 µl. The reaction was mixed gently and centrifuged briefly then incubated for 2 hours at 37 °C. 2 µl DNase I, RNase-free was added to remove template DNA then incubated for 15 min at 37 °C. 2 µl 0.2 M EDTA (pH 8.0) was added to stop the reaction.

To check the probe, 1 µl of reaction product was added to the same volume of RNA loading dye then run on the 1% agarose gel alongside with high range RNA ladder (Fermentas) which gives a detectable range of fragments 6000, 4000, 3000, 2000, 1500, 1000, 500 and 200 bp for quality and quantity assessment (Appendix 5.11 B). One RNA probe was prepared and used in this study, which was *HvBWMK1* (1737 bp) to check the expression of barley *HvBWMK1*.

To determine the labeling efficiency of the probe, the DIG-labeled RNA probe were diluted to several concentrations (10 ng, 1 ng, 100 pg, 10 pg and 1 pg), and a 1 µ1 spot from each concentration was applied on a piece of positively charged nylon membrane (Roche). The nucleic acid was fixed to the membrane by cross linking with UV-light, and then the nylon membrane was subjected to immunological detection with anti-digoxigenin-AP. After the washing step as described in Northern blotting (section 2.28), 500 µl of the chemiluminescent substrate solution CDP-Star® (Sigma) was used to detect the signal on the X-ray film (Kodak) as described in (section 2.28).

2.26 Complementary DNA (cDNA) synthesis

The RevertAid™ H Minus First strand cDNA synthesis kit (Fermentas) was used to create single strand DNA from RNA to confirm the target gene expression. To remove the genomic DNA from RNA preparations, 1 µg of RNA and 1 µl 10X reaction buffer with $MgCl_2$ were mixed in an RNase-free tube and the volume made up to 9 µl by adding DEPC-treated water then 1µl DNase I, RNase-free (1u/µl) enzyme was added and incubated at 37 °C for 30 minutes. Finally 1 µl of 25 mM EDTA was added and incubated at 65 °C for 10 minutes to stop the reaction. This treated RNA was used as a template for reverse transcriptase.

To create the first strand cDNA the instructions of the kit were followed as shown below. 0.1-5 µg of template RNA and 1 µl of random hexamer primer were mixed in a sterile, nuclease-free tube and the volume made up to 12 µl by adding DEPC-treated water. After that the following components were added in the indicated order: 4 µl 5X reaction buffer, 1 µl RNase Inhibitor (20 u/µl), 2 µl 10 mM dNTP mix and 1µl reverse transcriptase (200 u/µl). The mixture was mixed gently and incubated for 5 minutes at 25 °C followed by 60 minutes at 42 °C. The reaction was terminated by heating at 70 °C for 5 minutes. The reverse transcription reaction product can be directly used in PCR applications or stored at -70 °C for long term storage as recommended.

2.27 Real time PCR (SYBR green detection)

The Primer Design 2X precision™ Mastermix kit (Primer design) was used to measure the quantity of the gene expression. The PCR was conducted in the 7900 HT Fast-Real-Time PCR system (Applied Biosystem) using the SYBR green and the fixed reference UBC2. The real-time PCR was performed using the relative method. The

reaction of 20 µl per well consisted of 1 µl (final concentration 300 nM) of forward and reverse primers mix, 10 µl SYBR green PCR master mix, 5 µl of a 1:10 dilution of cDNA and 4 µl RNAse/ DNAse free water. The reactions were carried out in a 35 µl, 384-well plate (Star Lab) covered with an optically clear adhesive sheet ABsolute^{TM} QPCR Seal (ABgene). The PCR mixtures were incubated at 95 °C for 10 minutes to activate the enzyme followed by 50 amplification cycles of denaturation at 95 °C for 15 seconds and data collection at 60 °C for 60 seconds. The PCR reactions were subjected to a heat dissociation protocol present in the PCR system software for melting curve analysis. Following the final cycle of the PCR, the reactions were heat denatured at 0.03 °C/s over a 35 °C temperature gradient from 60 °C to 95 °C.

2.28 Northern blotting

Total RNA (10-20 µg per sample) was mixed with 2 volume of RNA loading buffer and denatured at 70 °C for 10 minutes. After cooling on ice, the sample was immediately pipetted into the gel and electrophoresed as mentioned above. A blot transfer was set up as follows, avoiding the formation of air bubbles between any two parts of the blot.

2 pieces of Whatman 3MM paper were soaked with 20 x SSC buffer (3 M NaCl, and 300 mM sodium citrate, pH 7**)** and placed on top of a pad of tissues. A piece of positively charged nylon membrane (Roche) at the same size of the gel was pre-wetted with sterile distilled water then soaked in the 20x SSC buffer for a few minutes, after which it was placed carefully on top of the Whatman 3MM paper and the air bubbles were eliminated. The gel was soaked twice (2 x 5 min) in 20x SSC buffer to remove formaldehyde (which can inhibit transfer) and placed, facing down, on the top of the membrane, the blot assembly was completed by adding two sheets of Whatman 3MM paper, cut to the size of the gel. 2 rectangular pieces of Whatman 3MM paper was used as a bridge to carry 20x SSC buffer from the tray to the blot assembly.

The blotting was left for 6 hours at room temperature and more 20x SSC buffer was added when needed. While the nylon membrane was still damp, the RNA was fixed to the nylon membrane by UV crosslinker (UVC-508, Anachem: 120,000 µJ of UV) for 1-3 minutes. The pre-hybridization process was carried out for the RNA blot for 30 minutes at 68 °C with 10 ml of pre-warmed hybridization buffer (6 M urea, 6x SSC, 1% (w/v) SDS and 50 mM Tris-HCl, pH 7.5). At the end of pre-hybridisation, the

appropriate amount of DIG-labelled antisense RNA probe was mixed with 100 µl of sterile distilled water in microfuge tube then denatured at 95 °C for 5 minutes and the recommended concentration 100 ng/ml was used for hybridisation of the blot overnight at 68 °C in a rotating oven.

After finishing the hybridization, the hybridization buffer was discarded and the membrane was washed twice with preheated low stringency 2x SSC, 1% (w/v) SDS buffer for 20 minutes at 68 °C in a rotating oven followed by another twice washing with pre-warmed high stringency 0.1x SSC, 0.1% (w/v) SDS buffer for 20 minutes at 68 °C. The membrane was then washed with autoclaved DIG1 buffer (100 mM Tris, and 1 M NaCl at pH 8), the blot was then sealed in a plastic bag filled with 10 ml autoclaved blocking buffer (maleic buffer (0.1M maleic acid and 1M NaCl, pH adjusted to 8 by NaOH), blocking reagent (0.5% Hammerstein casein, 0.3% Tween and 0.1% DEPC)), blocked for 1 hour at room temperature and followed by another incubation for 30 minutes at room temperature with 10 ml blocking buffer in presence of 1 µl (1:10.000) alkaline phosphates coupled DIG-antibody (Roche).

The membrane was moved to a clean tray and washed four times for 10 minutes each on the shaker with autoclaved DIG1 buffer, followed by washing for 3-5 minutes on the shaker with autoclaved DIG4 buffer (100 mM Tris and 100 mM NaCl at pH 9.5**)**. After which, both side of the membrane were wetted with 500 µl of the chemiluminescent substrate solution CDP-Star® (Sigma)**,** placed between two pieces of acetate sheet and put in the developing cassette.

In the dark room with a red safety light, the X-ray film (Kodak) was placed on the nylon membrane assembly for 10-60 minutes (the exposure time depends upon intensity of signal produced). The X-ray film was then washed first with the developer solution (Kodak) followed by rinsing with water and finally gently agitated in the fixer solution (Kodak).

2.29 Transgenic barley seeds dormancy breaking and germination

The healthy seeds of transgenic lines were sterilized in 70% ethanol for 1 minute then rinsed four times in sterile distilled water. The seeds were placed on two Whatman 3MM filter paper in petri dishes and treated with 0.75% of hydrogen peroxide to break dormancy and promote the germination (Fontaine *et al*. 1995, Çavusoglu and Kabar 2010) in addition to 100 µg/ml of hygromycin as a plant selectable marker (Hagio *et al*.

1995), The Petri dishes were kept on the bench at room temperature for 4-7 days under observation and adding water as needed. The healthy hygromycin resistant seedlings were moved to the soil in pots to carry on further experiments.

To determine the suitable concentration of hygromycin to use for transgenic seed segregation, a test was carried out. The seeds of wild type were placed on two Whatman 3MM filter paper in petri dishes and treated with several concentrations of hygromycin (0, 50, 75, 100, 150 µg/ml), 5 seeds for each concentration.

2.30 Measuring wild type susceptibility to abiotic stress

Abiotic stresses (drought and salinity) were imposed on wild type barley in order to: firstly, to understand the susceptibility of wild type barley to abiotic stresses (salinity and drought) and to determine the more important and easily quantifiable abiotic stress which will be used for assessing transgenic barley. Secondly, to understand the susceptibility of wild type barley to different concentrations of sodium chloride solutions to determine the concentration that will be used for assessing transgenic barley as well. Finally, to investigate the changes in physiological parameters that will be used to study the response of transgenic barley to abiotic stresses. Depending on the result of the wild type experiments, one abiotic stress, in case of salinity: one salt concentration and the best indicator parameters will be choosen for further experiments with transgenic lines.

Two week old seedlings from wild type were irrigated in triplicate with sodium chloride (NaCl) solution every other day for 14 days. Sodium chloride (NaCl) solutions were added in five concentrations of 0, 50, 100, 150 and 200 mM. Afterward the length of the shoot was taken then the shoots cut off, weighed and used immediately for analysis or freeze dried or stored in a freezer at -70 °C for further analysis.

2.31 Abiotic stresses (wounding, drought and salinity) performance

Mechanical wounding was carried out by gently crushing leaves with a hemostat tool to activate gene expression of *HvBWMK1*. The leaves were harvested after 30 minute (He *et al*. 1999, Agrawal *et al*. 2003c) to check the *HvBWMK1* mRNA level through northern blotting.

For salinity stress, two weeks old seedlings from wild type and each transgenic line were irrigated with 150 mM NaCl solution or tap water for controls every other day. Plants were periodically given the same saline solution to keep the salt concentration in the soil at a constant level (Verslues *et al*. 2006) for 14 days. Afterward the length of the shoot was taken then the shoots cut off, weighed and used immediately for analysis or freeze dried or stored at -70 °C for further analysis.

For drought stress, two weeks old seedlings from wild type and transgenic lines were exposed to drought stress by withholding water for 10 days. Then the shoot was cut off, the length measured, weighed (fresh weight) and stored at -70 °C for further analysis.

2.32 Standardization of hydrogen peroxide

Hydrogen peroxide (H_2O_2) concentration was standardized according to the procedure described by Patterson *et al*. (1984). H_2O_2 30% (w/v) was diluted to 10 mM in 50 mM phosphate buffer pH 7.0 and absorbance at 240 nm measured against a blank from which hydrogen peroxide had been removed by two successive additions of 1µg/ml catalase for 30 minutes at 20 °C. The absorbance due to the addition of catalase was corrected with a blank of catalase only in the buffer. The molar absorptivity of hydrogen peroxide at 240 nm and pH 7.0 is 40 mol/cm.

2.33 Measurement of hydrogen peroxide

Endogenous hydrogen peroxide was measured according to the titanium sulphate method as described by Jana and Choudhuri (1982). About 300 mg of weighed leaf tissue was homogenized with 3 ml of phosphate buffer (50 mM, pH 6.5) then centrifuged at 6000 g for 25 minutes. The supernatant was moved to a fresh tube and made up to 3 ml with phosphate buffer then mixed with 1 ml of 0.1% titanium sulphate in 20% (v/v) H_2SO_4. The mixture was centrifuged at 6000 g for 15 minutes. The intensity of the yellow colour of the supernatant was colorimetrically measured at 410 nm. H_2O_2 content was calculated from a standard curve which was prepared from known concentrations of hydrogen peroxide.

2.34 Measurement of proline

Proline content in the barley leaves was measured following the method described by Bates *et al*. (1973). Approximately 500 mg fresh leaves were homogenized in 10 ml of

3% sulphosalycylic acid using a clean mortar and pestle. The homogenate was filtered through Whatman filter paper, and then 2 ml of filtered extract mixed with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin (1.25 g ninhydrin, 30 ml glacial acetic acid and 6 M orthophosphoric acid). The reaction mixture was heated in a boiling water bath at 100 °C for 1 hour, after cooling on ice, 4 ml of toluene was added to the mixture then mixed vigorously by vortexing for 30-60 seconds and left to separate into two phases. The absorbance of the chromophore-containing toluene phase was read at 520 nm in spectrophotometer against the toluene blank. The proline concentration was estimated from a standard curve and calculated on a fresh weight basis depending on the following formula.

[(µg proline/ml × ml toluene)/115.5* µg/µmole]/[(g sample)/5] = µmoles proline/g FW*.
*115.5 = Molecular weight of proline.
*FW = Fresh weight of leaf tissue.

2.35 Measurement of chlorophyll

The chlorophyll was extracted from plant leaves according to Porra *et al*. (1989), Ashraf and Iram (2005). Fresh leaves were weighed and cut into 0.5 cm segments then immediately immersed into 5 ml of 80% acetone and the pigment was allowed to extract in dark at -10 °C for an overnight. Next day the samples were centrifuged at 14000 g for 5 minutes. The absorbance of the supernatant was measured spectrophotometrically against acetone at 663.6 nm and 646.6 nm. The following equations from Porra *et al*. (1989), Porra (2002) were used to compute the amount of chlorophyll a, b and total chlorophyll.

Chlorophyll a (µg/ml) = 12.25 ($A_{663.6}$) – 2.55 ($A_{646.6}$)
Chlorophyll b (µg/ml) = 20.31($A_{646.6}$) – 4.91($A_{663.6}$)
Total chlorophyll (µg/ml) = 17.76 ($A_{646.6}$) + 7.34 ($A_{663.6}$)

2.36 Measurement of relative water content (RWC)

Measurement of relative water content was conducted following the method used by Barrs and Weatherley (1962) where the leaves were excised then weighed (fresh weight, FW) and immediately soaked in distilled water and kept overnight in the dark. Next day, the leaves were dried between two pieces of tissue to remove any remaining water. Afterwards, the turgid weight (TW) was measured followed by incubation of the

samples in the incubator at 70 °C for 48 hours then the dry weight (DW) was taken. The followed formula was used to calculate the relative water content.

Relative water content RWC = (FW – DW) / (TW – DW) × 100.

2.37 Measuring of the shoot length, fresh and dry weight

The length of the shoots were measured from the junction of shoot and root to the shoot top using a measuring tape. The fresh weight was taken immediately after cutting the plant off whereas the dry weight was measured by drying the plants at 80 °C for 3 days to give a constant weight.

2.38 Metabolomics analysis

GC-MS was used to estimate the metabolomics compounds according to the method described by Foito *et al*. (2009). Freeze dried shoots were ground by using pestle and mortar to a fine powder. Approximately 20 mg was accurately weighed and recorded, afterward the samples were extracted with 3 ml 100% methanol following addition of 100 µl of each internal standard, which were ribitol 2 mg/ml for the polar, and methanolic methyl nonadecanoate 0.2 mg/ml for the non-polar compounds. The mixture was incubated on a shaker at 30 °C for 30 minutes. After which, 750 µl of distilled water and 6 ml of chloroform were added sequentially, and after each addition followed by incubation on a shaker at 30 °C for 30 minutes. Then 1.5 ml of distilled water was added and the solution was manually mixed vigorously and centrifuged for 10 minutes, generating a biphasic system. The upper layer (polar) and the lower layer (non-polar) were separated out by pipette and moved to clean tubes. The polar fraction could be stored at -20 °C awaiting further processing whereas the non-polar fraction was immediately subjected to the next process.

Non-polar fraction derivatisation: 4 ml of the non-polar fraction was evaporated to dryness in a centrifugal evaporator. Then, 1 ml of chloroform and 2 ml of 1% (v/v) methanolic sulphuric acid were added and the mixture incubated overnight at 50 °C for transesterification. The solution was manually mixed and left to separate into two layers. The upper layer was removed whereas the lower chloroform layer was shaken with 3 ml of 2% (w/v) potassium carbonate. The extract was left to separate into two layers, the lower chloroform layer was collected and dried by passage through a short column of anhydrous sodium sulphate in a Pasteur pipette and then evaporated to

dryness followed by dissolving in 40 µl of chloroform. 10 µl of pyridine and 80 µl of N-methyl-N-(trimethylsilyl)-trifluroacetamide (MSTFA) were added and incubated at 37 °C for 30 minutes for silylation. A subsample of 40 µl was added to an autosampler vial containing a retention standard mixture (evaporated 50 µl of undecane, tridecane, hexadecane, eicosane, tetracosane, tetratriacontane and octatriacontane; each at concentration of 0.2 mg/ml in isohexane). The sample was diluted (1:1) by adding 40 µl of pyridine and analysed by GC-MS.

Polar fraction derivatisation: 250 µl of the polar fraction was pipetted into a clean tube and incubated in centrifugal evaporator until totally dry. 80 µl of methoxylamine hydrochloride (20 mg/ml) in anhydrous pyridine was added and the sample was oximated at 50 °C for 4 hours. After cooling at room temperature, the samples were silylated at 37 °C for 30 minutes in presence of 80 µl of N-methyl-N-(trimethylsilyl)-trifluroacetamide (MSTFA). A subsample was prepared as described for the non-polar fraction.

Sample analysis: Metabolite profiles were estimated using a GC-MS (DSQII Thermo-Finnigan, UK) system. 1 µl of each sample was injected into a programmable temperature vaporising (PTV) injector with a split ratio of 80:1 according to the following conditions: injection temperature 132 ^{o}C for 1 min, transfer rate 14.5 ^{o}C/s, transfer temperature 320 ^{o}C for 1 min, clean rate 14.5 ^{o}C/s and the clean temperature 400 ^{o}C for 2 min. The process of chromatography was carried on a DB5-MS^{TM} column (15 m x 0.25 mm x 0.25 µm; J&W, Folsom, USA) using helium at 1.5 ml/min in constant flow mode. The GC temperatures were 100 ^{o}C for 2.1 min, then 25 ^{o}C/min to 320 ^{o}C, and then isothermal for 3.5 min. The interface temperature was 250 ^{o}C. Mass spectra were acquired at 70 eV over the mass range 35 - 900 a.m.u at 4 spectra/s with a source temperature 200 ^{o}C and a solvent delay of 1.3 min. Acquisition rates were set to give approximately ten data points across a chromatographic peak. The software package $X_{CALIBUR}^{TM}$ version 1.4 (Thermo Scientific, Waltham, MA, USA) was used to acquire and analyse the data. The software package AMDIS^{TM} (NST, Boulder, CO, USA) was used to process the raw data files to verify the presence of individual analytes to create a metabolite list. The compounds of barley metabolite were identified depending on comparisons with GC-MS libraries and literature data, analysis of standard and extrapolation of data from known compounds. All statistical analysis of metabolic compounds was conducted by using GenStat program version 9.2.0.153 (VSNi, Hemel Hempstead, UK).

CHAPTER 3 RESULTS

3.1 Bio-informatic database searching

The amino acid sequences from seven publicly available barley MAP kinase clones were compared with the amino acid sequences of the 17 rice MAP kinases by using the Clustal W program. The result is summarized as a phylogenetic tree (Fig. 3.1), which illustrates the relationship between the clones of barley and the rice MAP kinases genes. The relationships between these clones is complicated by the fact that the rice clones have been given several different names by different authors but these relationships are clarified in Table 3.1 which summarises the names from the phylogenetic tree and the published papers related to this subject. The deduced amino acid sequence of the barley clone with the Genebank accession number AK252439 was found to be very similar (88%) to the encoded protein from rice gene *OsBWMK1* (Appendix 5.2) which is also called *OsMPK17-1* (Hamel *et al*. 2006), and *OsMPK12* (Reyna and Yang 2006). This barley clone was chosen for further investigations in this study and the cDNA was named *HvBWMK1* because of the high sequence homology to the previously named and characterised rice *OsBWMK1* (see Appendix 5.1 for the full-length cDNA sequence).

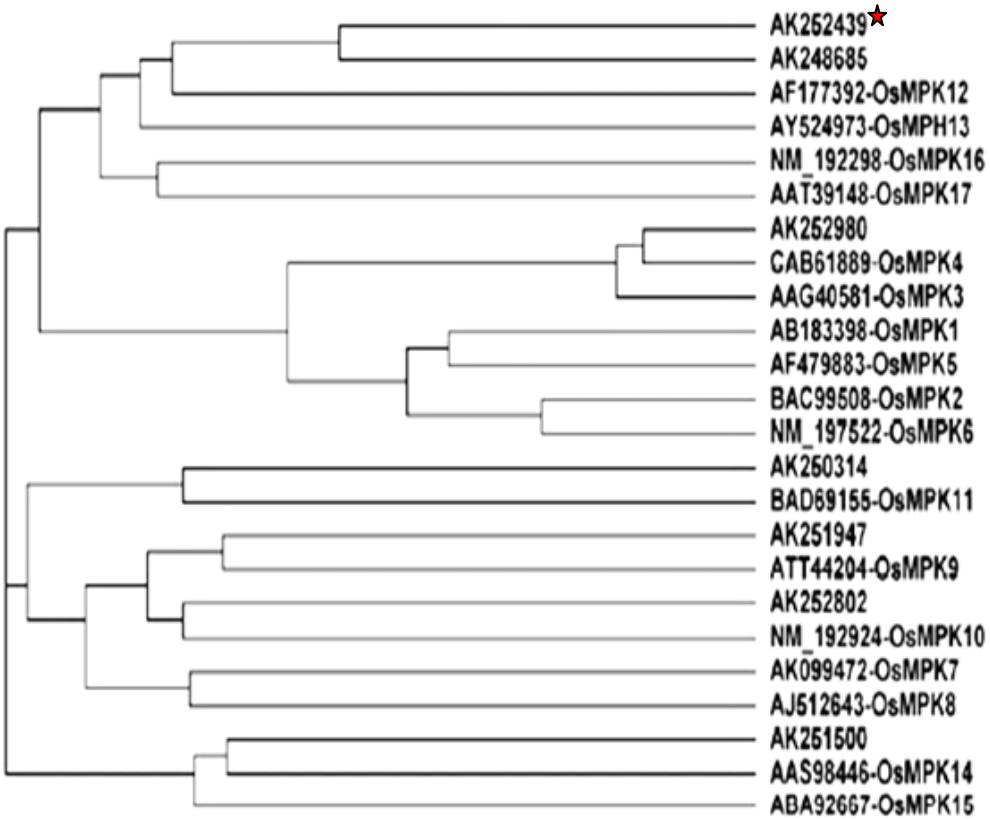


Figure 3.1: Phylogenetic tree illustrating the relationship of the encoded amino acid sequences from available barley cDNA clones (AK) and the 17 rice MAPK genes (Os). ★Barley clone AK252439 named *HvBWMK1*.

Table 3.1: The comparison between barley clones encoding MAP kinase and homologous rice MAP kinases genes.

Barley clone accession number	Information on rice MAP kinases genes					
	TIGR[1] accession number	Hamel[2] identification	NCBI[3] accession number	Reyna[4] identification	NCBI name	Rice chromosome No.
AK252439	Os06g4930	*OsMPK17-1*	AF177392	*OsMPK12*	*OsBWMK1*	6
AK248685	Os06g4930	*OsMPK17-1*	AF177392	*OsMPK12*	*OsBWMK1*	6
AK251947	Os05g50560	*OsMPK20-2*	AAT44204	*OsMPK9*	*OsMAPK9*	5
AK252802	Os01g43910	*OsMPK20-1*	NM-192924	*OsMPK10*	*OsMAPK10*	1
AK252980	Os06g48590	*OsMPK7*	CAB61889	*OsMPK4*	*OsMAPK4*	6
AK250314	Os06g26340	*OsMPK20-3*	BAD69155	*OsMPK11*	*OsMAPK11*	6
AK251500	Os011g17080	*OsMPK16*	AAS98446	*OsMPK14*	*OsMAPK14*	11

TIGR[1] : The Institute for Genomic Research (TIGR).
Hamel[2] : Hamel *et al*. 2006.
NCBI[3] : The Natural Center for Biotechnology Information (NCBI).
Reyna[4] : Reyna and Yang 2006.

3.2 *HvBWMK1* cloning and transformation

3.2.1 Amplification of *HvBWMK1* gene

The *HvBWMK1* coding region was amplified by PCR from the cDNA in pBluescript(pflc111)-flbaf180a19 plasmid using *Taq* polymerase enzyme first to check the ability of the program to amplify the open reading frame (ORF) of *HvBWMK1*, with an expected size of 1737 bp (Fig. 3.2 A). The same cycling parameters were then used but with the *Pfu* polymerase enzyme. The results of PCR products were visualized under UV light after gel electrophoresis (Fig. 3.2 B). The *Pfu* generated PCR products were digested with endonuclease restriction enzyme *Xba*I to obtain sticky ends and then purified from an agarose gel (Fig. 3.2 C).

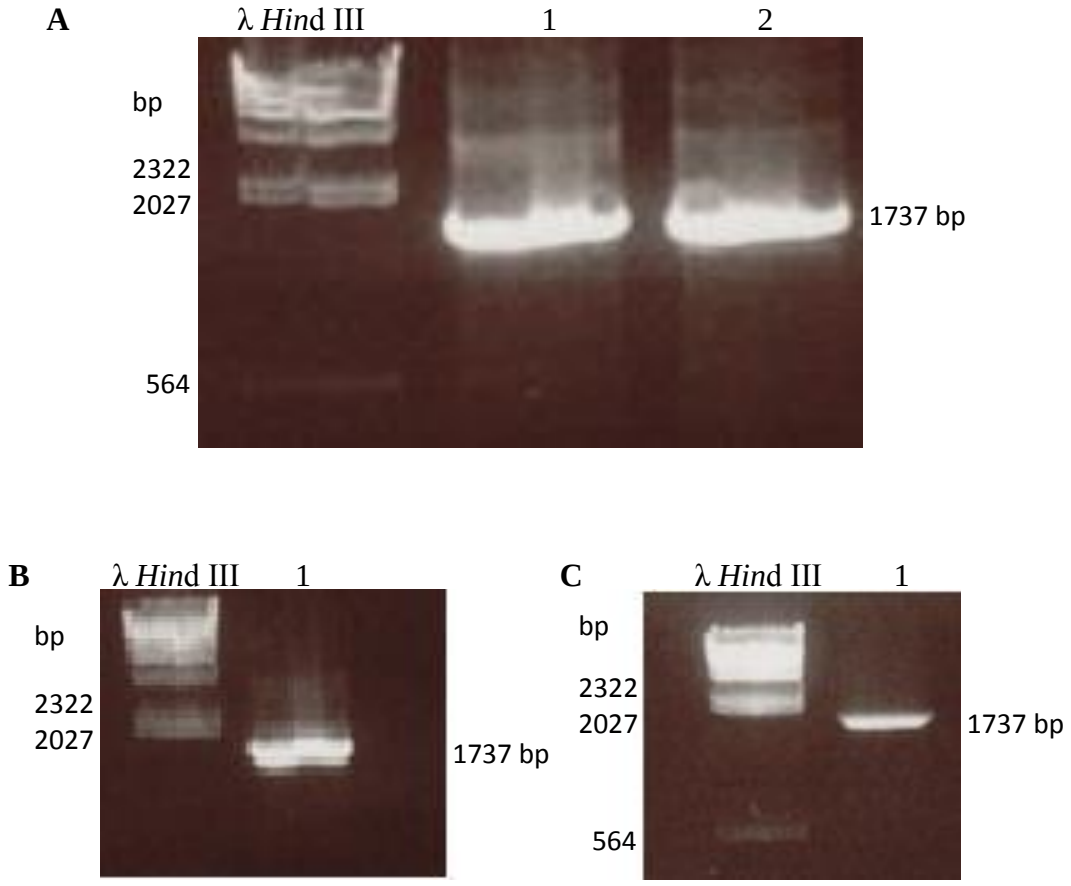


Figure 3.2: Agarose gel electrophoresis of *HvBWMK1*.
(**A**) *Taq* polymerase amplified PCR product using *HvBWMK1* cDNA as a template. λ *Hin*d III marker, 1 and 2: 5 µl each of two amplifications of *HvBWMK1*, expected size 1737 bp. (**B**) *Pfu* polymerase amplified PCR product using *HvBWMK1* cDNA as a template. λ *Hin*d III marker, 1: 5µl of amplification of *HvBWMK1*, expected size 1737 bp. (**C**) λ *Hin*d III marker, 1: purified *HvBWMK1*, 1737 bp.

3.2.2 Combination between *HvBWMK1*, promoter and terminator

The mini-prep DNA plasmid product of the vector pTopoActin-OCS (Fig. 3.3 A) which contains the rice *Actin* promoter and the *OCS* terminator was digested with the endonuclease restriction enzyme *Xba*I present in the multiple cloning site between actin and OCS to provide compatible sticky ends with the *HvBWMK1* gene and then purified. The expected size of 5810 bp was obtained with a concentration of 40 ng/µl (Fig. 3.3 B). To combine *HvBWMK1* to the promoter and terminator, 5810 bp and 1737 bp DNA fragments of vector and insert, respectively, were ligated with the molar ratio of 1:3 (vector : insert) to create the target constructions, overexpression and antisense, as the

insert can ligate in either orientation (Fig. 3.4). The following formula was used to calculate the amount of insert.

Insert mass in ng = 3 × (insert length in bp / vector length in bp) × vector mass in ng.

The new ligated DNA was transformed into *E. coli* competent cells to permit isolation of the desired constructs.

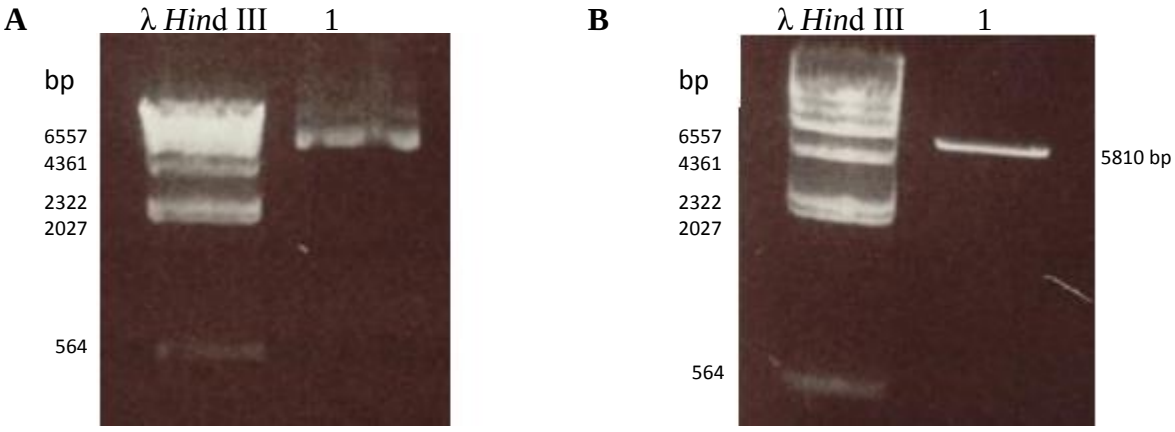


Figure 3.3: Agarose gel electrophoresis of DNA plasmid pTopoActin-OCS.
(A). Mini-prep plasmid DNA, λ *Hin*d III marker. 1: 5µl of undigested plasmid DNA. (B) λ *Hin*d III marker. 1: 5 µl of digested plasmid DNA with the unique endonuclease restriction enzyme *Xba*I, expected size 5810 bp.

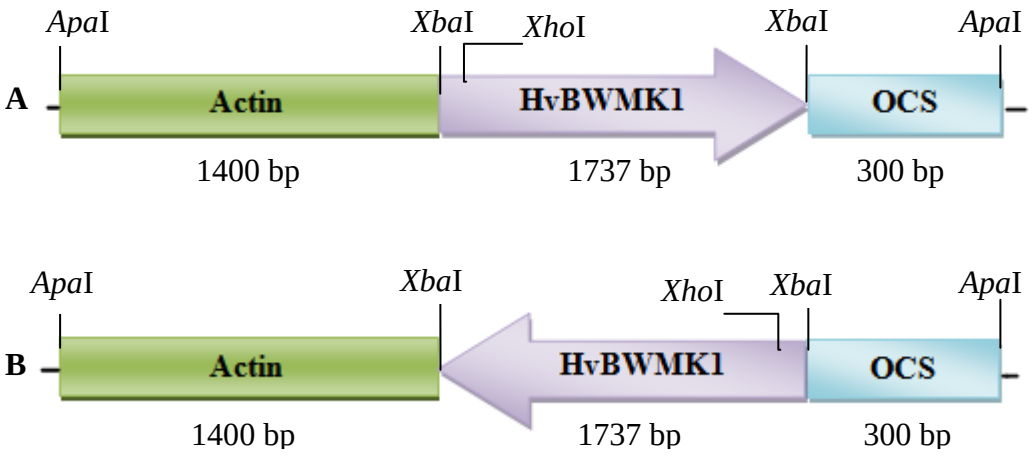


Figure 3.4: The combination between *HvBWMK1* gene, *Actin* promoter and *OCS* terminator.

A. The overexpression orientation.
B. The antisense orientation.

3.2.3 Gene detection and filter-hybridization

HvBWMK1 DNA was labeled with Digoxigenin by PCR (Fig. 3.5) and used as a probe for hybridization to detect colonies bearing the Topo-Actin-OCS vector which contain the target gene *HvBWMK1*. The positive colonies showed a purple color on the filter after hybridization (Fig. 3.6). 10 positive colonies were picked and grown in liquid LB, and analyzed by plasmid mini-prep and DNA restriction enzymes.

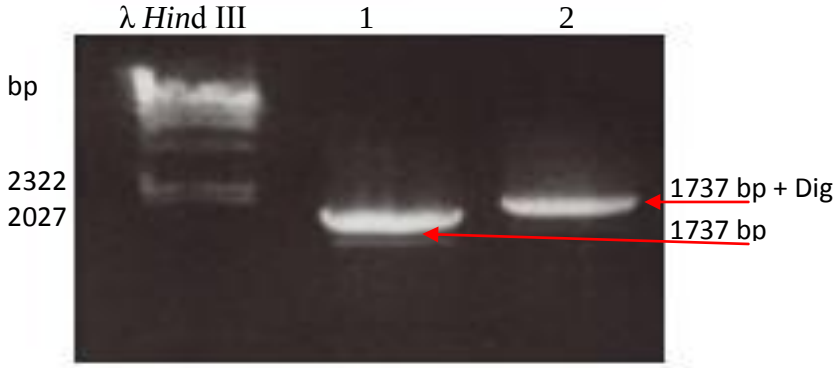


Figure 3.5: Agarose gel electrophoresis of digoxygenin labeled *HvBWMK1* DNA.
λ *Hin*d III marker. 1: 5 µl of amplified *HvBWMK1*, expected size 1737 bp. 2: 5 µl of amplified *HvBWMK1* DNA labeled with Digoxigenin. A size shift resulting from the incorporated digoxygenin can be seen.

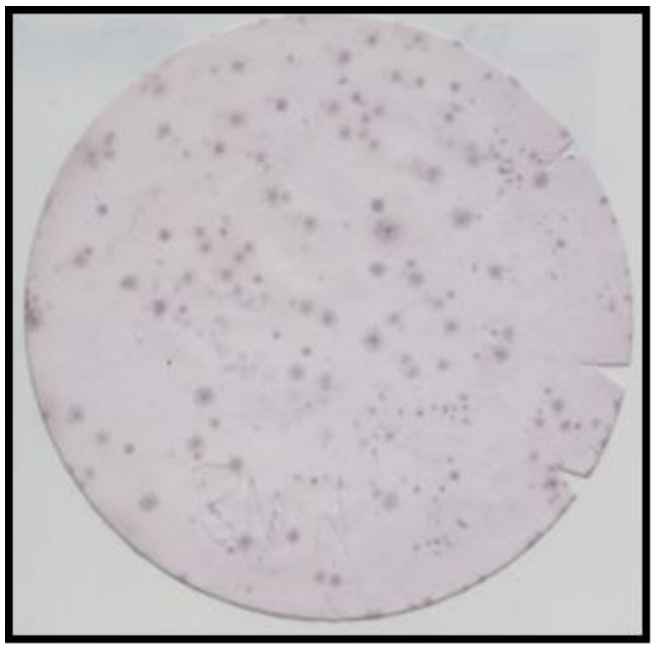


Figure 3.6: Filter-hybridization showed the positive colonies containing *HvBWMK1*.

3.2.4 Determination the orientation of *HvBWMK1*

The DNA of the recombinant plasmid pTopoActin-HvBWMK1-OCS (Fig. 3.7 A) was digested with *Apa*I to ensure the presence of *Actin-HvBWMK1-OCS* fragment where the band of 3400 bp was obtained (Fig. 3.7 B), then the DNA was digested with endonuclease restriction enzyme *Xho*I to determine the orientation of the gene, *Xho*I has restriction site at the beginning of actin promoter (Fig. 2.2) and restriction site at 118 bp within *HvBWMK1* fragment (Appendix 5.5), the predicted overexpression bands 1500 bp and 6000 bp and the predicted antisense bands 3000 bp and 4500 bp were obtained in plasmid DNA from individual colonies (Fig. 3.7 C).

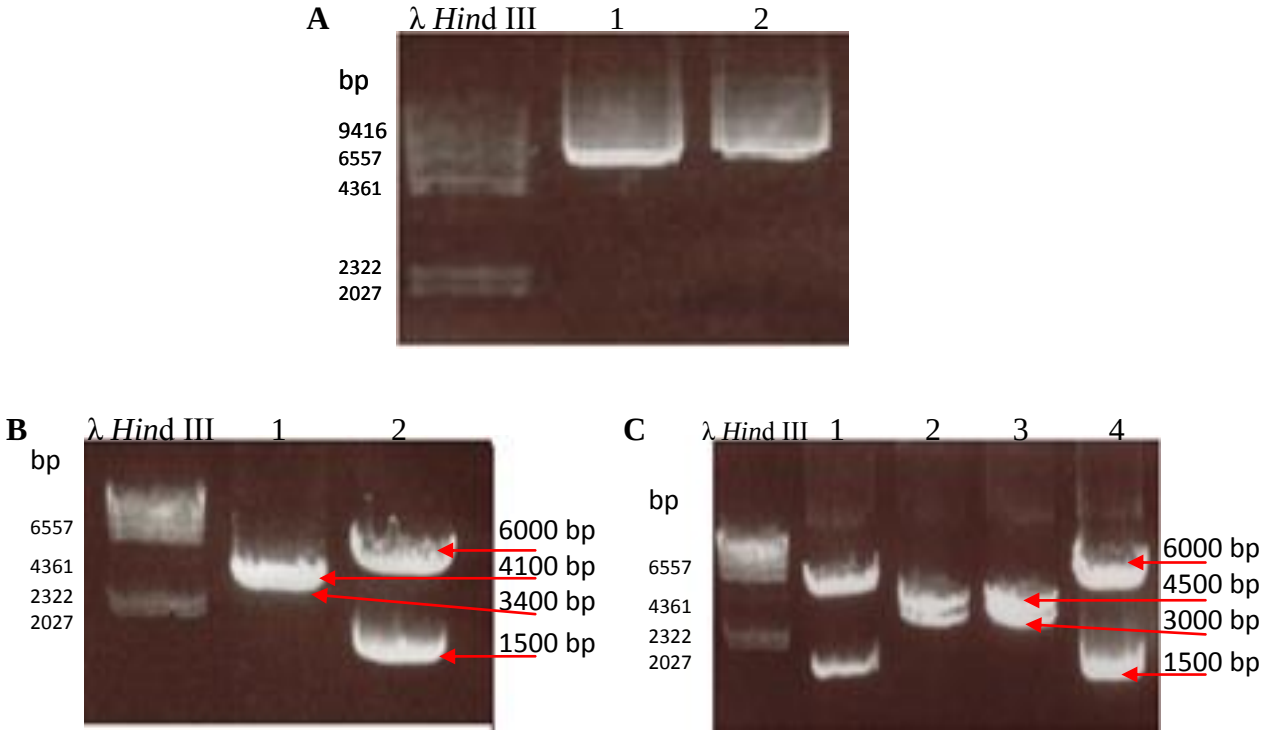


Figure 3.7: Agarose gel electrophoresis of plasmid DNA of recombinant plasmid pTopoActin-HvBWMK1-OCS.

(**A**) λ *Hind III* marker. 1 and 2 undigested plasmid DNA of pTopoActin-HvBWMK1-OCS. (**B**) λ *Hin*d III marker. 1 plasmid DNA of pTopoActin-HvBWMK1-OCS digested with *Apa*I, expected size 3400 bp and 4100 bp. 2 plasmid DNA of pTopoActin-HvBWMK1-OCS digested with *Xho*I, expected size 1500 bp and 6000 bp. (**C**) λ *Hin*d III marker. 1 and 4 plasmid DNA of pTopoActin-HvBWMK1-OCS digested with *Xho*I, expected size 1500 bp and 6000 bp confirmed positive overexpression. 2 and 3 plasmid DNA of pTopoActin-HvBWMK1-OCS digested with *Xho*I, expected size 3000 bp and 4500 bp confirmed positive antisense.

3.2.5 Sequencing of cloning *HvBWMK1* gene

The coding region of the *HvBWMK1* overexpression construct was fully sequenced in order to obtain a clone with the full native sequence. The sequencing result was showed 100% identity between the cloned *HvBWMK1* sequence and the original sequence of the gene (Appendix 5.3), thus the sequence alignment of amino acids were identical (Appendix 5.4).

3.2.6 Actin-HvBWMK1-OCS constructs joining with T-DNA binary vector

The plasmid DNA of T-DNA binary vector pWBVec8 (Figs. 2.3 for the map and 3.8 A for gel analysis) was digested with the unique endonuclease restriction enzyme *Apa*I and purified, expected size 11280 bp. (Fig. 3.8 B). Also the DNA plasmid of pTopoActin-HvBWMK1-OCS for both constructions, overexpression and antisense were digested with endonuclease restriction enzyme *Apa*I to cut out the fragment Actin-HvBWMK1-OCS, expected size 3400 bp.

50 ng/µl DNA of the overexpression and antisense Actin-BWMK1-OCS fragments were obtained and ligated into the T-DNA binary vector pWBVec8 with the molar ratio of 1:3 (vector : insert) to create the target T-DNA constructions, overexpression and antisense (Fig. 3.9). The ligated DNA was transformed into *E. coli* and then 1 µl of *E. coli* bacterial solution from selected colonies was used as a template for PCR amplification with *HvBWMK1* primers to determine the positive colonies (Figs. 3.10 A and 3.11 A). Purified mini-prep plasmid DNA from the positive colonies for both orientations were transformed into *Agrobacterium tumefaciens* and another PCR reaction was conducted using transformed *Agrobacterium* colonies as a template with *HvBWMK1* primers to choose the positive colonies for the next step (Figs. 3.10 B and 3.11 B)

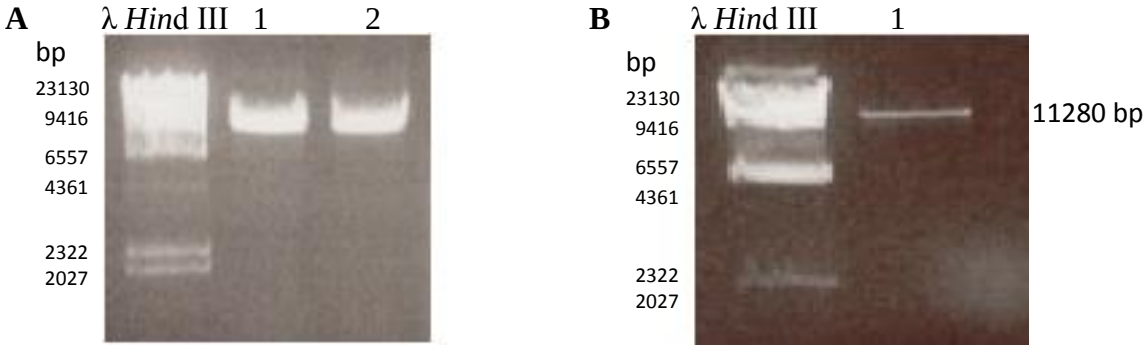


Figure 3.8: Agarose gel electrophoresis of plasmid DNA of the binary vector pWBVec8.

(**A**) Mini-prep of plasmid DNA. λ *Hin*d III marker. 1 and 2, digested DNA with *Apa*I, expected size 11280 bp. (**B**) digested and purified DNA. λ *Hin*d III marker. 1, digested and purified plasmid DNA of the binary vector pWBVec8, expected size 11280 bp.

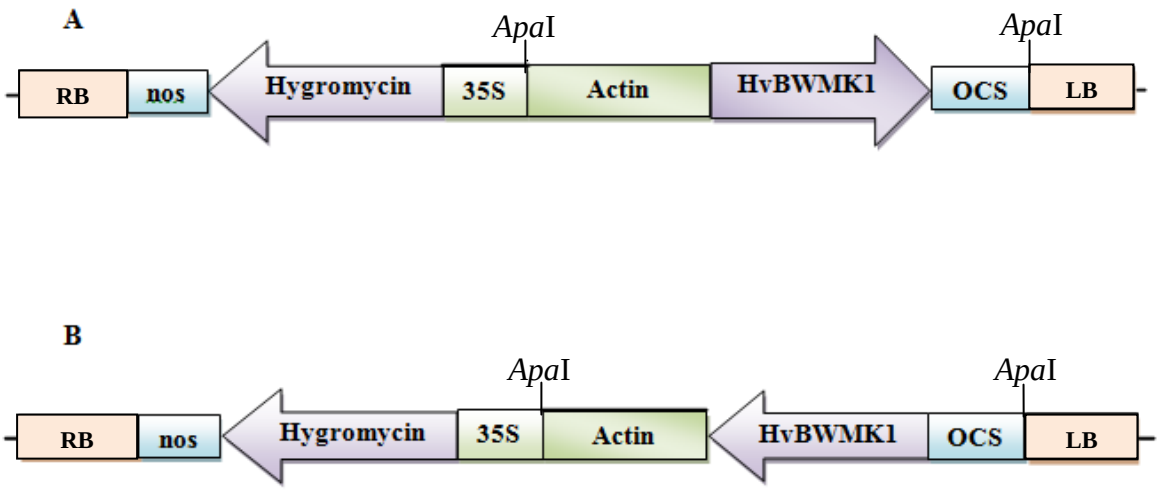


Figure 3.9: The T-DNA constructions containing the barley gene *HvBWMK1* driven by *Actin* promoter and *OCS* terminator.

A. The overexpression orientation construct.

B. The antisense orientation construct.

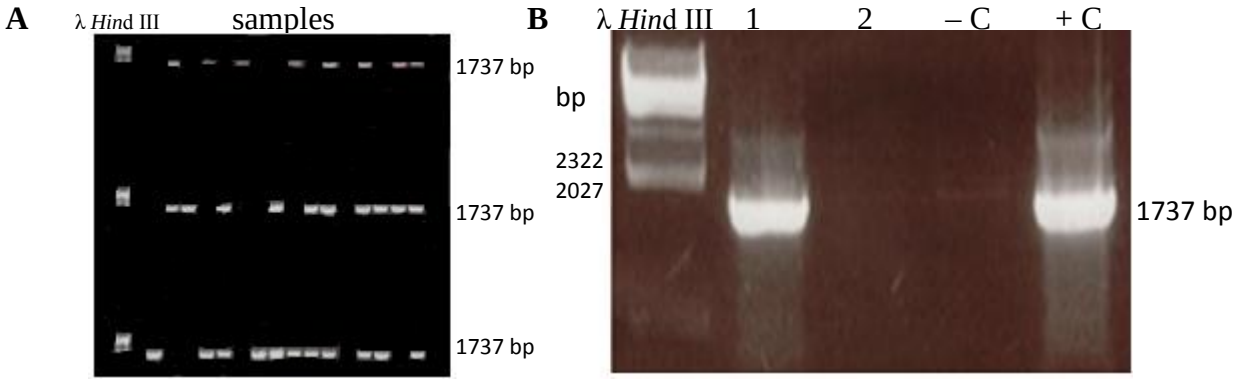


Figure 3.10: Agarose gel electrophoresis of PCR products of the overexpression *HvBWMK1* gene present in recombinant plasmid pWBVec8Actin-HvBWMK1-OCS.

A- λ *Hin*d III marker. Positive bands 1737 bp of *HvBWMK1* transformed into *E. coli.* **B**- λ *Hin*d III marker. 1, negative sample. 2, positive band 1737 bp of *HvBWMK1* transformed into *Agrobacterium tumefaciens.* – C = PCR negative control. + C = PCR positive control.

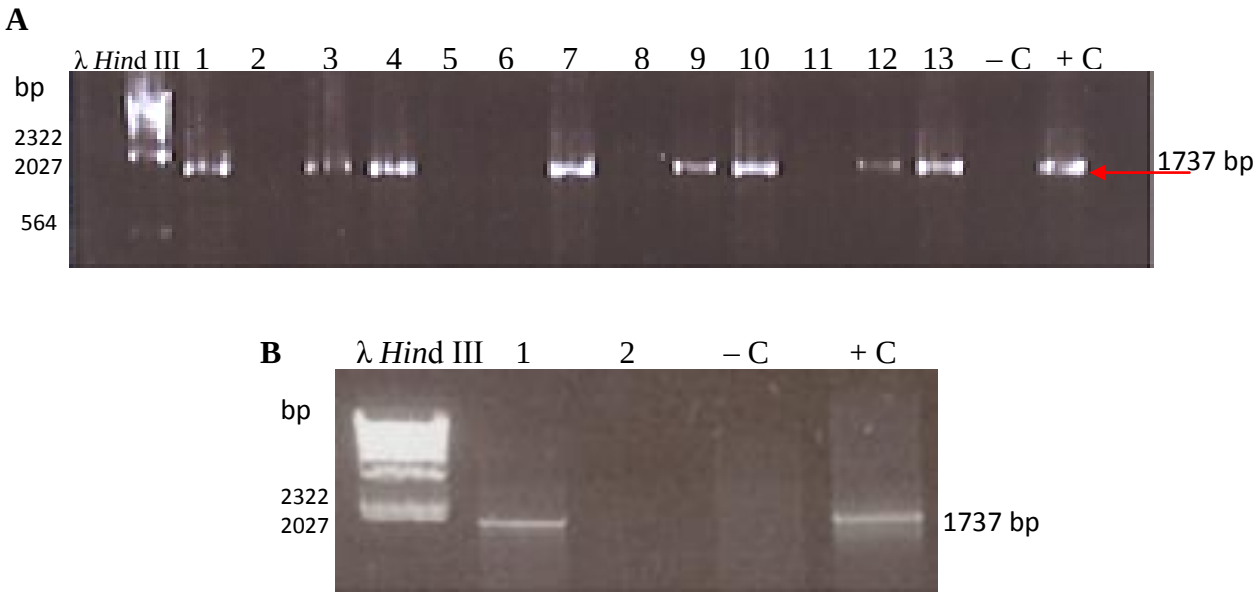


Figure 3.11: Agarose gel electrophoresis of PCR products of the antisense *HvBWMK1* gene present in recombinant plasmid pWBVec8-Actin-HvBWMK1-OCS.

A- pWBVec8-Actin-HvBWMK1-OCS transformed into *E .coli*. λ *Hin*d III marker. 1, 3, 4, 7, 9, 10, 12 and 13 positive bands 1737 bp of *HvBWMK1*. 2, 5, 6, 8 and 11 negative samples. – C = PCR negative control. + C = PCR positive control. **B**- pWBVec8-Actin-HvBWMK1-OCS transformed into *Agrobacterium tumefaciens.* λ *Hin*d III marker. 1, positive band 1737 bp of *HvBWMK1*. 2, negative sample. – C = PCR negative control. + C = PCR positive control.

3.3 Barley transformation

The supervirulent *Agrobacterium tumefaciens* strain EHA105 was used to perform the plant transformation. The *Agrobacterium* was first transformed with binary vector pWBVec.8 which contained the required gene *HvBWMK1* and the hygromycin gene as selectable marker for callus growth and roots and shoot differentiataion during the tissue culture process, and also later for seed segregation.

The percentage of successful transformation using *Agrobacterium*-mediated transformation was around 10%, where 28 transgenic plants were obtained from 270 immature cultured embryos (Table 3.2). The transformation steps were carried out according to the protocol of Harwood *et al.* (2009) with some modifications taken from Tingay *et al.* (1997) and Bartlett *et al*. (2008). After the infection of immature embryos with the *Agrobacterium* cells, different stages of tissue culture were carried out including callus selection, regeneration and rooting stage as shown in Fig. 3.12.

3.3.1 Morphology of transgenic barley plants

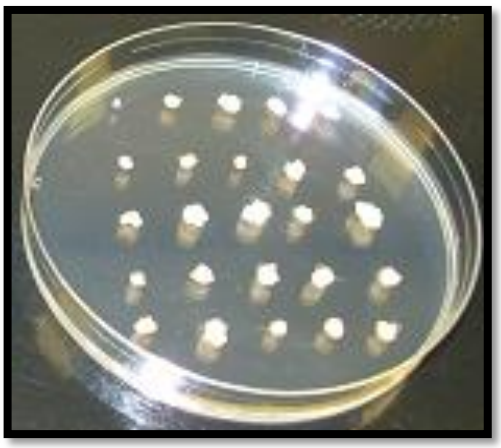
The primary transformants are termed T_0, and the first generation of seeds the T_1. All transgenic barley plants lines of (T_0 and $T_{1)}$ showed similar growth pattern compared with wild type plants during different developmental phases under growth room conditions, with no obvious phenotypic mutations.

Table 3.2: Transformation efficiency.

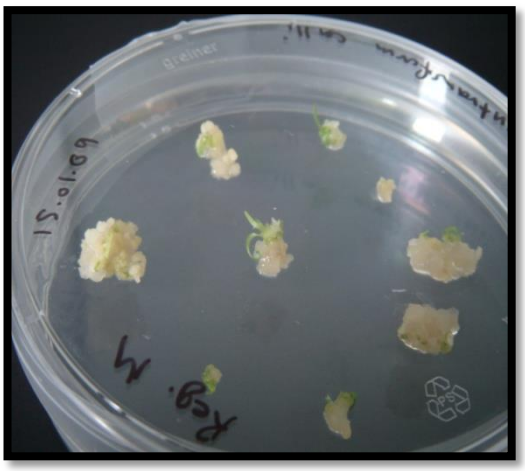
Construct	No. of cultured embryos	No. of plants regenerated*	No. of plants producing seeds	Transformation %
Overexpression	120	13	9	10.8
Antisense	75	8	7	10.6
Empty vector	75	7	3	9.3
Total	270	28	19	10.37

* All regenerated plants were healthy and performed good growth, but because of the growth room chamber size some of these plants were omitted before tillering stage. No infertile plants were recorded.

After infection with bacteria.

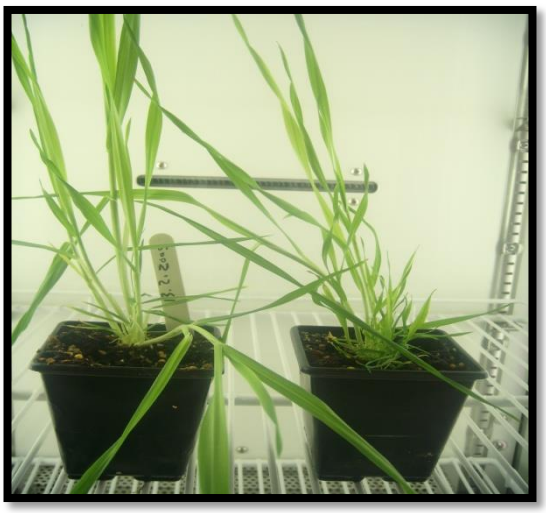


Selection stage.



Regeneration stage.

Rooting stage.



Adaptation stage.

Growth in soil.

Figure 3.12: The different stages of transformed barley from immature embryo infections until growth in soil.

3.4 Transgene detection

The presence of the T-DNA construction (the hygromycin phosphotransferase gene and *Actin-HvBWMK1-OCS*) in the genome of transgenic barley plants regenerated after transfection was detected by PCR analysis using plant genomic DNA as a template. The transgenic barley lines analysed included *HvBWMK1* overexpression transformants, *HvBWMK1* antisense transformants and empty vector pWBVec.8 transformants.

The genomic DNA was extracted from leaves from each line and to investigate its quality was checked with tubulin primers as showed in Figs. 3.13, 3.15 and 3.18 with the expected size 217 bp. Then to reveal the presence of the hygromycin phosphotransferase gene, the genomic DNA from all transgenic lines (overexpression, antisense and empty vector) were amplified by PCR with hygromycin primers where in each case a 1068 bp fragment was obtained (Figs. 3.14, 3.16, and 3.19). After that specific PCR reactions were conducted to ensure the presence the target transformed construct. The PCR amplification using *HvBWMK1* reverse primer and *OCS* reverse primer indicated the presence of *HvBWMK1* in antisense direction for antisense transgenic plants, the expected size was 2000 bp (Fig. 3.17), whereas the PCR amplification using *HvBWMK1* forward primer and *OCS* reverse primer indicated the presence of *HvBWMK1* gene in the sense direction for overexpression transgenic lines with expected size 2000 bp as shown in Fig. 3.20.

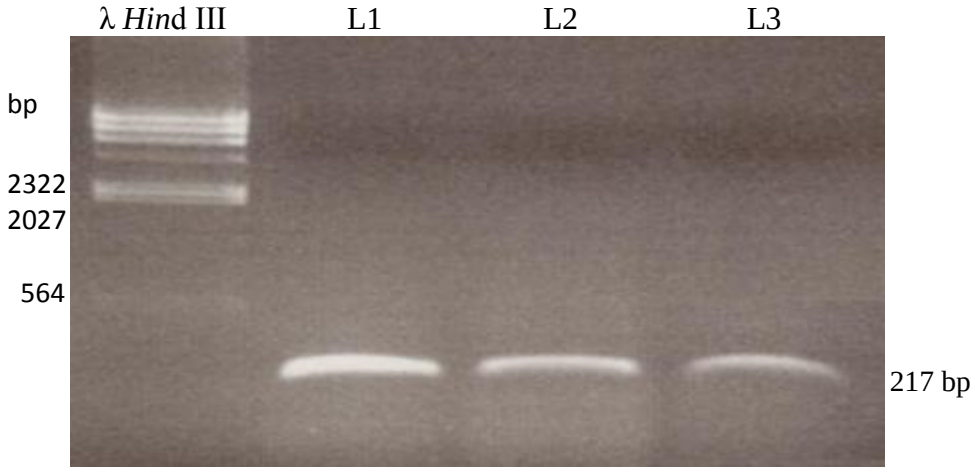


Figure 3.13: PCR products generated using genomic DNA from transgenic barley plants transformed with empty binary vector (pWBVec.8) lines (L1, L2, and L3) with tubulin gene primers (217 bp).

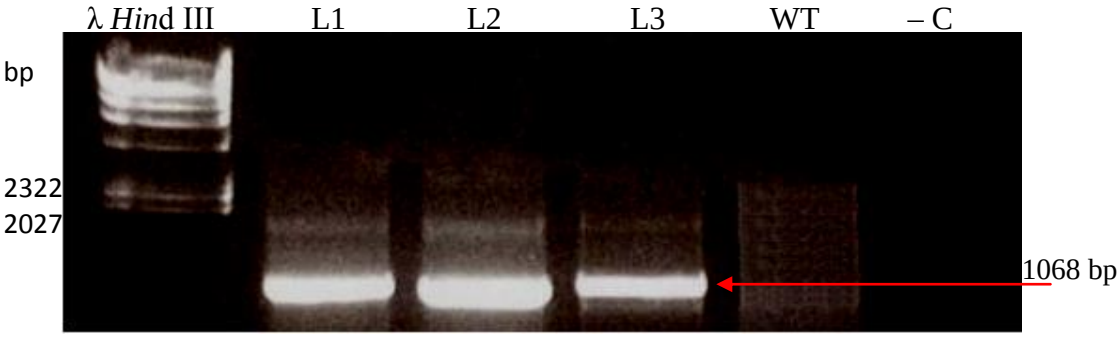


Figure 3.14: PCR products with hygromycin phosphotransferase gene primers (1068 bp) generated using genomic DNA from transgenic barley plants transformed with empty binary vector (pWBVec.8) lines (L1, L2, and L3).
WT = Wild type control (untransformed plant).
– C = PCR negative control.

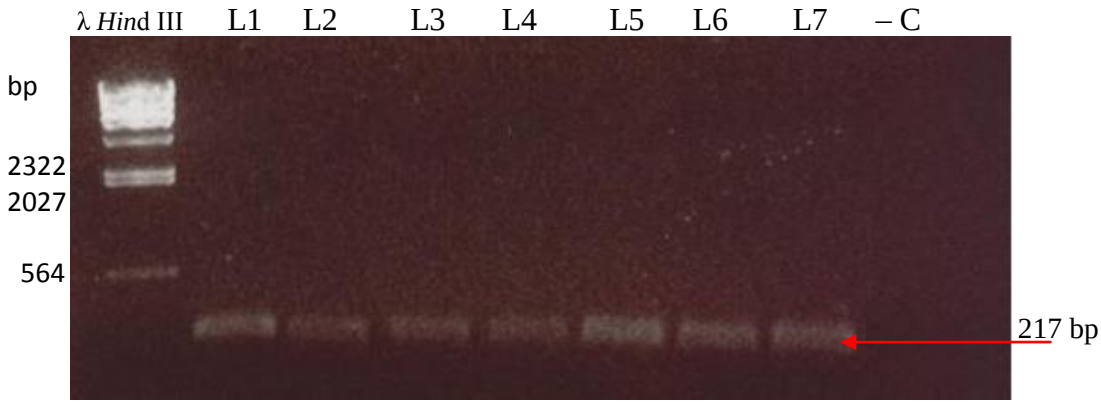


Figure 3.15: PCR products with tubulin gene primers (217 bp) generated using genomic DNA from transgenic barley plants transformed with antisense *HvBWMK1* gene.
L1, L2, L3, L4, L5, L6, and L7 represent different lines of antisense transgenic plants.
– C = PCR negative control.

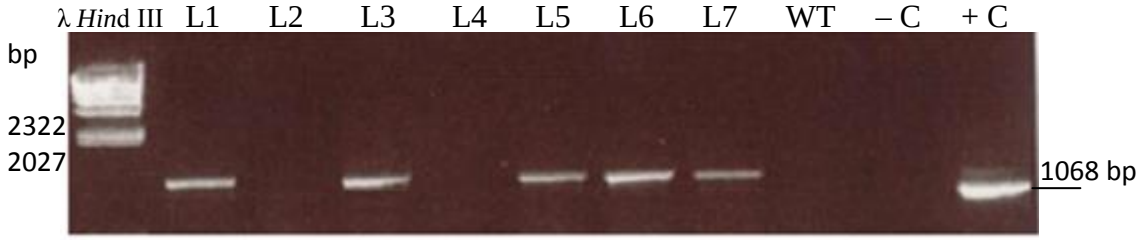


Figure 3.16: PCR products with hygromycin phosphotransferase gene primers (1068 bp) generated using genomic DNA from transgenic barley plants transformed with antisense *HvBWMK1* gene.

L1, L2, L3, L4, L5, L6 and L7 represent different lines of antisense transgenic plants.

WT = Wild type control (untransformed plant).

– C = PCR negative control.

+ C = PCR positive control.

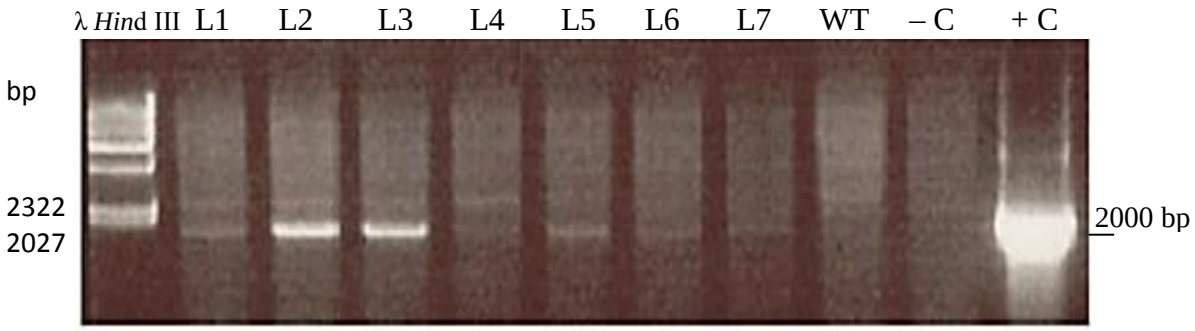


Figure 3.17: PCR products with *HvBWMK1* reverse primer and *OCS* reverse primer (2000 bp) generated using genomic DNA from transgenic barley plants transformed with antisense *HvBWMK1* gene.

L1, L2, L3, L4, L5, L6 and L7 represent different lines of antisense transgenic plants.

WT = Wild type control (untransformed plant).

– C = PCR negative control.

+ C = PCR positive control.

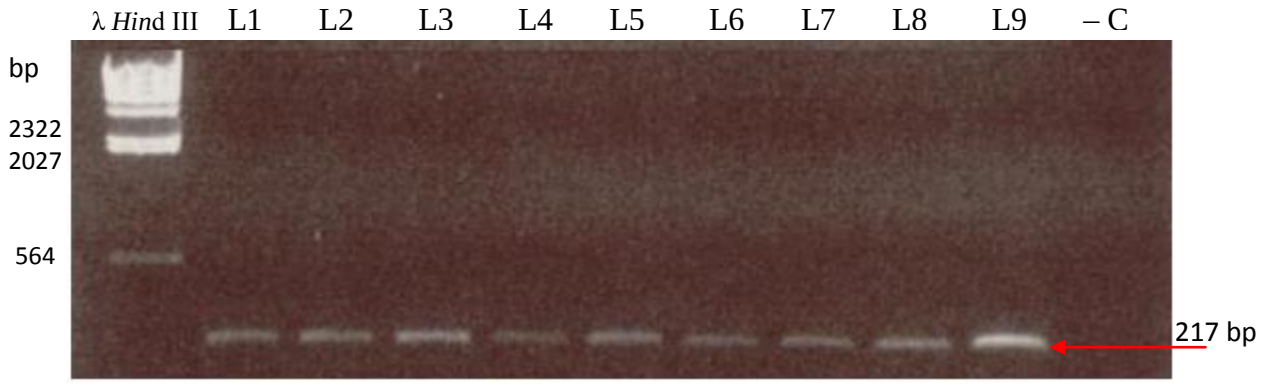


Figure 3.18: PCR products using tubulin gene primers (217 bp) generated using genomic DNA from transgenic barley plants transformed with overexpression *HvBWMK1* gene.

L1, L2, L3, L4, L5, L6, L7, L8 and L9 represent different lines of overexpression transgenic plants.

– C = PCR negative control.

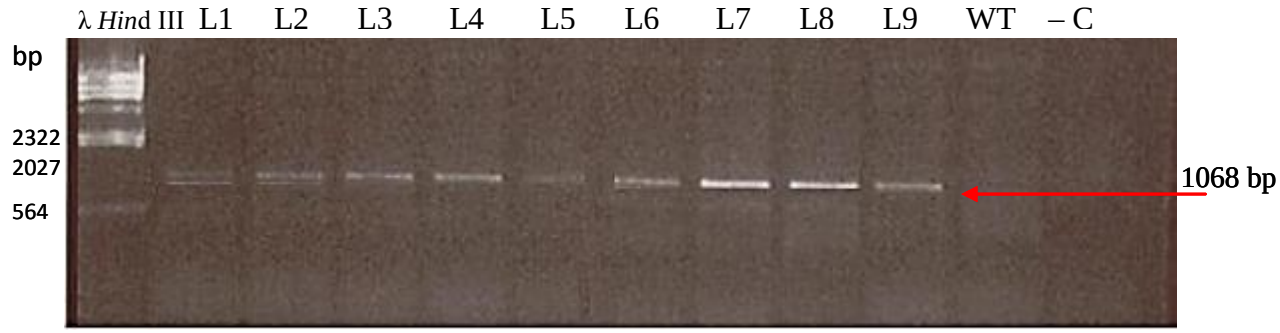


Figure 3.19: PCR products with hygromycin phosphotransferase gene primers (1068 bp) generated using genomic DNA from transgenic barley plants transformed with overexpression *HvBWMK1* gene.

L1, L2, L3, L4, L5, L6, L7, L8 and L9 represent different lines of overexpression transgenic plants.

WT = Wild type control (untransformed plant).

– C = PCR negative control.

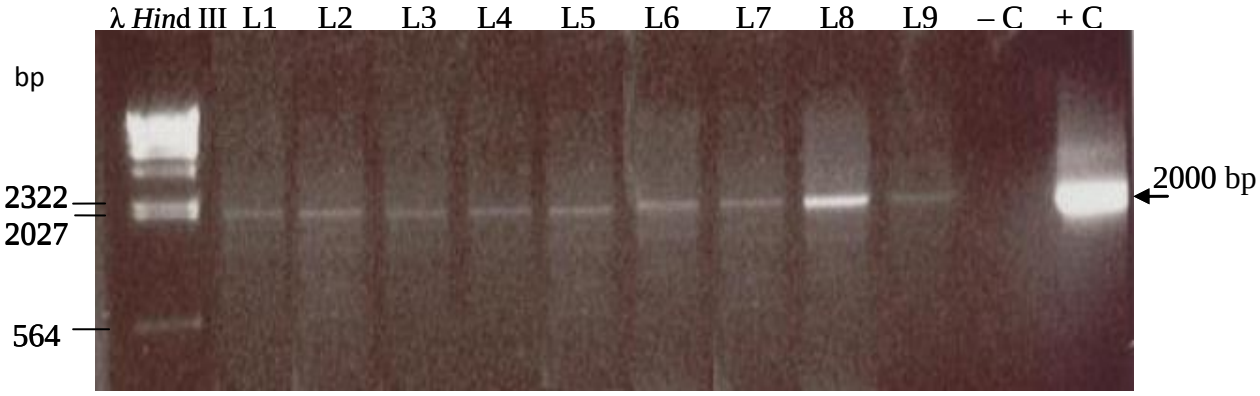


Figure 3.20: PCR products with *HvBWMK1* forward primer and *OCS* reverse primer (2000 bp) generated using genomic DNA from transgenic barley plants transformed with overexpression *HvBWMK1* gene.
L1, L2, L3, L4, L5, L6, L7, L8 and L9 represent different lines of overexpression transgenic plants. – C = PCR negative control. + C = PCR positive control.

3.5 Northern blot analysis for *HvBWMK1*

The wounding process was conducted to check the gene expression of transformed *HvBWMK1* as it is known that the *BWMK1* gene can be activated by wounding (He *et al*. 1999, Agrawal *et al*. 2003c). *HvBWMK1* gene expression was analysed for 7 T_0 lines of wound-stressed plants containing the *HvBWMK1* overexpression construct and another 7 T_0 lines of wound-stressed plants containing the *HvBWMK1* antisense construct alongside a wound-stressed wild type plant. 15 µg of total RNA extracted from the wounded leaves of each barley lines were separated on a denaturing agarose gel (Figs. 3.22 and 3.23), then blotted on positively charged nylon membranes and hybridised with an antisense single-stranded *HvBWMK1* probe (Fig. 3.21).

Northern blot analysis of all 7 wounded antisense primary transformants showed a weaker signal for *HvBWMK1* mRNA compared with the wild type, but with some differences between the lines (Fig. 3.22). Northern blot analysis of all 7 wound-stressed overexpression primary transformants showed a stronger signal for *HvBWMK1* mRNA compared with the wild type for some lines (Fig. 3.23).

From the northern blotting results, the antisense primary transformants lines 3, 5 and 7 alongside with the overexpression primary transformants lines 3, 4 and 5 were chosen to collect T_1 seeds to conduct further experiments.

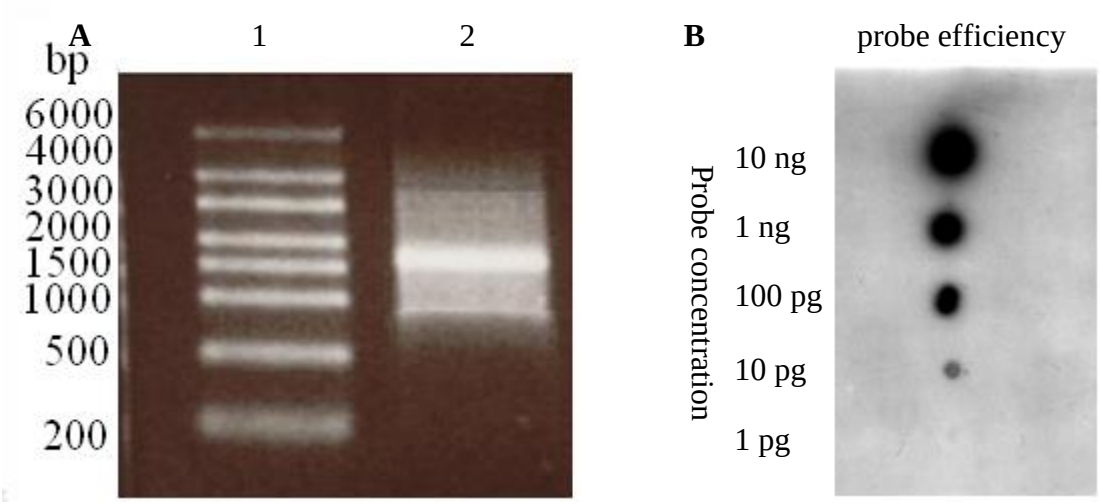


Figure 3.21: Antisense single-stranded *HvBWMK1* probe.

A- Agarose gel electrophoresis of digoxygenin labeled *HvBWMK1*. 1, high range RNA ladder (Fermentas). 2, *HvBWMK1* probe 1737 bp. **B**- X-ray film illustrated the probe efficiency.

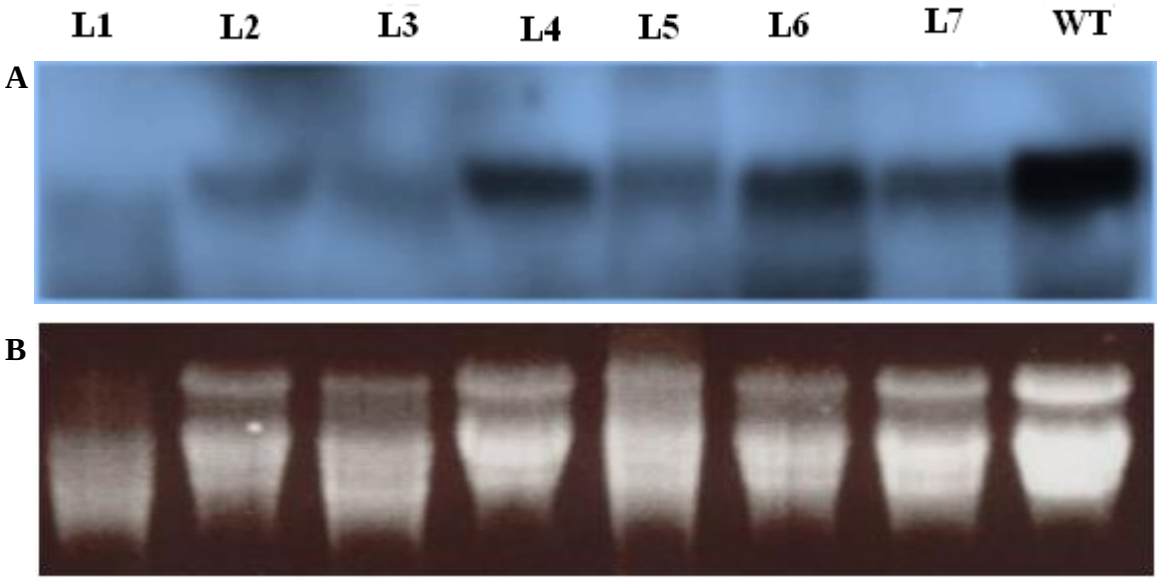


Figure 3.22: Detection of *HvBWMK1* expression in wound-stressed T_0 transgenic barley leaves.

A- X-ray film illustrated the northern blotting of wounded antisense primary transformants showed in all lines a weaker signal for *HvBWMK1* mRNA compared with the wild type. **B**- Denaturing gel of ethidium bromide stained RNA of wounded antisense primary transformants.

WT = Wild type (untransformed plant).

L1, L2, L3, L4, L5, L6 and L7 different lines of antisense *HvBWMK1* transgenic plants.

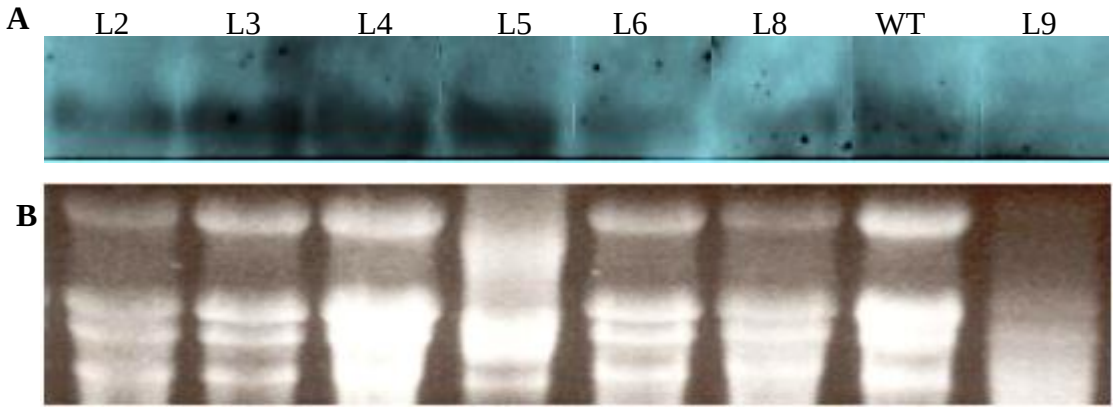


Figure 3.23: Detection of *HvBWMK1* expression in wound-stressed T_0 transgenic barley leaves.

A- X-ray film illustrated the northern blotting of wounded overexpression primary transformants showed a stronger signal of the levels of *HvBWMK1* mRNA in lines 3, 4 and 5 compared with the wild type. **B-** Denaturing gel of ethidium bromide stained RNA of wounded overexpression primary transformants.

WT = Wild type (untransformed plant).

L2, L3, L4, L5, L6, L8 and L9 different lines of overexpression *HvBWMK1* transgenic plants.

3.6 Real-time PCR result

Two weeks old seedlings of wild type and T_1 transgenic barley lines were exposed to 150 mM NaCl for 14 days, then RNA was extracted from leaves and successfully converted to cDNA which was checked in PCR using *HvBWMK1* foreward and reverse primers to amplify *HvBWMK1* gene, the result in Fig 3.24 confirms gene expression.

The real-time PCR was conducted following the method described in section 2.27. The data generated from the real-time PCR was analysed according to relative quantification method ($2^{-\Delta\Delta CT}$ method). The calculations were done according to the equations below.

Fold change = $2^{-\Delta\Delta CT}$

Where:

$\Delta C_T = C_T$ target - C_T reference

$\Delta (\Delta C_T) = \Delta C_T$ treated - ΔC_T untreated

The real-time PCR result is presented as fold changes compared with the reference (wild type), which is set to 1 as summarised in the Fig. 3.25. The lines 3 and 4 of *HvBWMK1* overexpression showed high 1.79 and 1.35 fold-expression respectively compared with wild type but line 5 was 0.89 less than wild type. The *HvBWMK1* antisense lines 3, 5 and 7 showed lower expression 0.86, 0.77 and 0.61 fold respectively compared with wild type.

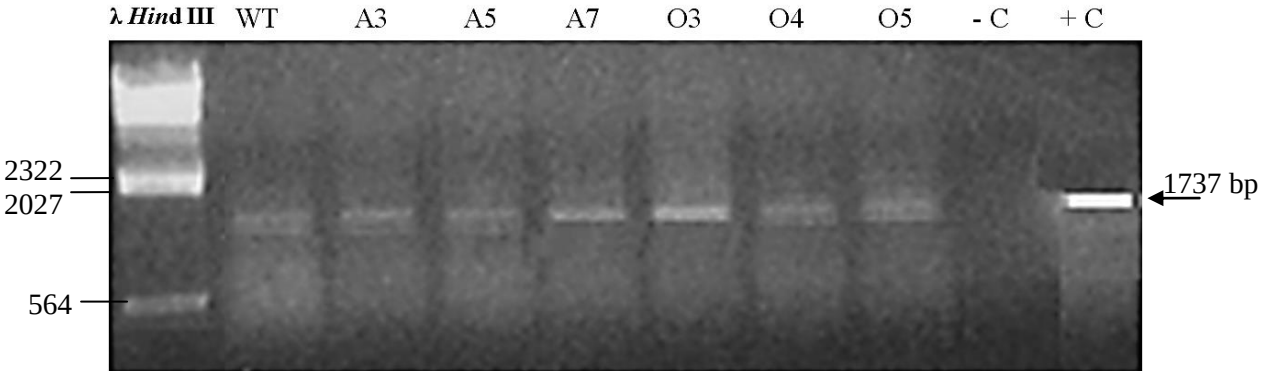


Figure 3.24: PCR products with *HvBWMK1* foreward and reverse primers (1737 bp) generated using cDNA from salt-stressed wild type and T_1 transgenic barley plants transformed with antisense and overexpression *HvBWMK1* gene.
λ *Hind* III marker. WT = wild type (untransformed plant). A3, A5 and A7 = antisense lines 3, 5 and 7. O3, O4 and O5 = overexpression lines 3, 4 and 5.
– C = PCR negative control. + C = PCR positive control (DNA).

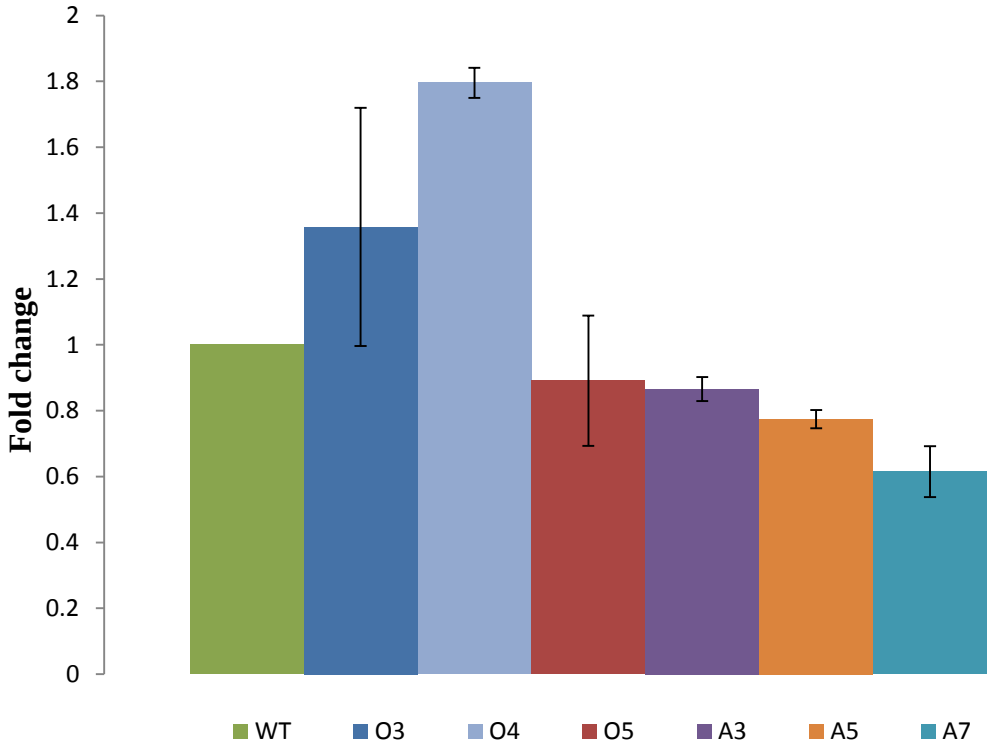


Figure 3.25: The real-time PCR result of detection of *HvBWMK1* expression in salt-stressed wild type and T_1 transgenic barley leaves.
3 replicates for each line. WT = Wild type (untransformed plant). A = Antisense transgenic lines 3, 5 and 7. O = Overexpression transgenic lines 3, 4 and 5.

3.7 The seeds of transgenic barley

3.7.1 Morphology of transgenic barley seeds

Mature dry seeds of T_0 transgenic plants lines and wild type plant which grow under the same conditions were observed by naked eye and dissecting microscope. The observation indicated no differences between transgenic lines and wild type related in seed shape, colour and size except *HvBWMK1* overexpression line 9 and *HvBWMK1* antisense line 4 for both of which the seeds were of a longitudinal shape and bigger size than others (Fig. 3.26).

The weight of 100 seed for each transgenic lines and wild type grown under the same conditions was taken. The statistical model: one-way analysis of variance (ANOVA) in SigmaStat program was used to analyse the results at $P < 0.05$. In case the differences were considered statistically significant followed by Tukey test to perform pairwise multiple comparisons. All antisense transgenic lines, all empty binary vector pWBVec.8 transgenic lines and 5 overexpression transgenic lines produced smaller seeds with

weights significantly less than wild type seed weight. No statistically significant differences were seen between 3 other overexpression transgenic lines and the wild type whereas only one overexpression transgenic line was produced seed significantly bigger than wild type seed (Fig. 3.27).

Figure 3.26: A and B illustrated the similarity and the differences in shape, colour and size between seeds of wild type and T_1 seeds of several transgenic lines.

1 = original wild type (Golden promise). 2 = wild type grown under plant room conditions. 3, 4 and 5 represent empty vector transgenic lines 1, 2, and 3 respectively. 6, 7, 8, 9, 10, 11, 12, 13 and 14 represent overexpression lines 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively. 15, 16, 17, 18, 19, 20 and 21 represent antisense lines 1, 2, 3, 4, 5, 6 and 7 respectively.

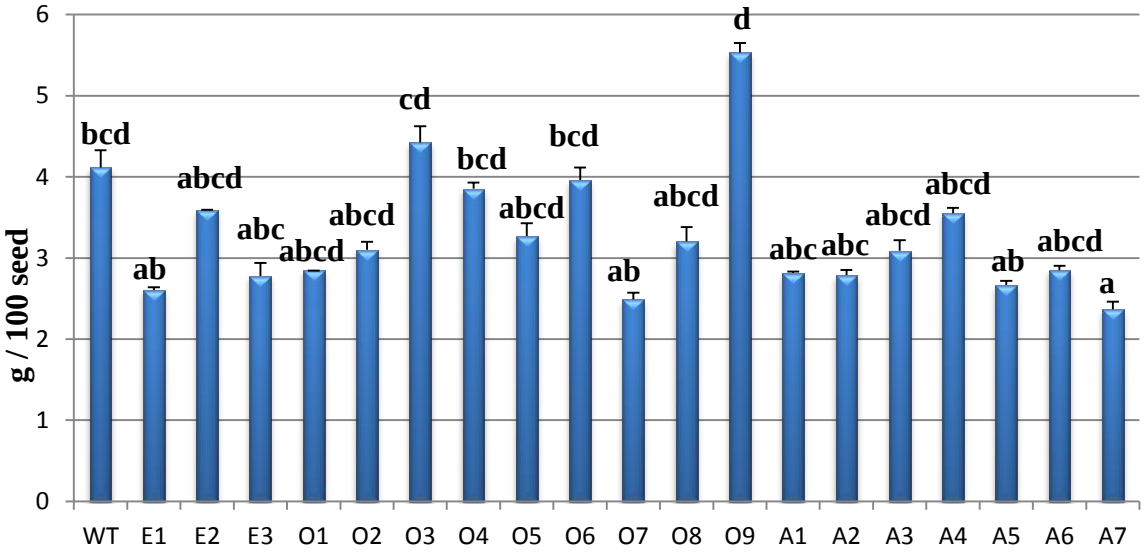


Figure 3.27: Illustrates the average weight of 100 seed of WT and the transgenic lines. Vertical bar illustrates the standard deviation. 3 replicates for each line. Different letters show statistically significant difference ($P < 0.05$). WT = wild type grown under plant room conditions. E = empty vector. O = overexpression transgenic lines. A = antisense transgenic lines.

3.7.2 Transgenic barley seeds segregation

Wild type seed germination was tested under several hygromycin concentrations to determine the suitable concentration needed to prevent germination of non-transgenic plants. The result showed that the concentration 100 µg/ml hygromycin was suitable to prevent the germination of non-transgenic plants (Fig. 3.28). The concentration of 100 µg/ml of hygromycin was used to test transgenic seed segregation.

The hygromycin compound was purchased from two suppliers, one compound prevent totally the germination of wild type seeds at 100 µg/ml (Fig. 3.28), whereas the other one at 100 µg/ml made the wild type seeds produce dwarf seedlings with short roots growing to upper side then convex down (Fig. 3.29).

The T_1 seeds from the T_0 transgenic lines were pre-treated with 0.75% hydrogen peroxide for 24 h to break the dormancy then germinated on two Whatman 3MM filter papers in petri dishes and moistened with water containing 100 µg/ml of hygromycin. The seedlings showed green leaves and developed elongated roots if they were resistant to hygromycin whereas the untransformed plantlets became dwarfed with short roots or developed a phenomenon where the roots grow like spider legs (Figs. 3.29 and 3.30).

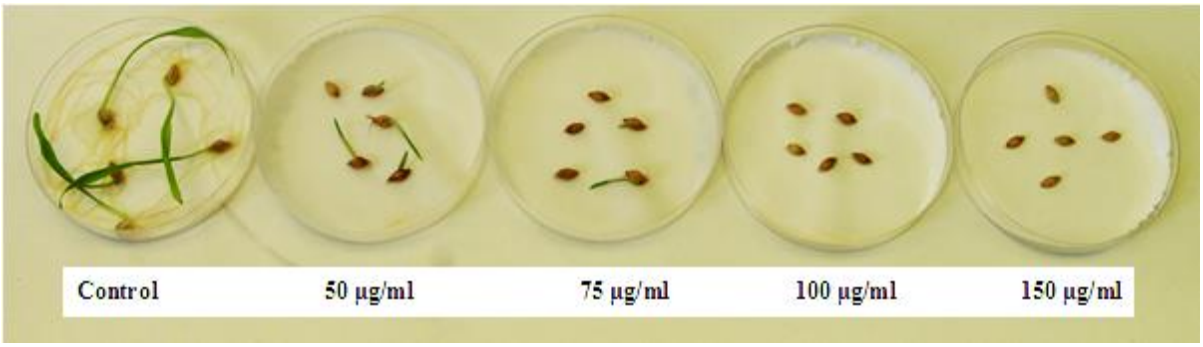


Figure 3.28: The germination of wild type seeds under concentrations of hygromycin for 5 days.

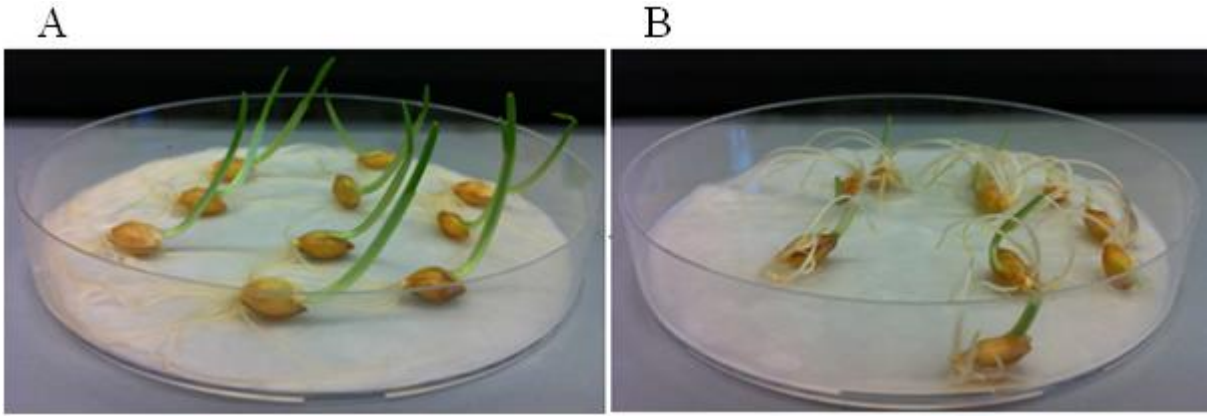


Figure 3.29: Plates illustrated the growth difference between untreated wild type seeds and treated wild type seeds with100 µg/ml of hygromycin for 3 days.
Plate (A) untreated. Plate (B) treated.

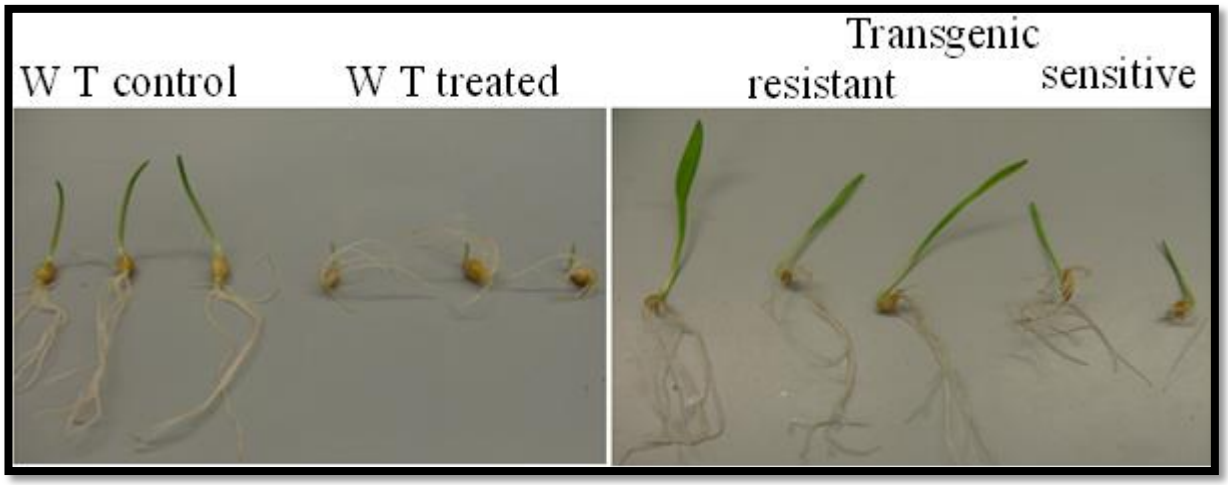


Figure 3.30: Segregation of transgenic seeds under hygromycin treatment compared with wild type, treated wild type and sensitive transgenic showed dwarfing and abnormal root growth.

The hygromycin phenotype of the T_1 progeny of T_0 seeds segregated into both Mendelian and non-Mendelian segregation ratios where three lines showed a higher ratio than expected from a Mendel segregation ratio of 3:1 and on the other hand three lines showed a lower ratio whereas three lines were produced the same Mendel segregation ratio 3:1 (Table 3.3).

Table 3.3: Segregation of barley T_1 lines growing on hygromycin.

T_0 lines name	No. of T_1 seeds tested	Rate of germination	Hygromycin resistance in T_1 generation		Segregation ratio
			Resistant seedlings	Sensitive seedling	
OX L 3	40	100%	31	9	3 : 1
OX L 4	120	100%	91	29	3 : 1
OX L 5	40	100%	26	14	2 : 1
AS L 3	40	100%	31	9	3 : 1
AS L 5	40	100%	26	14	2 : 1
AS L 7	120	100%	74	46	3 : 2
EV L 1	120	100%	72	48	3 : 2
EV L 2	20	100%	5	15	1 : 3
EV L 3	20	100%	17	3	6 : 1

3.7.3 Chi-square analysis of observed segregation ratio

The Chi-square test was used to compare the significance of observed segregation ratios with the expected segregation ratios derived from the Mendelian segregation ratio (3:1) according to the formula below.

X^2 = sum [(observed - expected)^2 / expected]

The critical value for 0.05 probability level with degree of freedom (df = 1) was 3.841 according to Chi-square distribution table. The results are shown in (Table 3.4) where 6

lines were shown that there is no statistical significant differ between the observed and expected values, and 3 lines showed significant departure from Mendelian expectations ratio at $P < 0.05$.

Table 3.4: Chi square (X^2) test of inheritance segregation ratio of transgenic barley lines

Line	Observed	Expected	O - E	$(O – E)^2$	$(O – E)^2 / E$	X^2 value
OX L 3	31	30	1	1	0.033	0.133^{ns}
	9	10	-1	1	0.1	
OX L 4	91	90	1	1	0.011	0.044^{ns}
	29	30	-1	1	0.033	
OX L 5	26	30	-4	16	0.533	2.133^{ns}
	14	10	4	16	1.6	
AS L 3	31	30	1	1	0.033	0.133^{ns}
	9	10	-1	1	0.1	
AS L 5	26	30	-4	16	0.533	2.133^{ns}
	14	10	4	16	1.6	
AS L 7	74	90	-16	256	2.844	11.377^{*}
	46	30	16	256	8.533	
EV L 1	72	90	-18	324	3.6	14.4^{*}
	48	30	18	324	10.8	
EV L 2	5	15	-10	100	6.666	26.666^{*}
	15	5	10	100	20	
EV L 3	17	15	2	4	0.266	1.066^{ns}
	3	5	-2	4	0.8	

(^{ns}) indicate not statistically significant differences from Mendelian ratio at $P < 0.05$.
(*****) indicate statistically significant differences from Mendelian ratio at $P < 0.05$.

3.8 Measuring wild type susceptibility to abiotic stress

In order to understand the susceptibility of commercially grown barley to abiotic stresses (salinity and drought) and to determine the best parameters to measure in order to monitor the responses to the drought and salt stress, an experiment was conducted by exposing two weeks old wild type plants to water shortage and also to different

concentrations of sodium chloride solutions (0, 50, 100, 150 and 200 mM) for a particular time (2 weeks). The plant responses were then measured and the data were analysed. SigmaStat program was used to conduct statistical analysis. The statistical model: one-way analysis of variance (ANOVA) was used to analyse the results at $P < 0.05$. In case the differences were considered statistically significant, this was followed by Tukey test to perform pairwise multiple comparisons.

3.8.1 Growth parameters investigation

The result of the shoot height of plants are summarised in Fig. 3.31. No statistically significant differences were seen between control and the first three treatments of salinity (50, 100 and 150 mM NaCl) where the average of the shoot height was between 34 and 40 cm. However the treatment of 200 mM NaCl showed a statistically significant difference compared with the control, as it produced shorter plants than the control. However no statistically significant differences in plant height were seen between control and the drought treatment.

In relation to fresh weight, the lowest weight was recorded after drought treatment which was 0.6 g per plant and this was a statistically significant difference between drought treatment and control treatment. The control treatment gave the highest fresh weight at an average of 2.48 g per plant whereas the average fresh weights of the increasing salinity treatments were 2.14, 1.9, 1.4 and 1.58 g respectively. No statistically significant differences were seen between the control and the lowest NaCl concentration treatments (50 mM and 100 mM) whereas there were statistically significant differences with the high NaCl concentrations treatments (150 mM and 200 mM) as illustrated in Fig. 3.32.

The comparisons between the averages of the shoot dry weight under all the treatments showed no statistically significant differences, as summarised in Fig. 3.33.

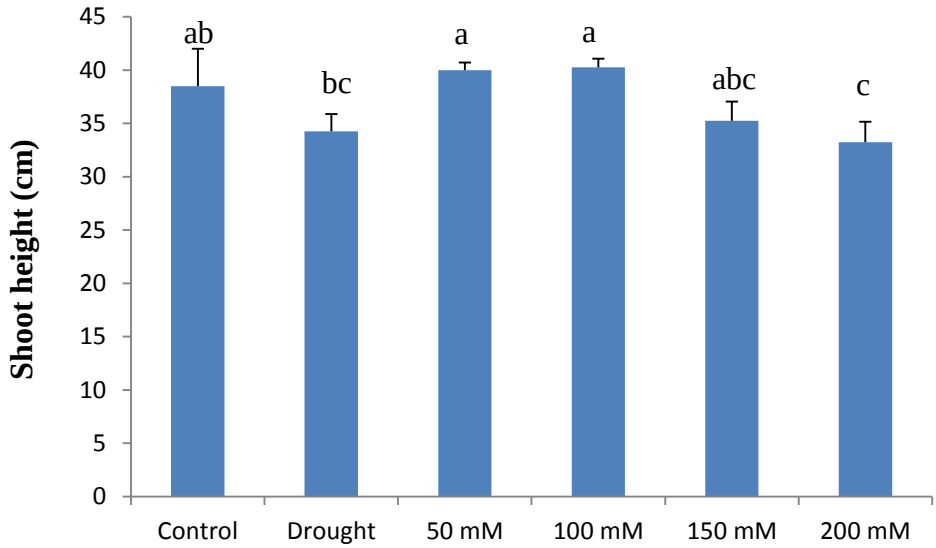


Figure 3.31: Illustrates the average height of wild type barley exposed to drought and salinity stresses.

50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.

3 replicates for each treatment. Vertical bar illustrates standard deviation.

Different letters show statistically significant difference ($P < 0.05$). At least one identical letter above the bars refers to no statistically significant differences.

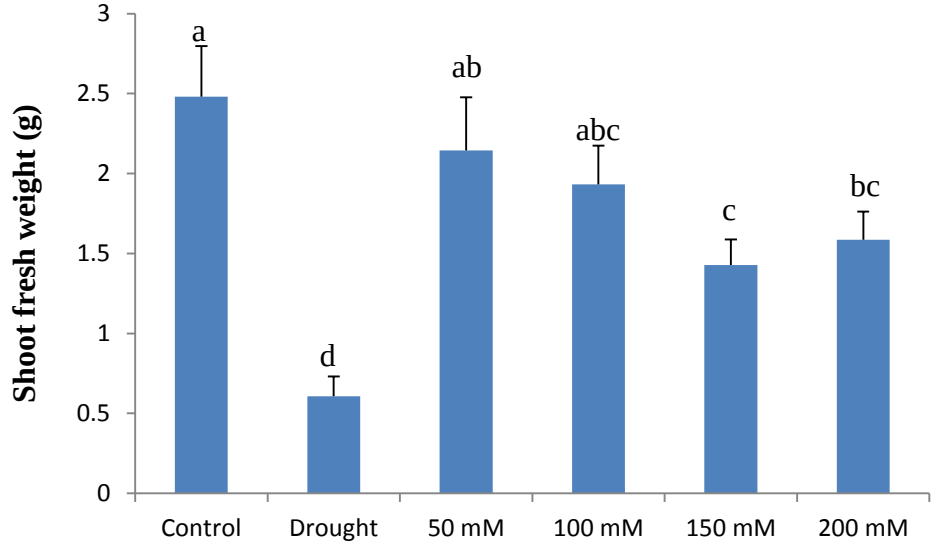


Figure 3.32: Illustrates the average fresh weight of wild type barley exposed to drought and salinity stresses.

50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.

3 replicates for each treatment. Vertical bar illustrates standard deviation.

Different letters show statistically significant difference ($P < 0.05$). At least one identical letter above the bars refers to no statistically significant differences.

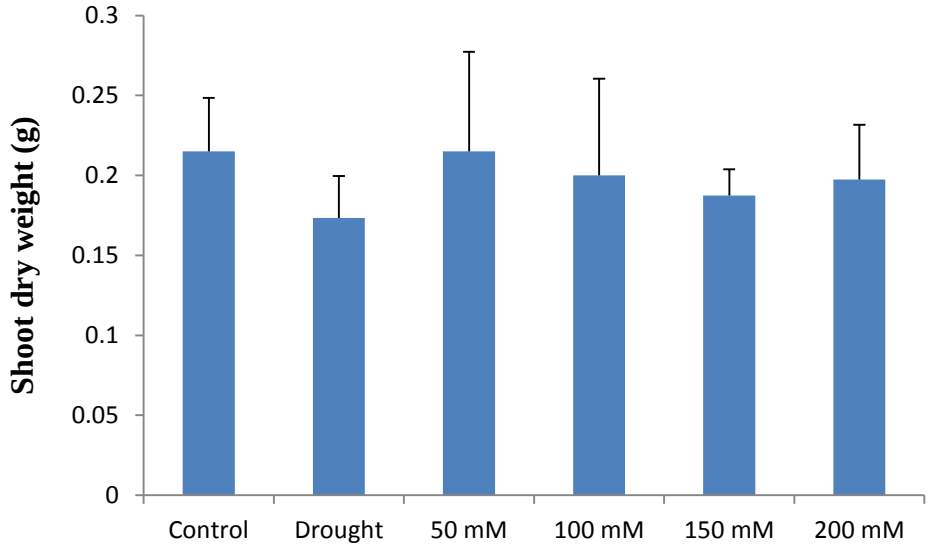


Figure 3.33: Illustrates the average dry weight of wild type barley exposed to drought and salinity stresses.
50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.
3 replicates for each treatment. Vertical bar illustrates standard deviation.
Means did not differ statistically by ANOVA test ($P < 0.05$).

3.8.2 Investigation of physiological parameters

3.8.2.1 Relative water content (RWC)

Based on the measured relative water content, it was shown that RWC% decreased in parallel with increasing salt concentration to 92.8, 88, 83 and 81% respectively whereas the control showed average of approximately 97% as illustrated in Fig. 3.34. No statistically significant differences were seen between the control and the lowest NaCl concentrations treatments (50 mM and 100 mM) as well as the drought treatment, whereas there were statistically significant differences with the highest NaCl concentration treatments (150 mM and 200 mM).

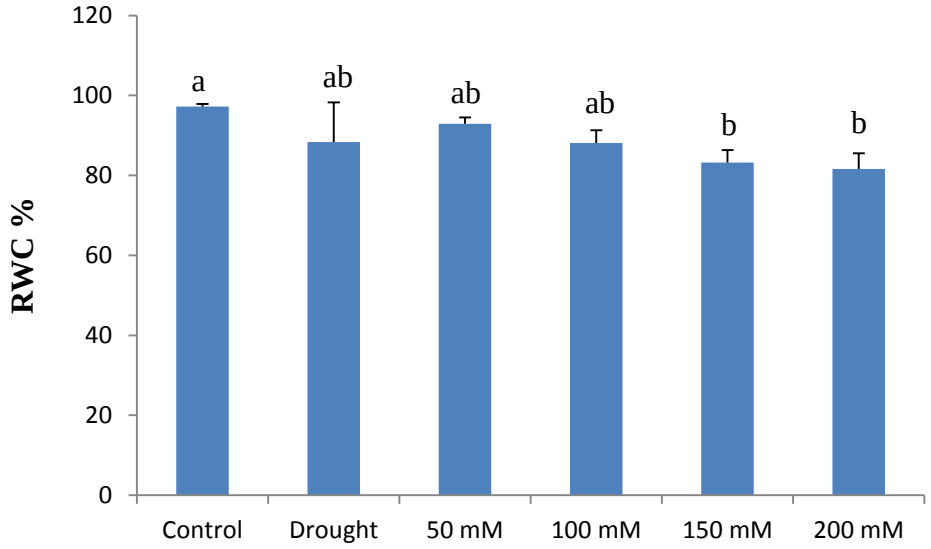


Figure 3.34: Illustrates the average RWC % of wild type barley exposed to drought and salinity stresses.
50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.
3 replicates for each treatment. Vertical bar illustrates standard deviation.
Different letters show statistically significant difference ($P < 0.05$). At least one identical letter above the bars refers to no statistically significant differences.

3.8.2.2 Chlorophyll content

The chlorophyll (a) content of leaves of the control showed a mean value of 344 µg/gFW. Despite no statistically significant differences between all treatments, the chlorophyll (a) content of leaves showed a trend to decrease in parallel with increasing salt concentration 342, 324, and 258 µg/gFW respectively and then increased to 285 µg/gFW with 200 mM treatment as showed in Fig. 3.35.

A different pattern was observed in the case of chlorophyll (b) content of leaves where there was a clear statistically significant difference between the control and all of the salt treatments, but no differences between the individual salt treatments, as exhibited in Fig. 3.36.

The total chlorophyll content of leaves gave a different pattern as well, with no statistically significant differences among all treatments (drought and salt concentrations) as well as among all treatments with control treatment except for the 150 mM treatment which gave statistically significant differences with control treatments as exhibited in Fig. 3.37.

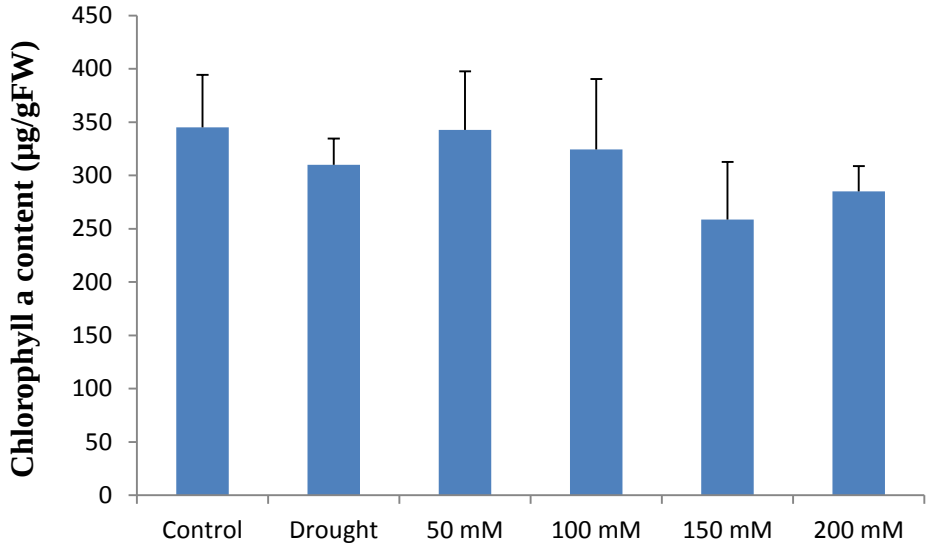


Figure 3.35: Illustrates the average chlorophyll (a) content (µg/gFW) of wild type barley exposed to drought and salinity stresses.

50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.

3 replicates for each treatment. Vertical bar illustrates standard deviation.

Means did not differ statistically by ANOVA test ($P < 0.05$).

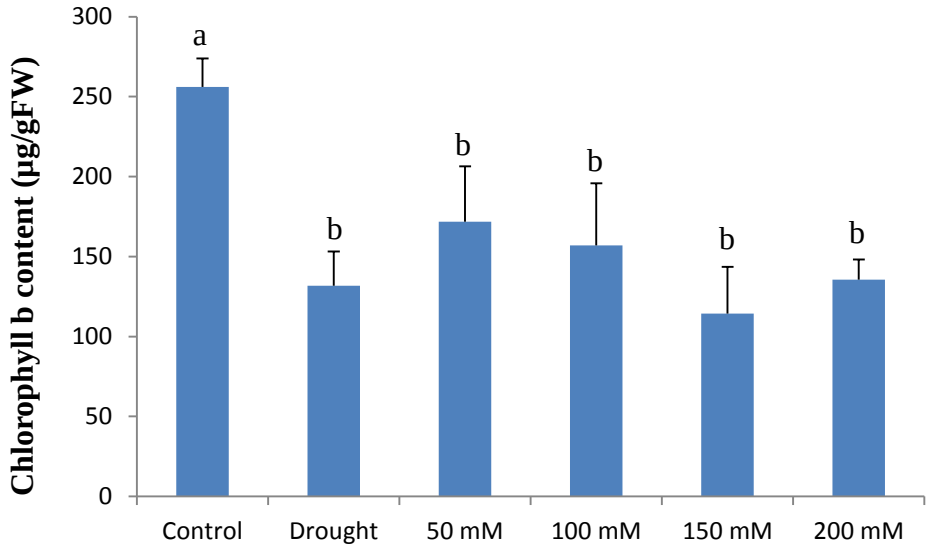


Figure 3.36: Illustrates the average chlorophyll (b) content (µg/gFW) of wild type barley exposed to drought and salinity stresses.

50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.

3 replicates for each treatment. Vertical bar illustrates standard deviation.

Different letters show statistically significant difference ($P < 0.05$).

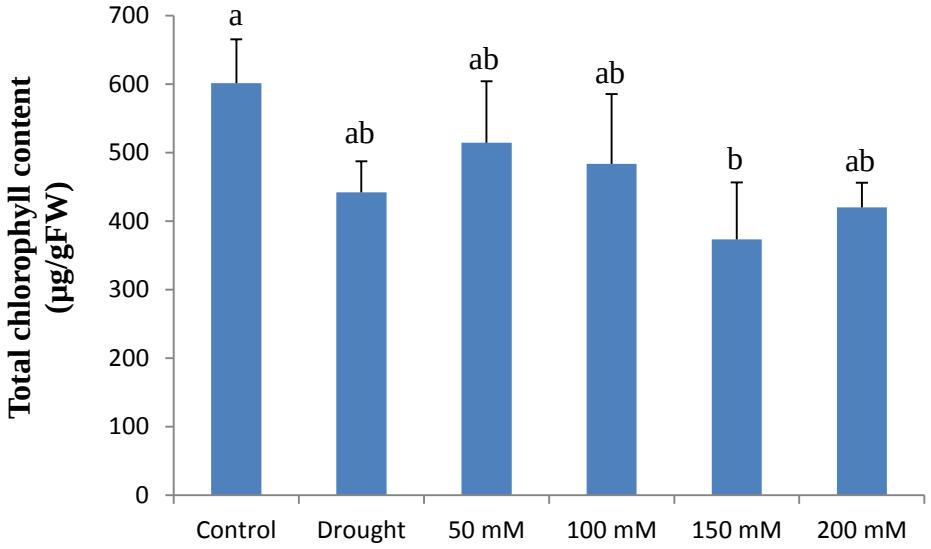


Figure 3.37: Illustrates the average total chlorophyll content (µg/gFW) of wild type barley exposed to drought and salinity stresses.
50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.
3 replicates for each treatment. Vertical bar illustrates standard deviation.
Different letters show statistically significant difference ($P < 0.05$). At least one identical letter above the bars refers to none statistically significant differences.

3.8.3 Investigation of chemical parameters

3.8.3.1 Proline

Proline levels in seedlings leaves increased in parallel with increasing salt concentration, with 1.36 µmol/gFW in the control treatment and 1.53, 2.69, 4.99 and 6.13 µmol/gFW after treatment with the increasing salt concentrations respectively. No statistically significant differences were seen between control and the low NaCl concentration treatments (50 mM and 100 mM) as well as the drought treatment whereas there were statistically significant differences with the high NaCl concentrations treatments (150 mM and 200 mM) as seen in Fig. 3.38.

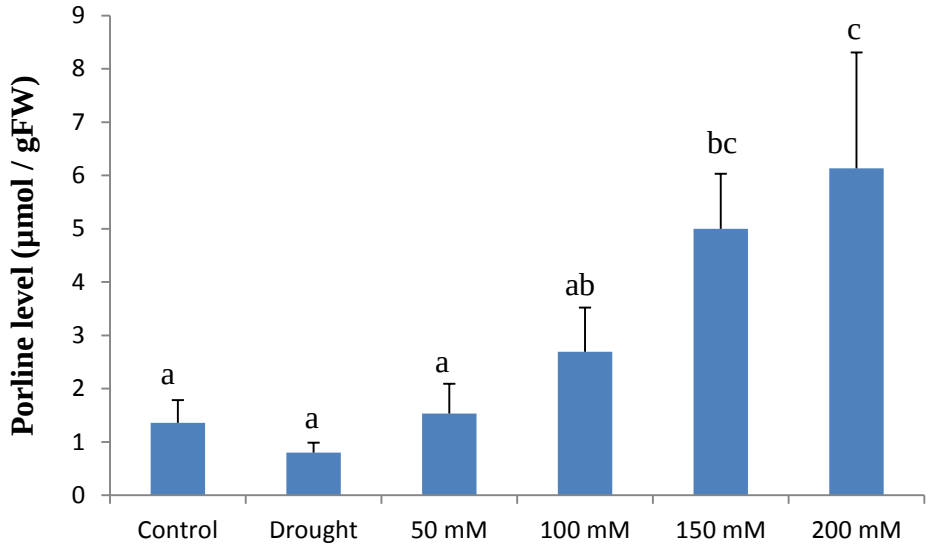


Figure 3.38: Illustrates the average proline level (µmol/gFW) of wild type barley exposed to drought and salinity stresses.
50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.
3 replicates for each treatment. Vertical bar illustrates standard deviation.
Different letters show statistically significant difference ($P < 0.05$). At least one identical letter above the bars refers to none statistically significant differences.

3.8.3.2 Hydrogen peroxide

Salt stress caused an increase in accumulation of H_2O_2, where the control contained an average of 5.3 µmol/gFW. Levels increased with drought treatment to 7.8 µmol/gFW and also increased in parallel with increasing of salt concentration to 6.3, 5.6, 8.5 and 12.3 µmol/gFW respectively. In spite of these changes no statistically significant differences were observed among all treatments, as the standard deviation were high as shown in Fig. 3.39.

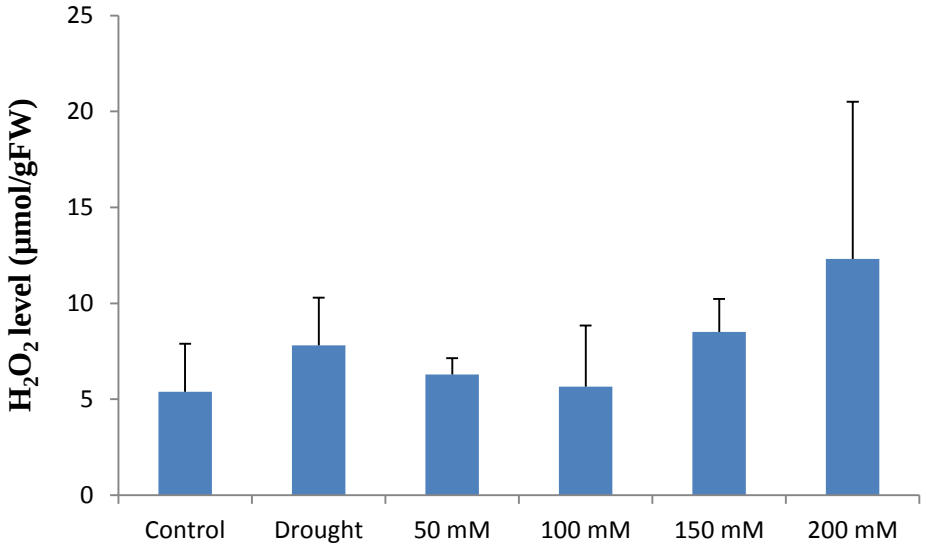


Figure 3.39: Illustrates the average H_2O_2 level (µmol/gFW) of wild type barley exposed to drought and salinity stresses.
50 mM, 100 mM, 150 mM and 200 mM = NaCl concentrations.
3 replicates for each treatment. Vertical bar illustrates standard deviation.
Means did not differ statistically by ANOVA test ($P < 0.05$).

3.9 Assessing response of transgenic barley lines to abiotic stress

Barley cultivar Golden Promise was transformed by using *Agrobacterium*-mediated transformation method, three different constructions were used to generate transgenic barley (empty binary vector pWBVec.8, overexpression Actin-*HvBWMK1*-OCS and antisense Actin-*HvBWMK1*-OCS). Salinity stress was imposed in order to assess the response of these transgenics to abiotic stress. The experiment was carried out on 3 replicates of wild type for each treatment (0 and 150 mM NaCl), 3 different lines of empty vector × 3 replicates for each line × 2 treatment, 3 different lines of overexpression *HvBWMK1* × 3 replicates for each line × 2 treatment and 3 different lines of antisense *HvBWMK1* × 3 replicates for each line × 2 treatment.

Two week old wild type and transgenic barley seedlings were subjected to two weeks of salt stress (0 and 150 mM NaCl) then several growth parameters, physiological and chemical parameters were measured and compared to unstressed control plants. After collecting the data, SigmaStat program was used to conduct statistical analysis. The statistical model: two-way analysis of variance (ANOVA) was used to analyse the

results at $P < 0.05$. In case, the differences were considered statistically significant, followed by Tukey test to perform pairwise multiple comparisons.

3.9.1 Growth parameters

Based on the measurements of the shoot height, no statistically significant differences were recorded among the plants to either control or the stress treatment. Stressed transgenic barley seedlings showed reduced average shoot height compared to stressed wild type plants but there were no statistically significant differences among them. Wild type and antisense lines showed a similar pattern of shoot height with no statistically significant differences between control and stressed treatment whereas empty vector line and overexpression line showed a similar pattern but opposite to the above. Overall, salinity stress (150 mM NaCl) caused a reduction in plants height compared with non-stressed plants as explained in Fig. 3.40.

Referring to the fresh weight results, the control treatment of overexpression line produced the highest average fresh weight of 2.42 g per plant compare with 2.1 g for antisense and wild type and 1.7 g for empty vector transgenics, but no statistically significant differences were observed. Salinity stress caused an extremely significant reduction in fresh weight of the stressed treatment compare with non-stressed, with a 53% reduction in fresh weight in empty vector lines, 38% in antisense lines and 49% in the overexpression lines, but the reduction was very low and non-significant at about 16% in the case of wild type. Except for stressed empty vector, no statistically significant differences were observed among stressed wild type, antisense and overexpression line as showed in Fig. 3.41.

Salinity stress caused a non-significant reduction in dry weight in salt stressed plants compare with non-stressed, except in the case of the wild type where the stressed treatment dry weight was higher by 5%. Overall, no statistically significant differences in dry weight were recorded among the plants to either control or stressed treatment except between stressed wild type and stressed empty vector line as illustrated in Fig. 3.42.

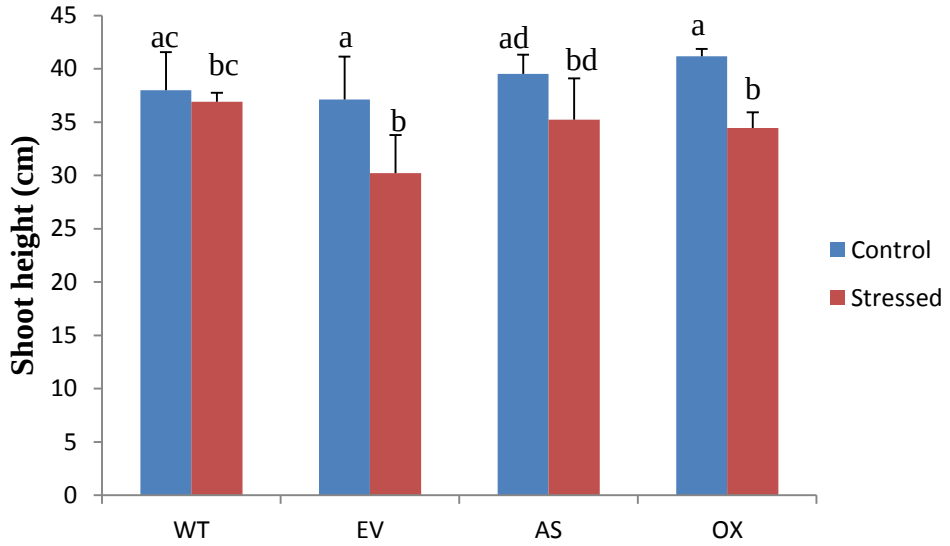


Figure 3.40: The impact of salinity (150 mM NaCl) on the shoot height of transgenic barley lines.

WT = Wild type. EV = Empty vector. AS = Antisense. OX = Overexpression. Vertical bar illustrates standard deviation. At least one identical letter above the bars indicates that the values are not significantly different at the $P < 0.05$ level according to Tukey's multiple comparisons. 3 replicates for wild type and 3 lines × 3 replicates for EV (line 1, 2, 3), AS (line 3, 5, 7) and OX (line 3, 4, 5).

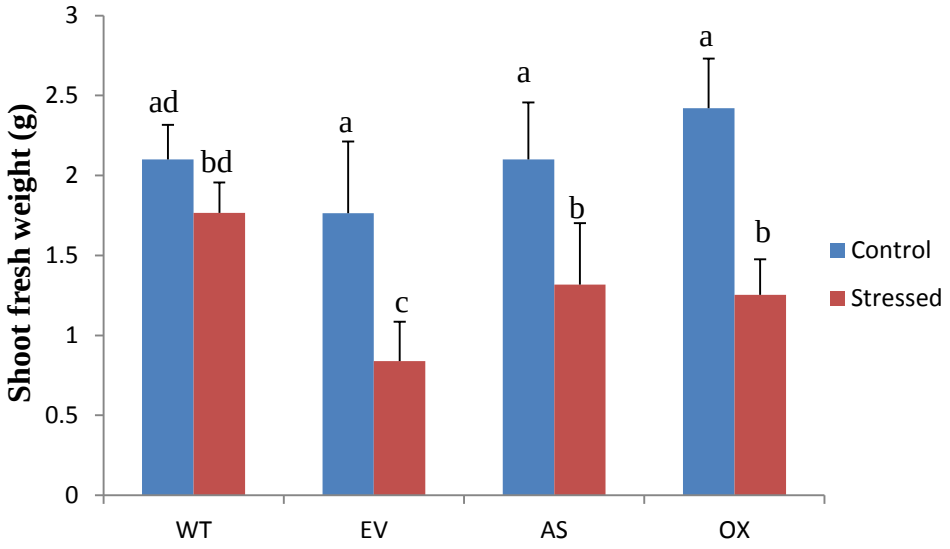


Figure 3.41: The impact of salinity (150 mM NaCl) on the FW of transgenic barley lines.

WT = Wild type. EV = Empty vector. AS = Antisense. OX = Overexpression. FW = fresh weight. Vertical bar illustrates standard deviation. Different letters show statistically significant difference ($P < 0.05$). 3 replicates for wild type and 3 lines × 3 replicates for EV (line 1, 2, 3), AS (line 3, 5, 7) and OX (line 3, 4, 5).

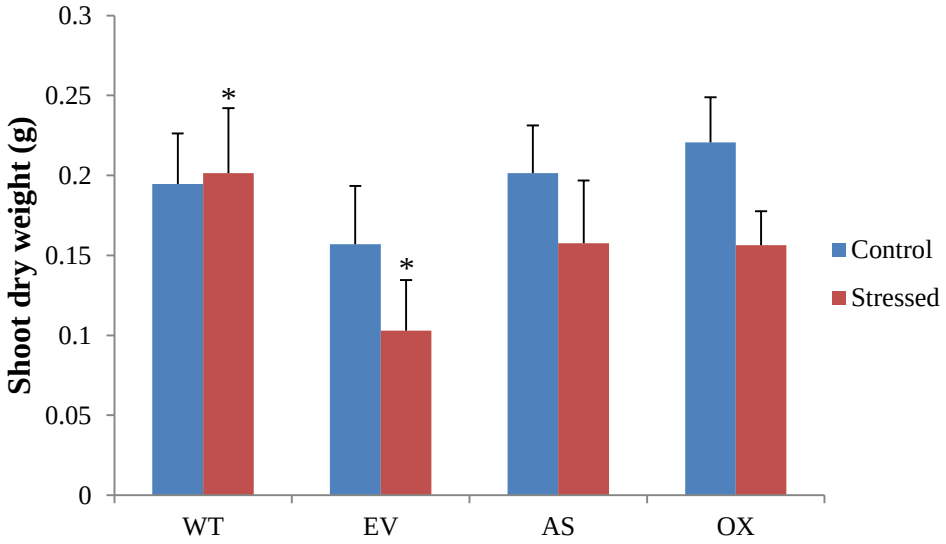


Figure 3.42: The impact of salinity (150 mM NaCl) on the dry weight of transgenic barley lines.

WT = Wild type. EV = Empty vector. AS = Antisense. OX = Overexpression.

Vertical bar illustrates standard deviation. Averages did not differ statistically by ANOVA test ($P < 0.05$) except that labelled by asterisk.

3 replicates for wild type and 3 lines × 3 replicates for EV (line 1, 2, 3), AS (line 3, 5, 7) and OX (line 3, 4, 5).

3.9.2 Investigation into physiological parameters of transgenic lines

3.9.2.1 Relative water content (RWC)

Salinity stress of 150 mM NaCl caused a significant reduction in RWC % of stressed treatment compared with non-stressed. The RWC of the stressed overexpression lines was significantly different from both stressed wild type and stressed antisense lines. Whereas RWC in the non-stressed overexpression lines was only significant different from non-stressed wild type as exhibited in Fig. 3.43.

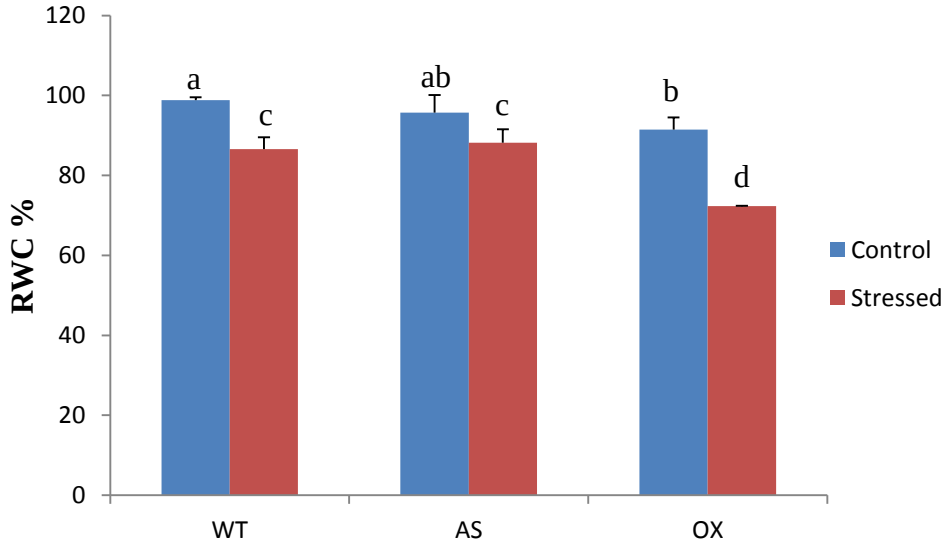


Figure 3.43: The impact of salinity (150 mM NaCl) on RWC % of transgenic barley lines.

WT = Wild type. AS = Antisense. OX = Overexpression. Vertical bar illustrates standard deviation. Different letters show statistically significant difference ($P < 0.05$). 3 replicates for each wild type, AS line 7 and OX line 4.

3.9.2.2 Chlorophyll content

Salinity treatment of 150 mM NaCl led to a non-significant decrease in chlorophyll (a) content by 25% in stressed wild type and 20% in stressed antisense lines compared with the non-stressed plants, whereas there was a noticeable significant increase by 50% was recorded in stressed overexpression line compared with its control. Overall, no statistically significant differences were recorded among the plants for either the control or stressed treatment, except the one which was mentioned above and shown in Fig. 3.44.

The reduction rate of chlorophyll (b) in stressed wild type was 47% less than the control, this was a statistically significant difference, and was 11% lower in stressed antisense relative to the control, but this difference was statistically non-significant. There was statistically significant increase of 38% in chlorophyll (b) recorded in stressed overexpression lines relative to the unstressed control. Chlorophyll (b) was at a lower level in non-stressed transgenic lines than non-stressed wild type but this was statistically significant only between the wild type and the antisense line. The chlorophyll (b) content of the stressed overexpression lines was statistically significant from either the stressed wild type or the stressed antisense line as shown in Fig. 3.45.

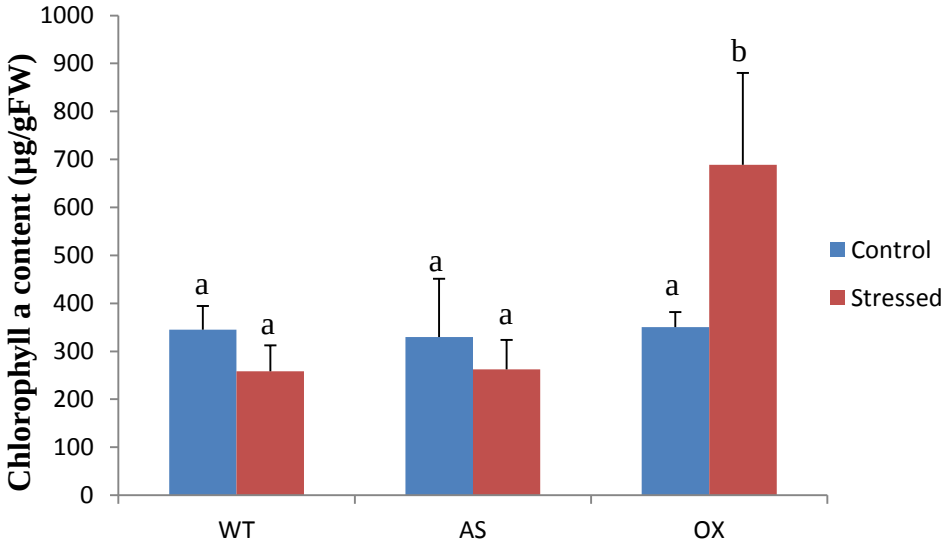


Figure 3.44: The impact of salinity (150 mM NaCl) on chlorophyll (a) content (µg/gFW) of transgenic barley lines.

WT = Wild type. AS = Antisense. OX = Overexpression. Vertical bar illustrates standard deviation. Different letters show statistically significant difference ($P < 0.05$). 3 replicates for each wild type, AS line 7 and OX line 4.

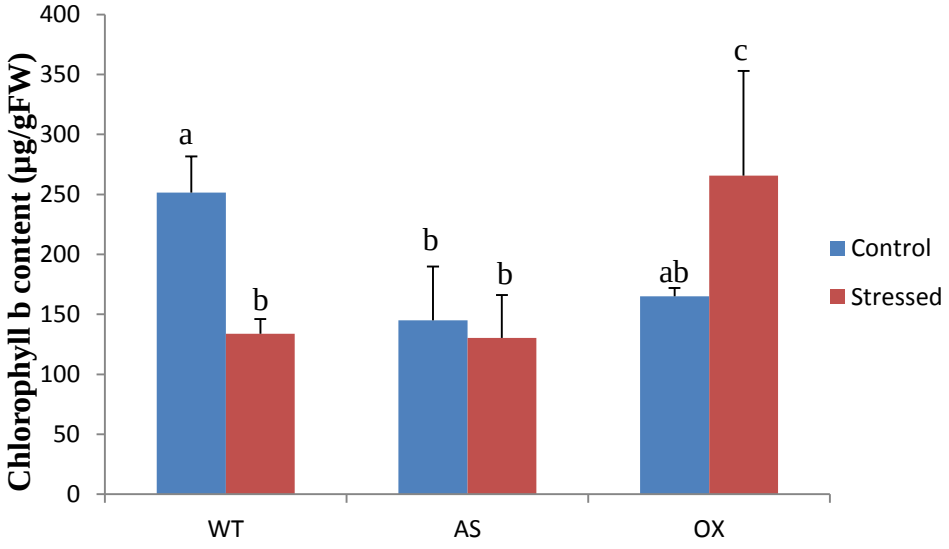


Figure 3.45: The impact of salinity (150 mM NaCl) on chlorophyll (b) content (µg/gFW) of transgenic barley lines.

WT = Wild type. AS = Antisense. OX = Overexpression. Vertical bar illustrates standard deviation. Different letters show statistically significant difference ($P < 0.05$). 3 replicates for each wild type, AS line 7 and OX line 4.

A very similar pattern to chlorophyll (a) content was observed in total chlorophyll content as a non-significant decrease in total chlorophyll content by 38% in stressed wild type and 30% in stressed antisense lines compared with unstressed control. There was a noticeable significant increase of 47% recorded in the stressed overexpression line when compared with its control. Overall, no statistically significant differences were recorded among the plants for either the control or stressed treatment, except the one which was mentioned above and illustrated in Fig. 3.46.

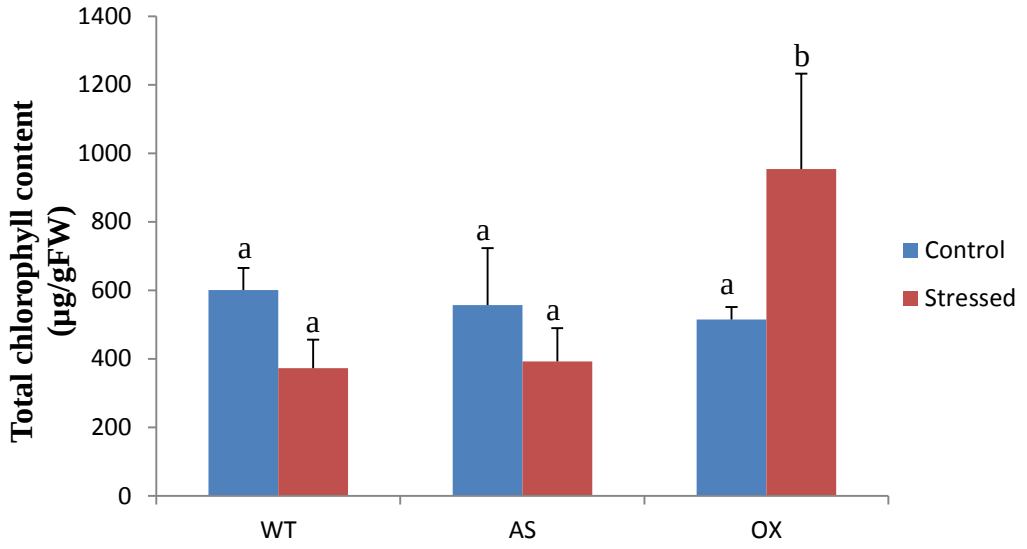


Figure 3.46: The impact of salinity (150 mM NaCl) on the total chlorophyll content (µg/gFW) of transgenic barley lines.
WT = Wild type. AS = Antisense. OX = Overexpression. Vertical bar illustrates standard deviation. Different letters show statistically significant difference ($P < 0.05$). 3 replicates for each wild type, AS line 7 and OX line 4.

3.9.3 Investigation into chemical parameters of stressed transgenic plants

3.9.3.1 Proline

Salinity stress resulted in a greater increase in proline content in the overexpression genotype than in wild type or the antisense line. There were statistically significant differences between the stressed and non-stressed plants in each line except for the antisense line, in which levels were very low and did not increase upon salt stress. The proline level was 51 fold higher in the stressed overexpression line than the unstressed control whereas there was a 5 fold increase in stressed wild type line relative to the unstressed control as shown in Fig. 3.47.

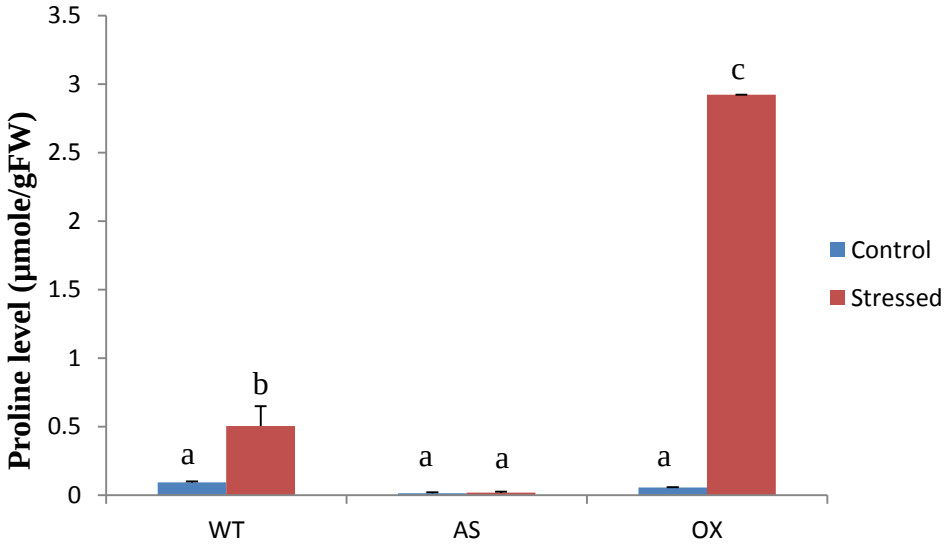


Figure 3.47: The impact of salinity (150 mM NaCl) on proline level (µmol/gFW) of transgenic barley lines.

WT = Wild type. AS = Antisense. OX = Overexpression.

Vertical bar illustrates standard deviation.

Different letters show statistically significant difference ($P < 0.05$).

3 replicates for each wild type, AS line 7 and OX line 4.

3.9.3.2 Hydrogen peroxide

Hydrogen peroxide levels were very high in the non-stressed overexpression line, about 60 and 40 fold greater than both the non-stressed wild type and antisense line respectively. A similar pattern was observed in the stressed treatments where the overexpression line showed about 27 and 22 fold more hydrogen peroxide than both wild type and antisense line respectively. Salinity stress caused a statistically significant decrease in hydrogen peroxide level in the overexpression line compared with its respective controls. Overall, no statistically significant differences were recorded among both wild type and antisense line to either control or stressed treatment as showed in Fig. 3.48.

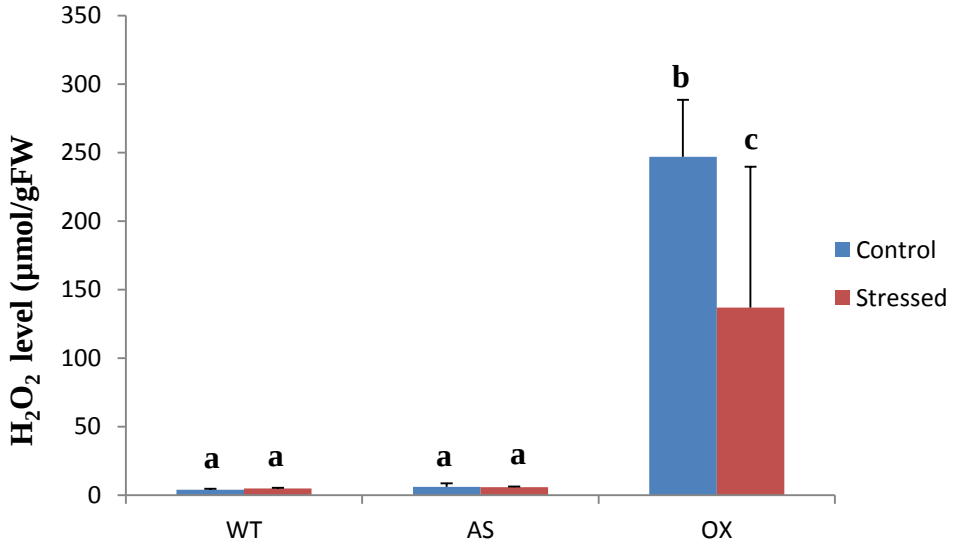


Figure 3.48: The impact of salinity (150 mM NaCl) on hydrogen peroxide level (µmol/gFW) of transgenic barley lines.
WT = Wild type. AS = Antisense. OX = Overexpression.
Vertical bar illustrates standars deviation.
Different letters show statistically significant difference ($P < 0.05$).
3 replicates for each wild type, AS line 7 and OX line 4.

3.9.4 Analysis of metabolite after salt stress

GC-MS analysis was used to show changes in 85 different metabolite compounds in freeze dried shoots extractions of barley. 17 of those were unknown compounds as they were not present in the machine's library of compounds. The total of 68 compounds of known structure comprised 28 organic and fatty acids, 20 amino acids and amines, 10 sugars and 10 other different compounds (Table 3.5).

GenStat program was used to conduct statistical analysis. The statistical model: two-way analysis of variance (ANOVA) was used to analyse the results at $P < 0.05$. ANOVA was carried out after a logarithmic transformation of the metabolite concentrations to normalise the data. In case the differences were considered statistically significant, Fishers protected LSD test was used to perform pairwise multiple comparisons. 32 compounds showed statistically significant differences among the tested lines as illustrated in the table in Appendix 5.8. For ease of analysis and presentation, Excel was used to create a graph for each compound as illustrated in Appendix 5.9.

Table 3.5: Metabolites found in freeze dried shoot extractions of barley.

Amino acids and amines	α-carbohydrate	α-linolenic
Asparagine	Sucrose	**Others**
Ethanolamine	**Organic and fatty acids**	Heneicosanol
γ-aminobutyric acid	2,3-Dihydroxypropanoic	n-docosanol
Glutamic acid	2-OH-Docosanoic acid methyl ester	n-hexacosanol
Glutamine	2-OH-tetracosanoic acid	n-octacosanol
Glycine	2-Piperidinecarboxylic	n-tetracosanol
L-aspartic acid	Caffeic acid	Octadecanol
Leucine	Citric acid	Phosphate
L-isoleucine	Fumaric acid	Phytilmethylether 1
L-lysine	Hexadecanoic acid	Phytilmethylether 2
L-methionine	Linoleic	β-sitosterol
L-proline	Malic acid	**Unknown code**
L-serine	n-docosanoic	Unknown-U1845
L-threonine	n-eicosanoic	Unknown-U2498
L-tyrosine	n-heptadecanoic	Unknown-UA-10-0
L-valine	n-hexacosanoic	Unknown-UA-2-88
Oxo-proline	n-hexadecanoic	Unknown-UA-2-92
Putrescine	n-octadecanoic	Unknown-UA-3-95
Tryptophan	n-pentadecanoic	Unknown-UA-4-23
β-alanine	n-tetracosanoic	Unknown-UA-4-72
Sugars	n-tetradecanoic	Unknown-UA-5-08
Carbohydrate	n-tricosanoic	Unknown-UA-7-32
Fructose	Octadecanoic acid	Unknown-1
Galactose	OH-Eicosanoic acid methyl ester	Unknown-4-67
Glucose	p-coumaric acid	Unknown-6
Glucose Gala glycerol	Quinic acid	Unknown-7-36
Glycerol	Succinic acid	Unknown-7-82
Inositol	Threonic acid	Unknown-X
Polysaccharide	Trans cinnamic acid	Unknown-UP3318

Principle component analyses (PCA) based on the correlation matrix were performed by using the SIMCA-P+ software in order to check the quality control of the experiment and to observe any outlier (Fig. 3.49) or separation (Fig. 3.50) and hence find the similarities and dissimilarities among the barley lines, 14 scores plot (for interpreting relations among samples) and 2 loading plot (For interpreting relations among variables) were created (Appendix 5.10).

Among the metabolites, most of the analysed amino acids were increased in all the transgenic lines and the wild type plants after salt treatment compared with the respective control plants, amino acid that increased were asparagine, ethanolamine, glutamine, glycine, leucine, isoleucine, lysine, methionine, proline, serine, threonine, tyrosine, valine, tryptophan and β-alanine. Amino acids at lower concentrations than the respective control plants were glutamic acid, aspartic acid, oxo-proline and putrescine. The rest of amino acids showed a fluctuating response.

Seven of the analysed sugars increased in all plants after salinity was imposed; these are fructose, galactose, glucose, glycerol, inositol and sucrose, whereas glucose-gala-glycerol, polysaccharide and α-carbohydrate decreased in concentration after salt treatment.

Only three compounds of organic and fatty acids, represented by piperidinecarboxylic, pentadecanoic and tricosanoic were increased after salt stress imposed on plants compared with unstressed controls whereas citric acid, dihydroxypropanoic acid, docosanoic acid, fumaric acid, hexadecanoic acid, linoleic acid, malic acid, octadecanoic acid, quinic acid, succinic acid and α-linolenic acid were decreased in addition to phosphate and β-sitosterol as well. The rest of organic and fatty acids showed a fluctuating response.

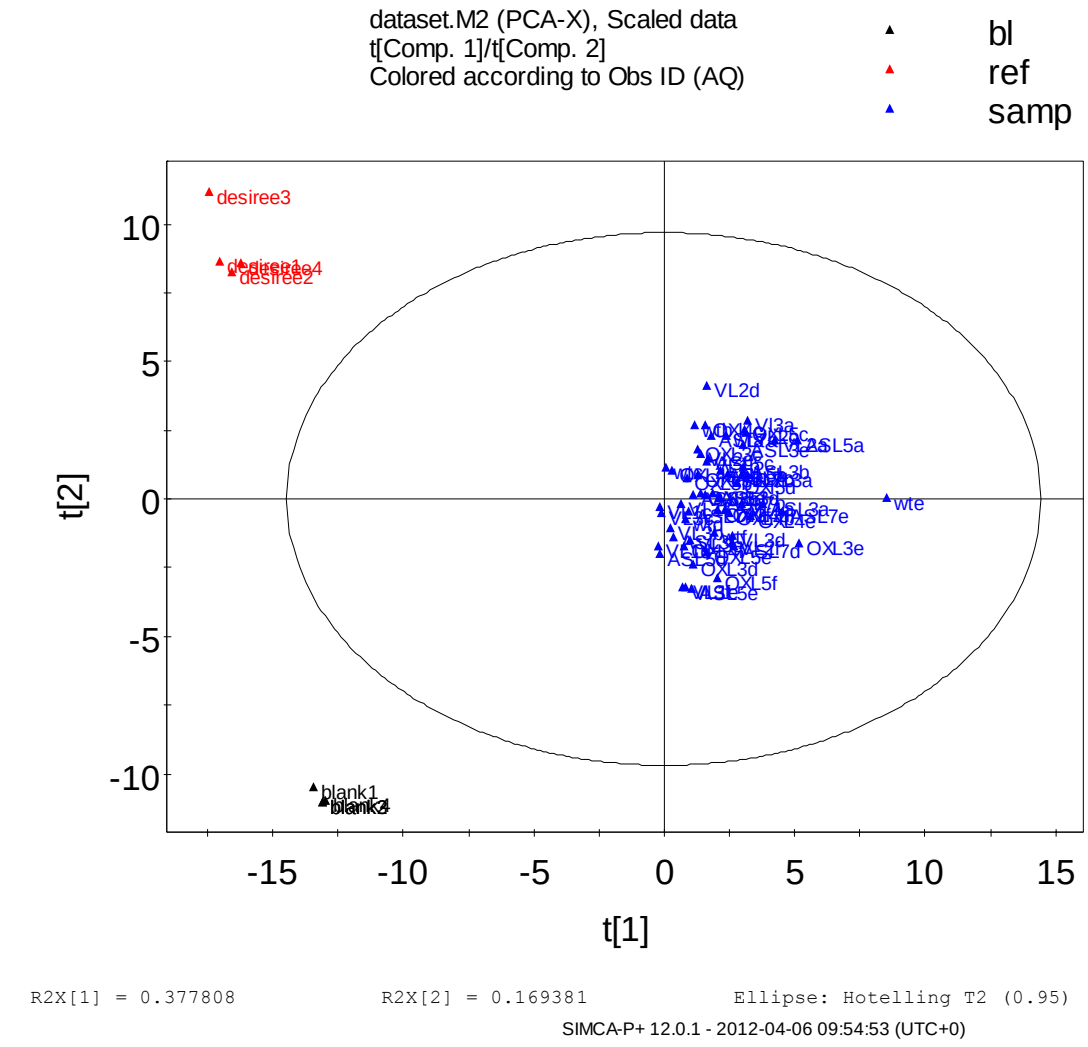


Figure 3.49: PCA score plot generated using the data from the metabolite profiles of the wild type and transgenic barley lines samples alongside with those from the biological reference (potato cultivar: Desiree) and blank injection. This PCA reflect the quality control of the experiment and showed clear segregation of the blank and reference from barley samples. Each data point represents the metabolite profile of a single sample.

▲ bl= blank. ▲ ref= reference. ▲ samp= samples.

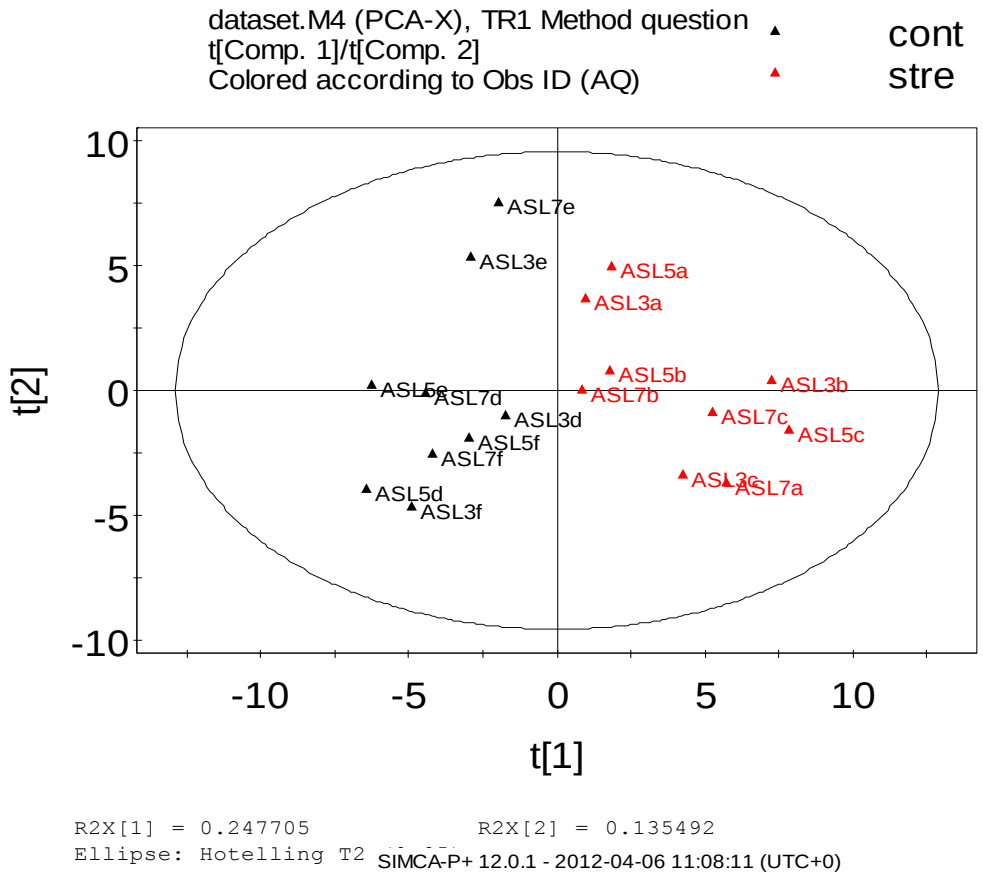


Figure 3.50: PCA score plot illustrates the separation between the control and salt stressed of antisense *HvBWMK1* lines (3, 5 and 7).

Each data point represents the metabolite profile of a single sample.

▲ cont= control. ▲ Stre= stressed.

CHAPTER 4 DISCUSSION

4.1 Identification and nomenclature of barley MAPKs genes

The result obtained from alignment between the amino acid sequences of all 17 rice MAPK open reading frames and that of the barley cDNA clone with Genbank accession number AK252439 showed 88% similarity between the barley clone and the rice gene with Genbank accession number AF177392, both containing a TDY activation domain. This rice gene was first isolated by He *et al*. 1999 and called *BWMK1*. Later, in multiple efforts to classify the rice MAPK genes, this gene was variously renamed *OsMPK17-1* (Hamel *et al*. 2006), *OsMPK12* (Reyna and Yang 2006), and *OsMPK7* (Liu and Xue 2007).

Searching in the NCBI database for barley MAPK revealed the presence of a cDNA clone with Genbank accession number AJ495775 extracted from barley Ingrid cultivar which was infected with powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*). The open reading frame of this gene was 1737 bp and showed very high similarity to our clone (except 11 nucleotides different, which altered 5 amino acids) as illustrated in Appendix 5.4, Eckey *et al*. (2004) called this gene *PWMK1* (powdery mildew fungus and wound induced MAP kinase), and it encodes a protein with 84% identity to the rice *OsBWMK1*.

Depending on the high sequence similarity to the previously named and characterised *PWMK1* from barley (Eckey *et al*. 2004) and *OsBWMK1* from rice (He *et al*. 1999), the barley clone AK252439 appears to encode a cDNA for the barley homologue of *OsBWMK1* and is called here *HvBWMK1*, adding a species acronym (Hv) from the nomenclature of barley (*Hordium vulgare*) as suggested by Ichimura *et al*. (2002).

Several researchers have investigated the response of *OsBWMK1* to biotic and abiotic stresses (He *et al*. 1999, Agrawal *et al*. 2003c, Cheong *et al*. 2003, Hong *et al*. 2007, Mashiguchi *et al*. 2008, Koo *et al*. 2007, 2009) the result showed that gene expression of *OsBWMK1* can be induced by several biotic and abiotic agents such as fungal elicitor, wounding, cold, dark, drought, heavy metals, high salt and sucrose, O_3, SO_2, H_2O_2, protein phosphatase inhibitors, SA, JA and ethephon (Table 1.7) and thus this gene and encoded protein plays an important role in regulating the plant defence against pathogens and abiotic stress.

4.2 Preparation of the plasmids and transformation into bacteria

The open reading frame of *HvBWMK1* was placed between the rice actin promoter and the octopine synthase (*OCS*) terminator, and then cloned in the binary vector pWBVec.8. The 5' region of the rice actin (Act1) gene, i.e. the actin (Act1) promoter has been used as a strong constitutive promoter for controlling foreign gene expression in cereal plants (McElroy *et al*. 1990, 1991, Zhang *et al*. 1991, 1999, Chibbar, *et al*. 1993). The octopine synthase (*OCS*) terminator from the Ti plasmid of *Agrobacterium tumefaciens* is widely used in plant expression constructs (Wesley *et al*. 2001, Chung *et al*. 2005). The binary vector pWBVec.8 was used in barley transformation research previously (Murray *et al*. 2004, Lange *et al*. 2006, Holme *et al*. 2006, 2008).

The rice actin promoter is active in many different plant, thus in transformed protoplasts of the moss *Physcomitrella patens* the rice *Act1* promoter was found to drive the highest induction of luciferase activity, on average 10 fold higher than the *CaMV 35S* promoter (Horstmann *et al*. 2004). A quantification of promoter strength in transgenic barley lines measured the specific GUS activity generated under the control of several different promoters (*ZmUbi1* promoter, *OsAct1* promoter, *CaMV d35S* promoter, and the *TaGstA1* promoter), the strongest activity was obtained by the *ZmUbi1* promoter, followed by the *OsAct1* promoter, the *TaGstA1* promoter and the *CaMV d35S* promoter (Himmelbach *et al*. 2007). Similarly, suspension culture cells from immature embryos of rice were transformed by plasmids encoding the *gus* gene under the control of the *CaMV 35S*, *Emu*, *Act1* or *Ubi1* promoter. The constitutive promoters *Act1* and *Ubi1* gave the highest expression levels (Li *et al*. 1997).

In this study, the transformed barley was obtained by using a hypervirulent EHA105 strain of *Agrobacterium tumefaciens* carrying a disarmed helper Ti plasmid pTiBo542 which carries an additional virulence gene (*virG*) and the binary vector pWBVec.8 containing the intron-interrupted hygromycin resistance gene *hpt* as the selectable marker for plant conferring resistance to the antibiotic hygromycin placed between the CaMV *35S* promoter and a transcription terminator sequence from the *Agrobacterium tumefaciens* nopaline synthase (*nos3*) gene. The open reading frame of the *HvBWMK1* cDNA was placed between the *actin* promoter and the octopine synthase (*ocs*) terminator and cloned into pWBVec8. In addition pWBVec.8 carries the T-DNA L+R borders and a spectinomycin resistance gene (*spec*) for bacterial selection.

The *Agrobacterium*-mediated transformation is a reliable method to obtain transgenic plants with lower copy number of target gene, intact foreign gene and stable gene expression (Dai *et al*. 2001, Travella *et al*. 2005, Rivera *et al*. 2012). In a comparison of rice transgenic plants obtained by *Agrobacterium*-mediated and particle bombardment transformation, the average gene copy numbers were 1.8 and 2.7, respectively and the fertility of transgenic plants obtained from *Agrobacterium*-mediated transformation was better. The percentage of transgenic plants containing intact copies of foreign genes was higher for *Agrobacterium*-mediated transformation and gene expression level was reported to be more stable as well (Dai *et al*. 2001). The *Agrobacterium*-mediated transformation is now the preferred method for cereal researchers. A search on the Web of Science literature database for citations of genetic transformation of plants gave for *Agrobacterium*-mediated transformation 1038 hits during 1985-1999 and 3604 hits during 2000-2011 while other methods such as electroporation, biolistics, electrophoresis, microinjection and silicon carbide fibers show a moderate increase (Rivera *et al*. 2012). Two independent studies show a doubling of the efficiency of *Agrobacterium*-mediated transformation as compared to biolistics. It was reported that the transformation efficiency of Golden Promise after biolistic transformation was, at 1.59%, more than twice as low compared to the 3.47% of *Agrobacterium*-mediated (Zalewski *et al*. 2012). Similarly, it was also reported that the efficiency of *Agrobacterium*-mediated transformation of barley was, at 2%, double that obtained with particle bombardment (1%), and while 100% of the *Agrobacterium*-derived lines integrated between 1 to 3 copies of the transgene, 60% of the transgenic lines derived by particle bombardment integrated more than 8 copies of the transgene (Travella *et al*. 2005). High copy numbers of transgenes in plants are thought to lead to genomic instability through recombination.

4.3 Barley transformation

Several methods can be used to produce fertile transgenic barley, but its efficiency is still low, to overcome this problem, *Agrobacterium*-mediated transformation can be an alternative (Roussy *et al*, 2001). The first transgenic plants from barley (Tingay *et al*. 1997) and wheat (Cheng *et al*. 1997) were obtained by this method. The barley cultivar Golden Promise with a high amenability to transformation was used in this study. It is the most readily transformed cultivar and the most widely used in transformation experiments (Table 1.3). It has excellent regeneration from immature embryo target

tissues as well as good amenability to *Agrobacterium* infection (Harwood 2012). Several published papers revealed that the transformation efficiency was much higher for Golden Promise as compared with, other genotypes (for example Haruna, Nijo, Dissa) (Hagio *et al*. 1995), (Schooner, Chebec and Sloop) (Murray *et al*. 2004), 5 spring barley genotypes (Koprek *et al*. 1996), 9 winter and spring lines (Hensel *et al*. 2008), 4 spring barley cultivars (Holme *et al*. 2008), and cultivar Scarlett (Zalewski *et al*. 2012).

In this project, the protocol of Harwood *et al*. (2009) was followed with some modification depending on advice provided by Ms. Jill Williams, a tissue culture technician from the James Hutton institute, Dundee. Harwood incubated the embryos scutellum side up at first and then after inoculation with *Agrobacterium* turned them scutellum side down, in our case the embryos were kept scutellum side down from the beginning and 1µl of *Agrobacterium* suspension dropped by pipette on the top of scutellum instead of immersing it in the suspension, thus avoiding over-growth of bacteria which leads to damage of the tissue. In addition the transition step (2 weeks) of Harwood was omitted and embryos moved from the co-cultivation step (2 days) directly to the selection stage (4 weeks). The other differences were: increasing the amount of phytagel in the medium from 3.5 g to 3.9 g per liter, and decreasing the amount of some other components (myo-inositol from 350 mg to 250 mg and proline from 690 mg to 650 mg) in the callus induction media in addition to adding 10 mM MES buffer. The cupric sulphate was only used in the selection medium. Dicamba is a broadleaf weed control agent, that is used as a plant growth regulator to induce callus regeneration. Harwood has used Dicamba in the early stages (co-cultivation and cultivation) of experiment, then later used 2,4 D (transition stage) while in the present study Dicamba was only used in the co-cultivation and selection stages as recommended by Trifonova *et al*. (2001) who reported that the induction medium containing Dicamba led to significantly more transformation events than the 2,4-D-containing medium. For all differences between the media see Appendix 5.6 and 5.7.

The selection medium was supplemented with 1.25 mg cupric sulphate at 50 fold higher than the concentration in Murashige and Skoog (MS) salts (0.025 mg/l). This was recommended by several researchers who showed that the amount of copper in (MS) medium is not adequate for the growth of cereal plants (Wojnarowiez *et al*. 2002, Nuutila *et al*. 2000). It was also reported that the addition of extra amount of copper led

to approximately double the number of regenerated shoots from each immature embryo as well as showing faster and larger growth (Bartlett *et al*. 2008).

It is very important to add a selectable marker such as an antibiotic to all types of media for barley tissue culture protocol to guarantee the successful selection and ensure that there is no non-transgenic escape during regeneration. In this work, 50 mg/l hygromycin was used as a plant selectable marker according to Bartlett *et al*. 2008. Hygromycin has been used as the selective agent for various monocot plants (Horn *et al*. 1988, Shimamoto *et al*. 1989, Zheng *et al*. 1991, Hiei *et al*. 1994) but the first report of using it with transgenic barley was in 1995 (Hagio *et al*. 1995) then followed by Cho *et al*. 1998, Fang *et al*. 2002, Murray *et al*. 2004, Bartlett *et al*. 2008). In comparison between two selectable markers, the *hpt* gene gave successful transformation rate of 5.5% relative to the number of bombarded wheat calli whereas the *bar* gene (which confers resistance to the herbicide Basta) gave 2.6% (Ortiz *et al*. 1996).

The first key requirement for a successful transformation system is a highly regenerable target tissue (Harwood 2012). Despite using various types of tissues for barley transformation such as apical meristem (Zhang *et al*. 1999), microspore (Carlson *et al*. 2001, Obert *et al*. 2008), ovule (Holme *et al*. 2006, 2008), according to published research papers (Table 1.3), the immature embryos (scutellum, calli derived from immature embryos, embryogenic cells suspension cultures, immature embryos protoplast, and mature embryos) have been the most successful target tissue for barley transformation (Harwood 2012). Among all the different types of tissue in wheat tissue culture, immature embryos have the highest regeneration capacity (Li *et al*. 2012). So the immature embryo (in particular the scutellum) was the target tissue in this work.

4.4 Analysis of transgenic barley plants

The transformation efficiency in this study was 10.37% (independent transgenic plant per embryo) for all the three constructs, which is in line with the results published in several research papers, for example 4.2% (Tingay *et al*.1997), 6% (Patel *et al*. 2000), 1.7-6.3% (Trifonova *et al*. 2001), 4.4-9.2% (Murray *et al*. 2004), 2.6-6.7% (Shrawat *et al*. 2007), 3.47% (Zalewski *et al*. 2012), 0.7-9.6% (Kapusi *et al*. 2013), on the other hand it was low compared with some other papers such as 21.7% (Hensel *et al*. 2008), 24.7% (Bartlett *et al*. 2008), 20-86% (Hensel *et al*. 2009). These high transformation efficiency of those researchers above might be as a result to the high number of

immature embryos that were used (600, 1750 and 300 respectively) because the logistics of tissue culture become easier.

Despite the antibiotic selection process which is carried out on the tissue culture media, it is crucial to conduct further confirmatory tests on the transgenic plants to ensure that the target gene present has integrated into the plant cells. In respect of that, PCR tests were conducted to check the presence of the T-DNA construction (hygromycin phosphotransferase gene and *HvBWMK1-OCS*) in the genome of the transgenic barley plants. The transgenic plants that showed negative result in PCR for the presence of the hygromycin phosphotransferase gene or *HvBWMK1-OCS* gene were omitted from the real-time PCR and physiological tests (Table 5.1).

For all lines of the transgenic plants, the germination rate was 100% for T_1 seeds which produce normal seedlings under the control condition. For segregation analysis, T_1 seeds were grown on filter papers supplemented with the antibiotic hygromycin. Four lines showed the same Mendel segregation ratio 3:1, two lines showed a higher ratio and three lines showed a lower ratio than expected from a Mendel segregation ratio. However, Chi-square analysis revealed that 6 lines were not statistically significantly different when compared with a Mendelian segregation ratio of 3:1 whereas the other 3 lines showed significant departure from Mendelian expectation of 3:1. These differences in ratios may be in part as a result of the number of copies of transgene integration into host genome, and in part due to differences in expression of the transgenes. Higher numbers of unlinked transgenes would be expected to give higher ratios of hygromycin resistant plants whereas a reduction in transgene expression would give a lower ratio.

The northern blot analysis of *HvBWMK1* gene expression in the wounding T_0 transgenic lines found at least four *HvBWMK1* overexpression lines and four *HvBWMK1* antisense lines showing clear differences in the signal for *HvBWMK1* level when compared with the wild type Golden Promise cultivar, where the lines of *HvBWMK1* overexpression showed, as expected, up-regulation in mRNA level, conversely the lines of *HvBWMK1* antisense expression showed clear down-regulation.

The levels of transgene expression were re-examined in the T_1 generation using real-time Q-PCR. The relative quantification method ($2^{-\Delta\Delta CT}$ method) was used to analyse real-time PCR data. This method quantifies the change in expression of the target gene

relative to a calibrator, reference sample and control sample. Because the control sample (wild type) is compared to itself, the expression level for the control sample is always set at one (Livak and Schmittgen 2001). Two lines of T_1 *HvBWMK1* overexpression showed higher expression compared with wild type, whereby the expression of lines 4 and 3 were increased by 79% and 35% respectively, whereas line 5 showed an unexpected result as it decreased by 11% with respect to the wild type. On the other hand, the *HvBWMK1* antisense construct successfully reduced the endogenous gene expression partially, thus all three transgenic antisense lines (3, 5 and 7) showed lower expression of *HvBWMK1* as compared with wild type, whereby the expression was decreased by 14%, 23% and 39% respectively compared with wild type. The average level of reduction in expression is 25% for all antisense lines. These results from real-time PCR in part confirm the northern blot results showing that *HvBWMK1* is up-regulated in the overexpression lines and *HvBWMK1* is down-regulated in the antisense lines, however there were some important differences. The level of up or down-regulation in the T_1 plants was not so striking as in the T_0 plants, which may be indicative of some transgene silencing in the T_1 generation.

Table 4.1: The summary of results of transformation PCR confirmation tests and expression tests.

Transgenic lines	PCR Hygromycin	PCR *HvBWMK1-OCS*	Northern blot signal level	Real-time PCR*	Physiol-ogical test*
WT	-	-	High	**1**	Yes
OX L2	+	+	Weaker t. WT	NT	No
OX L3	+	+	Stronger t. WT	**1.35**	Yes
OX L4	+	+	Stronger t. WT	**1.79**	Yes
OX L5	+	+	Stronger t. WT	**0.89**	Yes
OX L6	+	+	Weaker t. WT	NT	No
OX L7	+	+	NT	NT	No
OX L8	+	+	Weaker t. WT	NT	No
OX L9	+	+	Weaker t. WT	NT	No
AS L1	+	+	unclear	NT	No
AS L2	-	+	Weaker t. WT	NT	No
AS L3	+	+	Weaker t. WT	**0.86**	Yes
AS L4	-	-	Strong	NT	No
AS L5	+	+	Weaker t. WT	**0.77**	Yes
AS L6	+	+	strong	NT	No
AS L7	+	+	Weaker t. WT	**0.61**	Yes
EV L1	+	NT	NT	NT	Yes
EV L2	+	NT	NT	NT	Yes
EV L3	+	NT	NT	NT	Yes

WT = Wild type. OXL = Overexpression line. ASL = Antisense line.
EVL = Empty vector line. NT = not tested. (-) = negative result. (+) = positive result. Physiological test =Assessing tests. t. WT = than wild type.
*Real-time PCR and physiological tests were carried out on T_1 generation.

4.5 Measuring susceptibility of wild type barley to abiotic stress

Drought and salinity stress both lead to osmotic stress and water loss from plant cells, and ultimately affect the growth of plants. In this work, drought treatment of wild type barley resulted in measurements of 7 criteria out of 9 showing no statistically significant

differences compared with untreated plants whereas when salinity stress was carried out, only 3 criteria out of 9 showed no statistically significant differences compared with the control, thus under these conditions, using salinity as an abiotic stressor to assess the behaviour of transgenic barley is better than using drought stress.

Treatment with salinity concentrations of 150 and 200 mM showed in 6 out of 9 measured parameters a statistically significant difference compared with the control. There were no significant differences between both 150 and 200 mM in all 9 measured parameters. The lower salt concentrations of 50 mM and 100 mM only showed for 1 out of 9 parameters a statistically significant difference as compared with the control, therefore the salinity concentration of 150 mM NaCl was used in present study as an abiotic stress for assessing transgenic barley.

Despite the relatively short time treatment with salt (two weeks), barley was able to manifest differences in many growth parameters. Interestingly, there were statistically significant differences between control and stressed plants related to fresh weight that are the opposite of the dry weight data. The average of fresh weight for the control was 2.48g which then non-significantly decreases to 2.14g and 1.9g for 50 and 100 mM NaCl stress respectively and significantly decreases to 0.6g, 1.42g and 1.58g for the drought treatment, 150 and 200 mM NaCl stress respectively. For the dry weight, the control and 50 mM NaCl treatment gave similar dry weight of 0.215g whereas a non-statistically significant decrease was seen for the other treatments: the figures are 0.173g, 0.2g, 0.187g and 0.197g for drought, 100, 150 and 200 mM NaCl treatment respectively. This difference might be because that the plant needs a long term of growth to show differences in the dry matter as compared with the fresh weight, which reflect the plant ability to absorb water, regardless of the growing period. Overall, the results revealed that salinity caused a decrease in shoot fresh and dry weight, in agreement with the findings of Ligaba and Katsuhara (2010) who reported 15% and 40% fresh weight reduction in the tolerant barley K305 cultivar and sensitive barley I743 cultivar respectively under 100 mM NaCl compared with unstressed control, treatment with 200 mM NaCl lead to further inhibition in plant fresh weight by 50% and 65% in the tolerant and sensitive cultivar respectively. These findings also agree with those of Chen *et al*. (2007) who reported a decrease in shoot fresh and dry weight of four salt-stressed barley cultivars as compared with the control. Similar findings have been reported in barley (El-Tayeb 2005, Widodo *et al*. 2009, Seckin *et al*. 2010,

Tavakkoli *et al*. 2011, Movafegh *et al*. 2012), rice (Zhou *et al*. 2009, Cha-um *et al*. 2010), wheat (Azadi *et al*. 2011, Ashraf *et al*. 2012) and maize (Çiçek and Çakirlar 2002, Carpici *et al*. 2010). One of the reasons behind the decrease in plant growth is the lowering of available CO_2 which is caused by stomatal closure (Amirjani 2010, Seckin *et al*. 2010). Pérez-López *et al*. (2009) found that under salt stress combined with elevated CO_2, the relative water content and the leaf area of barley plants were higher with all tested salt concentrations compared with plants under salt stress combined with ambient CO_2, because the presence of elevated CO_2 reduces the harmful effects of salinity on water status by reducing the dehydration thanks to a lower transpiration rate. Çiçek and Çakirlar (2002) considered that as osmotic stress makes it hard for root cells to obtain sufficient water from soil, this thus affect the plant ability to uptake sufficient mineral nutrients dissolved in water, therefore growth and development of plants are inhibited due to defects in metabolism.

The studies presented here show that salt stress has a significant impact on the height of seedlings especially in higher salt concentrations where the plant length decreased by 8.5% and 13.7% from 38.5 cm for the control to 35.2 and 33.2 cm for 150 mM and 200 mM NaCl respectively. Widodo *et al*. (2009) similarly reported decreases in shoot height of two barley cultivars under 100 mM NaCl for 6 weeks, a reduction of 19% in Clipper and 14% in the Sahara cultivar. One of the reasons behind that is the limitation of cell leaf elongation. Elongation velocity was reduced to half that in non-salinized plants after one day of salinity stress, possibly due to the rate of solute accumulation to achieve osmotic adjustment in expanding leaf cells being the growth-limiting factor (Fricke *et al*. 2006). The decrease in the length of salt-stressed plants was also reported by several researchers, in barley (Chen *et al*. 2007, Seckin *et al*. 2010, and Movafegh *et al*. 2012), maize (Çiçek and Çakirlar 2002) and rice (Amirjani 2010, Cha-um *et al*. 2010).

It was obvious that the stressed plants were not able to absorb sufficient water, which was reflected in the decrease in fresh weight and that was also apparent in the case of RWC as well, where the 150 mM NaCl stressed plants showed 83% RWC compared with 97% RWC of the control plants. These results are in accord with the ones of Pérez-López *et al*. (2009) who found that non-salinized barley plants maintained high relative water content value about 97% but decreased in salinized barley plants to 85% in 240 mM NaCl. Also El-Tayeb (2005) who tested barley under a range of 0, 50, 100, 150 and

200 mM NaCl for 15 days, found that the RWC decreased with increasing NaCl concentration, RWC values were approximately 90%, 85%, 75% and 63% respectively. These results are also in agreement to what were reported in several crops like wheat (El-Bassiouny and Bekheta 2005, Azadi *et al*. 2011), rice (Amirjani 2010) maize (Çiçek and Çakirlar 2002).

Barley water relations under saline conditions might be improved by several factors such as decreasing the transpiration rate and cellular osmotic adjustment, and in this context Pérez-López *et al*. (2009) suggested that elevated CO_2 mitigates the negative effects of salt stress on water status by reducing passive dehydration thanks to a lower transpiration rate.

Chlorophyll content in plant leaves is one of the most widely used parameters to evaluate the plant response to environmental stress, as it reflects the plants ability to carry out photosynthesis, and thus accumulate sufficient nutrients and energy for growth. It is obvious that treatment with higher levels of salts resulted in a marked decline in photosynthetic pigments (Ashraf and Ashraf, 2012). Both drought and salinity caused a decrease in the chlorophyll content of the plant, and this was enhanced by increasing salt concentration. The highest amount of chlorophyll a, b and total chlorophyll was observed in the unstressed control witht 344.9, 256, 601 µg/gFW respectively. With salinity treatment the greatest degree of chlorophyll decrease was observed, at 258, 285 µg/gFW for chlorophyll a, 114, 135 µg/gFW for chlorophyll b and 373, 420 µg/gFW for total chlorophyll for salt at 150 mM and 200 mM respectively. These results are in agreement with those of Tavakkoli *et al*. (2011) who reported reductions in chlorophyll content between 20% to 36% in four barley cultivars grown under 120 mM NaCl. Similar results have been reported in barley (Fedina *et al*. 2002, 2009, Seckin *et al*. 2010, Movafegh *et al*. 2012), maize (Carpici *et al*. 2010), rice (Zhou *et al*. 2009, Cha-um *et al*. 2010, Amirjani 2010, Kanawapee *et al*. 2012) and wheat (Azadi *et al*. 2011, Ashraf and Ashraf 2012). The increase in activity of the chlorophyll-degrading enzyme chlorophyllase might be behind the decrease in chlorophyll concentration in salt-stressed plans (Amirjani 2010). It was noted by Arjenaki *et al*. (212) that producing reactive oxygen species (ROS) such as O_2^- and H_2O_2, can lead to lipid peroxidation and consequently, chlorophyll destruction.

Plant adapt to environmental stresses by increasing accumulation of compatible soluble osmotic molecules such as proline (Munns 2005, Munns and Tester 2008).

Proline accumulation during salt stress occurs as a result of increased proline synthesis and reduced proline degradation (Verbruggen and Hermans 2008). Glutamate is a precursor for proline, and the pyrroline-5- carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) enzymes are able to convert glutamate to proline. P5CS and P5CR encoded genes are upregulated by osmotic stress (Verbruggen and Hermans 2008, Szabados and Savoure 2009).

In the present study, the proline level of wild type barley leaves was increased in parallel with increasing salt concentration. Proline concentrations were 1.36 µmol/gFW for the unstressed control, and this increased to 1.53, 2.69, 4.9 and 6.13 µmol/gFW after treatment with 50, 100, 150, 200 mM NaCl respectively. This result is in line with those of Carpici *et al*. (2010) who found that proline in non-stressed maize seedlings of four cultivars varied from 0.14 to 0.43 µg/gFW whereas the levels in 100 mM NaCl stressed seedlings increased, and ranged from 0.75 to 1.7 µg/gFW. Similarly, such findings have been also reported in different crops like barley (Fedina *et al*. 2002, 2009, El-Tayeb 2005, Chen *et al*. 2007, and Movafegh *et al*. 2012), wheat (Ashraf *et al*. 2012), maize (Çiçek and Çakirlar 2002) and rice (Zhou *et al*. 2009, Kanawapee *et al*. 2012). It is assumed that proline is acting as a compatible solute in osmotic adjustment, which could help to maintain the relatively high water content necessary for growth and cellular functions (Çiçek and Çakirlar 2002, El-Tayeb 2005). In addition, it was also reported that proline acts as a ROS scavenger (Chen *et al*. 2007 and Ashraf *et al*. 2012).

In plant cells, H_2O_2 is produced in the chloroplasts, cytosol, mitochondria, peroxisomes and the apoplastic space. Salt stress triggers production of H_2O_2 that could causes damage to lipid, protein degradation and DNA mutation (Vinocur and Altman 2005, Ahmed *et al*. 2008, Bhattacharjee 2012). However, plants have developed efficient antioxidant system that can protect plants from the harmfull effects of H_2O_2 such as: superoxide dismutase (SOD), catalase (CAT) peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) and non-enzymatic antioxidants such as ascorbic acid (Sairam *et al*. 2005, Seckin *et al*. 2010) and more than 150 genes encode for different ROS-detoxifying mechanisms (Ahmed *et al*. 2008).

The results of this study showed that both drought and salt stress increased the endogenous H_2O_2 in stressed barley leaves compared with control (although this was statistically non-significant). However at the same time, salt stress could causes a rise in the activities of some antioxidant enzymes (Sairam *et al*. 2005), in addition to the high

level of proline which might be act as a ROS scavenger (Ashraf *et al*. 2012). Since the plants were stressed for 2 weeks, it should be considered that high levels of increase in ROS may be relatively transient and the steady state levels determined after 14 days reflects the combined effect of enhanced ROS and enhanced antioxidants. In the same way Chen *et al*. (2007) referred to the notion that the osmo-protectants, such as proline and mannitol may efficiently scavenge ROS compounds. Findings of Ashraf *et al*. (2012) revealed high activity in some key antioxidant enzymes (CAT, SOD, POD, and APX) at all growth stages in a salt tolerant wheat cultivar in conjunction with lower levels of H_2O_2. El-Tayeb (2005) reported that peroxidase (POX) activity increased progressively as the salinity level increased in the shoots of barley seedlings sown under 50, 100, 150 and 200 mM NaCl for 15 days, and that was associated with a decrease in membrane damage. It has also been reported that simultaneous treatments of barley leaves with 50 µM sodium nitroprusside, a nitric oxide donor, prevent the increase of ion leakage, and malendialdehyde (MDA), carbonyl, and hydrogen peroxide content in barley leaves thus alleviated the damage of salt stress. The activities of superoxide dismutases (SOD), ascorbate peroxidases (APX), and catalases (CAT) were increased in the presence of the nitric oxide donor (Li *et al*. 2008).

In this work, after two weeks of salt stress imposition, the H_2O_2 level was 5.3 µmol/gFW in control and increased in parallel with increasing salt concentration, it was 7.8 µmol/gFW for drought stress and about 6.3, 5.6, 8.5 and 12.3 µmol/gFW for 50, 100, 150, and 200 mM NaCl stress respectively. The data of Kim *et al*. (2005) support these results, where they found that hydrogen peroxide content increased by 12% from 2.6 µmol/gFW for control to 2.9 µmol/gFW in the shoot of barley after 5 days growing in 200 mM NaCl. Also several publications have reported similar findings in different crops, for example wheat (Sairam *et al*. 2005), rice (Amirjani 2010) and barley (Fedina *et al*. 2009).

4.6 The analysis of assessing the stress responses of transgenic barley

Two weeks old wild type and transgenic (empty binary vector pWBVec.8 lines, *HvBWMK1* antisense lines and *HvBWMK1* overexpression lines) barley seedlings were subjected to two weeks of salt stress (150 mM NaCl) then the criteria as determined through the analysis of the wildtype plants were measured and analysed.

Transgenic lines of empty binary vector pWBVec.8 were assessed using only growth parameters and metabolite compounds because most of the hygromycin resistant seedlings produced dwarf roots system. These seedlings died after moving them to soil, resulting in a shortage in seeds for the wider range of experiments.

4.6.1 The analysis of growth parameters

Salinity stress (150 mM NaCl) caused different rates in the reduction of plant shoot height compared with the control, the reduction was about 2.9% for wild type, 18.7% for empty binary vector pWBVec.8 lines, 10.9% for the *HvBWMK1* antisense lines and 16.3% *HvBWMK1* overexpression lines. The results for the transgenics are in agreement with those of Widodo *et al*. (2009) who exposed two wildtype barley cultivars to 100 mM NaCl for 6 weeks which resulted in decreasing in shoot height for both cultivars by 14% and 19%, however the data for the wildtype plants is difficult to explain. Salt stress did not show remarkable effect on the shoot lengths of both wildtype and *HvBWMK1* antisense line, about 2.9% and 10.9% length reduction respectively, while it was obvious in both transgenic lines empty binary vector pWBVec.8 and *HvBWMK1* overexpression, about 18.7% and 16.3% length reduction respectively.

Salinity stress caused a significant reduction in the shoot fresh weight of all transgenic lines (empty binary vector pWBVec.8 lines, *HvBWMK1* antisense and *HvBWMK1* overexpression) compared with the non-stressed controls, whereas stressed wild type showed non-significant reduction of about 16% compared with its control. It is worth noting that the dry weight of stressed wild type was higher by 5% than its control while all transgenic lines showed non-significant reduction in dry weight of stressed treatment compare with non-stressed. These findings are in agreement with Chen *et al*. (2007) who imposed severe salt stress (320 mM NaCl) on barley seedlings for 4 week, and reported a strong impact on plant growth (height, fresh and dry weight) which all showed significant reduction.

The decrease in plant growth (shoot height, fresh and dry weights of shoot) as a result of salt stress has been reported in several cereal crops such as maize (Çiçek and Çakirlar 2002), rice (Amirjani 2010, Cha-um *et al*. 2010) and wheat (Azadi *et al*. 2011, Ashraf *et al*. 2012).

Salt stress in plants causes an increase in the concentrations of Na^+ and Cl^- in their tissues, it is known that accumulation of such inorganic ions may cause a toxic effect

and cell injury, and lead to an inactivation of photosynthesis and respiration (Amirjani 2010). The growth reduction may be due to the slowing in the rate of cellular functions such as division, elongation and differentiation which consequently reduces the cell number and size (Kumar *et al*. 2012). Furthermore, the growth decline may be attributed to ion imbalance, as it is known that high levels of Na^+ inhibit the K^+ uptake which lead to an increase in the Na^+/K^+ ratio, Çiçek and Çakirlar (2002) reported a significant reduction in shoot length, fresh and dry weights associated with a significant increase in the Na^+/K^+ ratio of maize sown under salt stress for 30 days. It was also reported that salt stress (200 mM NaCl) for 14 days caused a reduction in leaf area in salt-stressed rice genotypes, which ranged from 34 to 73% with respect to the control (Cha-um *et al*. 2010), the low leaf area may be due to the early senescence of leaves enhanced by salinity (Kumar *et al*. 2012), also have been reported that the reduction of leaf area was associated with decreasing shoot length, fresh and dry weights of shoot of maize under salt stress for 30 days (Çiçek and Çakirlar 2002).

4.6.2 Relative water content

RWC is an appropriate parameter to study the physiological effects of plant cells after exposure to salinity stress. RWC of both wild type and transgenic barley leaves were significantly decreased under salt stress, in wild type this was reduced by 12%, *HvBWMK1* antisense by 7.8% and *HvBWMK1* overexpression by 20.9% as compared with their controls. This phenomenon of decline in RWC by salinity stress is common and reported by several researchers (El-Tayeb 2005, Pérez-López *et al*. 2009, Amirjani 2010 and Azadi *et al*. 2011). It is known that some metal ions such as K^+ play a significant role in the stomatal activity and water relation of plants, it was revealed that drought resistant wheat genotypes had the highest value of K^+ and less Na^+ than sensitive genotypes, and that was associated with increase in RWC of the tolerant genotypes (Arjenaki *et al*. 2002). The study of Chen *et al*. (2007) showed that superior K^+ retention is crucial for barley salt tolerance.

The lower RWC value of the stressed *HvBWMK1* overexpression lines was significantly different from both the stressed wild type and stressed *HvBWMK1* antisense while the RWC of non-stressed *HvBWMK1* overexpression lines was only significantly different from the non-stressed wild type. The implication of this is that *HvBWMK1* is a negative regulator of water retention after salt stress.

4.6.3 Chlorophyll

Chlorophyll a, b and total chlorophyll were non-significantly decreased by 25%, 47% and 46.7% respectively for wild type and 20%, 11% and 29.5% respectively for *HvBWMK1* antisense under 150 mM NaCl stress compared with their control. It is commonly known that salinity stress causes a decline in the chlorophyll content in leaves of different crops as reported by several researchers (discussed in section 4.5). Santos (2004) concluded that the reason behind the decrease of chlorophyll is because salinity induces the decline of 5-aminolevulinic acid synthesis which acts as a precursor for protochlorophyllide, which in turn acts as a precursor of chlorophyll biosynthesis, in addition to the lower levels of glutamic acid which is required for the synthesis of 5-aminolevulinic acid. One of the other reasons for a decrease in chlorophyll could be the presence of high concentrations of sugars in mesophyll cells which act as a kind of feedback limitation on chlorophyll synthesis (cited in Movafegh *et al*. 2012).

No significant difference was seen between non-stressed *HvBWMK1* overexpression and both non-stressed wild type and *HvBWMK1* antisense but a higher content of chlorophyll a, b and the total chlorophyll of the stressed *HvBWMK1* overexpression line was statistically significant different from either stressed wild type or the stressed *HvBWMK1* antisense line. A noticeable significant increase by 50%, 38% and 85.4% for chlorophyll a, b and total chlorophyll respectively were recorded in stressed *HvBWMK1* overexpression lines compared with its control. It thus appears that overexpression of the gene *HvBWMK1* might confer tolerance to salinity stress, as reflected on the increase of chlorophyll content in leaves after stress compared with unstressed control. These results suggest that *HvBWMK1* could play a positive role in salt tolerance. This is in line with the finding of Zhang *et al*. (2012) who tested tolerant and the moderately tolerant rice cultivars grown in saline soil with an average electrical conductivity (EC) of about 9.8 dS/m for 20 and 30 days, the chlorophyll a, b and the total chlorophyll in plant leaves were higher under salt stress than under control condition by about 17%, 94% and 32% respectively, in addition the tolerant cultivar showed higher concentration of chlorophyll a and higher chlorophyll a/b ratio, but lower concentration of chlorophyll b compared with moderately tolerant cultivar, they suggested that the tolerance of salt stress is related to the ability to maintain higher concentration of chlorophyll a and greater chlorophyll a/b ratio in plant tissues under salt stress. Also Zhou *et al*. (2009) found that transgenic rice expressing the salt

tolerance-related gene (*TaSTRG*) from wheat, contained a higher chlorophyll content in leaves under salt stress than in those of its respective control. Kanawapee *et al*. (2012) tested 106 of rice genotypes to screen for salt tolerance and from 94 surviving, 34 showed a decrease in chlorophyll while 60 showed increasing chlorophyll after salt stress for 7 days.

4.6.4 Hydrogen peroxide

The data generated in this study showed no statistically significant differences between wild type and *HvBWMK1* antisense lines in either the control or stress treatment. Both the wild type and *HvBWMK1* antisense lines under control or stressed treatments yielded a similar result to what was seen in the preliminary experiments for measuring wild type susceptibility to abiotic stress (section 4.5), the result of that experiment (responses of wild type barley to salt stress, section 4.5) was 5.3 µmol/gFW of H_2O_2 for non-stressed and 8.5 µmol/gFW after stress by 150 mM NaCl, whereas in this experiment (responses of transgenic barley to salt stress) H_2O_2 levels were 4.1 and 5 µmol/gFW for control and stressed wild type respectively, and 6 µmol/gFW for control and 5.9 µmol/gFW for stressed *HvBWMK1* antisense. These findings demonstrate the idea that salinity stress causes an increase in H_2O_2 level in plant leaves (as discussed in section 4.5), on the other hand it demonstrates that down-regulation of expression of the gene *HvBWMK1* didn't significantly affect the H_2O_2 levels in barley leaves.

Hydrogen peroxide level was high in non-stressed *HvBWMK1* overexpression plants about 60 and 40 fold higher than both non-stressed wild type and non-stressed *HvBWMK1* antisense line respectively. A similar pattern was observed in stressed treatments where *HvBWMK1* overexpression about 27 and 22 fold higher than both wild type and *HvBWMK1* antisense line respectively. This result is consistent with the findings of Koo *et al*. (2009) who reported that the concentration of H_2O_2 in *35S-OsBWMK1* transgenic tobacco plants was increased approximately 5 fold compared to those of wild-type plants.

Salinity stress caused a statistically significant decrease by 44.6% in hydrogen peroxide level in the *HvBWMK1* overexpression line compared with its respective controls. This reduction in H_2O_2 might be caused by proline, which showed higher levels in this line, about 51 fold higher than the control. Proline is considered as a ROS scavenger (Chen *et al*. 2007 and Ashraf *et al*. 2012). Similar findings were also reported

by Ruan *et al*. (2011), who reported a decline in H_2O_2 content of about approximately 36% and 10% in two transgenic rice plants carrying *OsCYP2* (a salt-induced rice cyclophilin gene that confers salt tolerance in rice seedlings when overexpressed) in response to salt stress (200 mM NaCl) compared with its control and that was accompanied by increasing in SOD, CAT and APX, ROS scavenging enzymes in the treated line.

4.6.5 Proline

The results from this study showed that salinity stress at 150 mM NaCl caused an increase in proline levels in stressed barley leaves compared with the unstressed controls. Proline accumulation is widely observed in various plants under salt stress, in addition to the reasons discussed in 4.5, where it was indicated that plants tend to increase proline levels as a defence system against stress, it was demonstrated by several researchers that proline acts as an osmo-protectant and ROS scavenger (Chen *et al*. 2007 and Ashraf *et al*. 2012). The level of proline accumulation in plants is highly variable from one species to another and even between some varieties of crop plants and can be 100 times higher in stressed plants than in control situation (Verbruggen and Hermans 2008). However, small amount (µmol/gFW) of compatible solutes such as proline in the plant tissues are sufficient for salt-tolerant cultivars to survive in severe salinity, by contrast, hyper-accumulation of compatible solutes appeared to be a symptom of injury (Chen *et al*. 2007).

There were no statistically significant differences among all non-stressed lines, but in contrast there were statistically significant differences between stressed and non-stressed for each line except for the *HvBWMK1* antisense line. The proline level was 51 fold higher in the stressed *HvBWMK1* overexpression line than its control whereas for wildtype proline levels were 5 fold higher in stressed plants compared to the control. The very high increase in proline level in the stressed *HvBWMK1* overexpression could be one of the reason behind the decline H_2O_2 by 44.6% in this line, as it is known that salt-tolerant plants produce larger amounts of the antioxidant such as proline to scavenge H_2O_2 (Ashraf *et al*. 2012), so, this suggest that the gene *HvBWMK1* positively regulates proline levels. In contrast, *HvBWMK1* in the antisense conformation is able to silence the endogenous *HvBWMK1* and the stressed *HvBWMK1* antisense line showed significantly lower accumulations of proline than stressed wild type. These results suggest that *HvBWMK1* could play a positive role in salt tolerance.

In this study, the overexpression *HvBWMK1* line revealed a negative correlation between proline and H_2O_2 in leaves. Similar finding were reported by earlier Ashraf *et al*. (2012) who found negative correlation between proline and H_2O_2 in salt-stressed wheat, they reported that proline content increased associated with decline of H_2O_2 in salt-tolerant wheat cultivar compared with sensitive cultivar. This again is consistant with a role for proline in acting as an antioxidant.

Overall, the findings on chlorophyll content, proline levels and hydrogen peroxide in the stressed overexpression lines are in contrast to the data for RWC, which indicates that the response of the plant to salt is not a simple linear pathway, but that *HvBWMK1* regulates different aspects of the plant response in different ways. Thus *HvBWMK1* appears to be a positive regulator of chlorophyll, proline and hydrogen peroxide accumulation under stress, but the relative water content of the salt stressed plants is reduced in comparison to the controls.

4.6.6 Metabolites

Two weeks old wild type and transgenic (empty binary vector pWBVec.8 lines, *HvBWMK1* antisense and *HvBWMK1* overexpression) barley seedlings were subjected to two weeks of salt stress (150 mM NaCl) then the shoots freeze-dried and ground to fine powder which was used to estimate the metabolomics compounds by GC-MS. Reference sample from tubers of the potato (*Solanum tuberosum*) cultivar Desiree and a blank sample containing only internal standards and retention standards were extracted and processed simultaneously with barley samples.

Changes in the concentration of 85 different metabolite compounds were found in freeze dried shoots extractions of barley, comprised 28 organic and fatty acids, 20 amino acids and amines, 10 sugars, 10 other different compounds and 17 unknown compounds, these findings are in range with which have been reported by several researchers such as Wu *et al*. (2013) who reported 82 metabolites in comparison between wild and cultivated barley cultivars after 21 days of 300 mM salinity treatment. In other research to compare salt sensitive and tolerant barley cultivars, 72 compounds were identified in leaf extracts comprising 23 amino acids, 20 organic acids and 29 sugars (Widodo *et al*. 2009). In the halophytic grass *Aeluropus lagopoides*, 52 compounds were found under salt stress comprising 20 amino acids, 16 nucleotides and

amino acids intermediates and 16 TCA cycle, glycolysis and photosynthesis compounds (Sobhanian *et al*. 2010).

Biosynthesis of amino acids are one of the most important indicators might be assessed the plants tolerance to abiotic stresses. Sobhanian *et al*. 2010 suggest that up-regulation of energy production, amino acid biosynthesis, C_4 photosynthesis, and detoxification are the main strategies for salt tolerance in *Aeluropus lagopoides*. In the experiments shown here, the wildtype line showed a higher level of content over all the transgenic lines in the following amino acids: asparagine, ethanolamine, glutamine, glutamic acid, glycine, lysine, serine, threonine, valine, putrescine, β-alanine and γ-aminobutyric acid. The reasons for this are unclear. Empty vector pWBVec.8 lines showed higher levels of methionine compared to wild type plants, *HvBWMK1* antisense lines and *HvBWMK1* overexpression lines. *HvBWMK1* antisense lines did not show any increase in amino acids compared on any other lines. *HvBWMK1* overexpression lines showed higher levels over the wild type, empty binary vector pWBVec.8 lines and *HvBWMK1* antisense lines in the following amino acids: leucine, isoleucine, proline, tyrosine, oxo-proline, tryptophan by the rate of 19.6%, 9%, 40%, 37%, 14.8% and 74.9% respectively than wild type line, these traits could have partially contributed to its salt tolerance. The content of proline was increased 53 fold in stressed *HvBWMK1* overexpression lines than unstressed control, which compares with an increase of 51 fold after NaCl treatment in the previous experiments when proline was measured spectrophotometrically. Similarly Sobhanian *et al*. 2010 reported 27 times increased in proline content when proline was measured by GC-MS and it was 32 times when measured by using a spectrophotometer for *Aeluropus lagopoides* shoots treated with 450 mM NaCl for 10 days. It is noteworthy that the proline (GC-MS measurements) was increased by 113, 23 and 39 fold approximately for stressed wild type, empty vector and antisense lines respectively compared with relative control, but on the other hand it was increased by 5 fold for wild type and 3 fold for antisense line compared with relative control when proline was measured spectrophotometrically. On the other hand the *HvBWMK1* overexpression lines showed higher levels in the rest of amino acids tested compared with the respective control except for aspartic acid, oxo-proline, putrescine and glutamic acid, which were reduced by 14%, 4.6%, 28% and 18% respectively compared with the control. Some amino acids might be decreased because they are precursors for other compounds up-regulated by salt stress, for example glutamate (carboxylate anion and salt of glutamic acid) is a known precursor for

synthesis of several amino acids such as proline (Verbruggen and Hermans 2008, Szabados and Savoure 2009) asparagine, glutamine and γ-aminobutyric acid synthesis (Forde and Lea 2007, Krasensky and Jonak 2012), so the increases in proline, glutamine and/or γ-aminobutyric acid synthesis after stress lead to reductions in glutamic acid content, the following amino acids, proline, asparagine, glutamine and γ-aminobutyric acid were increased by 53 fold 4 fold 2 fold and 1.2 fold respectively than the control after salt stress in the *HvBWMK1* overexpression lines in parallel with glutamic acid decreasing.

With respect to sugars, the levels of carbohydrate, fructose, galactose, glucose, glycerol and sucrose were increased by different rate in all lines after salt stress compared with unstressed controls whereas glucose gala glycerol, inositol and α-carbohydrate were decreased, polysaccharide was incrased in the wild type and empty binary vector pWBVec.8 lines and decreased in the *HvBWMK1* antisense lines and *HvBWMK1* overexpression lines. The stressed *HvBWMK1* overexpression lines accumulated higher content of sugars than the unstressed control but lower contents compared with other stressed lines, which agrees with the previous observations of Pattanagul and Thitisaksakul (2008) who reported an increase in concentrations of sugar only in the salt-sensitive rice cultivar, but not in moderately-tolerant and tolerant cultivar after exposure to 0, 50, 100 and 150 mM NaCl for 9 days. They suggested that accumulation of these sugars was probably as a result of the reduction of demand of sugars due to growth limitation of the sensitive cultivar.

TCA cycle-related compounds such as citric acid, fumaric acid, maleic acid and succinic acid were decreased in all wild type and transgenic lines after salt treatment, indicating that energy production in TCA cycle was affected by high salinity (Wu *et al*. 2013). It was reported by Sohanian *et al*. (2010) that some metabolites in the TCA cycle (citrate, aconitate, 2-oxoglutarate, succinate, and fumarate) were down-regulated in salt-stressed shoots of *Aeluropus lagopoides*. Also Widodo *et al*. (2009) reported in the salt adapted barley cultivar (Sahara) that the TCA cycle metabolites, such as citrate, fumarate, malate, and succinate were at -1.5 to -15 fold levels lower in salt treated lines than those in control plants.

Generally, in plants, stress tolerant genotypes have higher contents of stress-related metabolites under normal growth conditions, furthermore under stress conditions these varieties accumulate higher contents of protective metabolites, such as proline and

soluble sugars, indicating that their metabolism is prepared for adverse growth conditions (Krasensky and Jonak 2012).

4.7 Possibility of gene silencing in T_1 plants

The differences between the level of *HvBWMK1* expression in the T_0 and T_1 transgenics was very obvious, for example, the gene expression in wild type in the northern blot was much higher compared with T_0 antisense *HvBWMK1* lines 3, 5 and 7 whereas in real-time PCR of T_1, the expression of antisense *HvBWMK1* lines 3, 5 and 7 was lower than wild type by 14%, 23% and 39% respectively, which may be indicative of some transgene silencing in the T_1 generation.

There are two types of gene silencing systems, First, transcriptional gene silencing (TGS), occurs in nucleus via promoter inactivation resulting in the silencing of a transgene or an endogenous gene by methylation and histone modification. Second, post-transcriptional gene silencing (PTGS), presumably occurs in the cytoplasm when the promoter is active but the mRNAs fail to accumulate due to mRNA cleavage or inhibition of translation, that could be for transgenes and endogenous genes (Stam *et al*. 1997, Eamens *et al*. 2008).

The *Agrobacterium tumefaciens*, which was used in this study, integrates T-DNA at different chromosomal locations. If it inserted into euchromatin, in a transcriptional active region, the expression could be affected by regulatory sequences of nearby host genes, whereas the T-DNA can be inactivated if it insert in or near repetitive DNA or heterochromatin. Also *Agrobacterium tumefaciens* able to integrate two or more T-DNA at the same chromosomal locations, which can be arranged (head-to-tail) as a direct repeat (DR), and (head-to-head) or (tail-to-tail) as an inverted repeat (IR) (Stam *et al*. 1997). The last position IR often shows low expression indicating that the genes are silenced to some degree (Stam *et al*. 1998).

The presence of introns is a common feature in endogenous genes that might protect the endogenous genes from silencing. Conversely, transgenes which mostly depending on cDNA are often lacking the introns. Christie *et al*. (2011) was used an intronless construct to transformed *Arabidopsis* plants, 85% of T_1 plants show gene silencing, in contrast, introducing an intron into a transgene reduced silencing by more than fourfold compared with intronless transformed plants.

4.8 Future work

Future work would include further experiments to understand and clarify the role of the gene *HvBWMK1* in barley response to biotic and abiotic stress as explained below:

- Investigate the transgenic barley response to abiotic stresses using more different selection criteria.
- Investigate the transgenic barley responses to necrotrophic pathogen such as the fungus *Botrytis cinerea* and comparison with responses to biotrophic pathogen such as the fungus *Magnaporthe grisea.*
- Carry out immunoblot analysis using antibody to detect the level of *HvBWMK1* protein in plant tissues.
- Carry out proteomic analysis by using two-dimensional polyacrylamide gel electrophoresis, followed by protein identification.

APPENDICES

Appendix 5.1: The ORF sequence of *HvBWMK1* of barley

Salem BWMK1.strt From 1 to 1737.
Translation 578 a.a. MW=65278.85

```
   1 ATG GGG GGA GGG AAC GGC ATC GTC GAC GGC TTC CGC CGC TTG TTC CAC CGC CGC ACG CCC
   1  M   G   G   G   N   G   I   V   D   G   F   R   R   L   F   H   R   R   T   P

  61 TCC GGC TCC GTG CTC GGC AGC TCC AAC CAG TCC TCC GCC GGC GAG GAC TCC TCG GAG CTC
  21  S   G   S   V   L   G   S   S   N   Q   S   S   A   G   E   D   S   S   E   L

 121 GAG GCC GTC GAG GAC CTG GAT CTC GTG GGC CTC CGC CCC ATC CGC GTC CCC AAG CGC AAG
  41  E   A   V   E   D   L   D   L   V   G   L   R   P   I   R   V   P   K   R   K

 181 ATG CCG CTC CCC GTC GAG AGC CAC AAG AAG AAC ATA ATG GAG AAA GAA TTC TTC ACT GAG
  61  M   P   L   P   V   E   S   H   K   K   N   I   M   E   K   E   F   F   T   E

 241 TAC GGA GAG GCA AGC CAG TAC CAA ATC CAA GAA GTT GTT GGC AAA GGG AGT TAT GGA GTG
  81  Y   G   E   A   S   Q   Y   Q   I   Q   E   V   V   G   K   G   S   Y   G   V

 301 GTT GCT GCT GCA ATA GAT ACC CGC ACC GGC GAG CGG GTT GCG ATT AAG AAG ATC AAT GAT
 101  V   A   A   A   I   D   T   R   T   G   E   R   V   A   I   K   K   I   N   D

 361 GTG TTT GAG CAT GTC TCG GAT GCC ACA CGC ATC CTC CGT GAG GTC AAG CTC CTT CGG CTG
 121  V   F   E   H   V   S   D   A   T   R   I   L   R   E   V   K   L   L   R   L

 421 CTC CGT CAT CCG GAC GTG GTA GAG ATC AAG CAC ATA ATG CTC CCC CCT TCT CGG AGG GAA
 141  L   R   H   P   D   V   V   E   I   K   H   I   M   L   P   P   S   R   R   E

 481 TTC CAA GAT ATA TAT GTT GTT TTC GAG CTC ATG GAG TCG GAT CTC CAT CAG GTT ATC AGA
 161  F   Q   D   I   Y   V   V   F   E   L   M   E   S   D   L   H   Q   V   I   R

 541 GCT AAT GAT GAC CTC ACG GCG GAG CAT TAC CAG TTT TTC CTT TAC CAG CTT CTC CGC GCT
 181  A   N   D   D   L   T   A   E   H   Y   Q   F   F   L   Y   Q   L   L   R   A

 601 CTC AAG TAC ATC CAT GGG GCT AAT GTA TTT CAT CGC GAT CTG AAG CCC AAG AAT ATA CTG
 201  L   K   Y   I   H   G   A   N   V   F   H   R   D   L   K   P   K   N   I   L

 661 GCC AAC GCA GAC TGC AAA CTG AAA ATT TGT GAC TTT GGA CTT GCG CGT GTA TCA TTT AAT
 221  A   N   A   D   C   K   L   K   I   C   D   F   G   L   A   R   V   S   F   N

 721 GAT GCA CCT TCA GCT ATA TTT TGG ACG GAT TAT GTA GCA ACA AGG TGG TAC AGA GCT CCT
 241  D   A   P   S   A   I   F   W   T   D   Y   V   A   T   R   W   Y   R   A   P

 781 GAA TTA TGT GGC TCC TTT TTC TCG AAA TAC ACT CCT GCT ATT GAT ATT TGG AGT ATT GGA
 261  E   L   C   G   S   F   F   S   K   Y   T   P   A   I   D   I   W   S   I   G

 841 TGT ATA TTT GCC GAG CTT CTC ACT GGA CGA CCA CTT TTT CCT GGG AAG AAT GTT GTA CAC
 281  C   I   F   A   E   L   L   T   G   R   P   L   F   P   G   K   N   V   V   H

 901 CAG TTA GAT ATA ATA ACA GAT CTT CTT GGA ACT CCA TCA TCA GAA ACC TTA TCT CGG ATT
 301  Q   L   D   I   I   T   D   L   L   G   T   P   S   S   E   T   L   S   R   I

 961 CGA AAC GAG AAG GCC AGG AGG TAC TTA AGT TGC ATG CGA AAA AAA CAT CCT GTG CCC TTG
 321  R   N   E   K   A   R   R   Y   L   S   C   M   R   K   K   H   P   V   P   L

1021 ACT CAG AAA TTT CCT AAT GCT GAT CCG TTG GCG GTT CGC CTA CTG GGG CGT TTA CTT GCA
 341  T   Q   K   F   P   N   A   D   P   L   A   V   R   L   L   G   R   L   L   A

1081 TTT GAT CCT AAA GAC CGG CCT TCA GCT GAA GAG GCT TTG GCA GAC CCA TAT TTC GCA TCT
 361  F   D   P   K   D   R   P   S   A   E   E   A   L   A   D   P   Y   F   A   S

1141 CTT GCT AAT GTG GAA CGT GAG CCT TCA AGG CAT CCA ATT TCG AAA CTT GAG TTT GAG TTT
 381  L   A   N   V   E   R   E   P   S   R   H   P   I   S   K   L   E   F   E   F
```

```
1201 GAG AGA CGA AAG GTG ACA AAA GAT GAT GTT AGA GAA TTG ATC TAT CGA GAG ATT TTG GAG
 401  E   R   R   K   V   T   K   D   D   V   R   E   L   I   Y   R   E   I   L   E

1261 TAC CAT CCA CAA ATG CTG GAG GAG TAC ATG AAA GGG GGA GAT CAG ATT AGC TTC CTC TAT
 421  Y   H   P   Q   M   L   E   E   Y   M   K   G   G   D   Q   I   S   F   L   Y

1321 CCA AGT GGG GTT GAC CGC TTT AAG CGG CAG TTT GCG CAC CTG GAG GAG CAT TAC AGC AAA
 441  P   S   G   V   D   R   F   K   R   Q   F   A   H   L   E   E   H   Y   S   K

1381 GGA GAA CGA GGT TCT CCA CTG CAA AGA AAG CAT GCC TCT TTA CCG AGG CAG AGA GTA GGT
 461  G   E   R   G   S   P   L   Q   R   K   H   A   S   L   P   R   Q   R   V   G

1441 GCA TCG AAC GAC GGT AAT AAT GAA CAG CAT ATT AGT GAT CAG GAG ATG AGT GCA GAG CCT
 481  A   S   N   D   G   N   N   E   Q   H   I   S   D   Q   E   M   S   A   E   P

1501 GAT GCC CGT GGT GCA GTG AGC CCT CAA AAG CCA CAG GAT GCA CCC GGT GTT GGC CAG AAT
 501  D   A   R   G   A   V   S   P   Q   K   P   Q   D   A   P   G   V   G   Q   N

1561 GGT CTG AGC CCC ACC AGC TTG AGC TCG CGG ACC TAC CTC AAG AGC GCG AGC ATT AGT GCT
 521  G   L   S   P   T   S   L   S   S   R   T   Y   L   K   S   A   S   I   S   A

1621 TCC AAG TGT GTC GTT GTC AAC CCG AAT AAA CAG CCA GAG TAT GAC GAT GCG ATC TCT GAG
 541  S   K   C   V   V   V   N   P   N   K   Q   P   E   Y   D   D   A   I   S   E

1681 GAA ACG GAA GGG GCC GTC GAC GGA CTA TCC GAG AAG GTC TCC AAG ATG CAT GCC TAG
 561  E   T   E   G   A   V   D   G   L   S   E   K   V   S   K   M   H   A   *
```

The highlighted letters in bold refer to the phosphorylation motif site TDY.

Appendix 5.2: Result of TBlastN analysis using rice *OsBWMK1* amino acids sequence as query

```
>dbj|AK252439.1|  Hordeum vulgare subsp. vulgare cDNA clone:
FLbaf161m06, mRNA sequence
Length=2321

 Score =927 bits(2395),Expect= 0.0,Method:Compositional matrix adjust.
 Identities = 447/506 (88%, Positives=471/506(93%), Gaps =4/506(0%)
 Frame = +3

Query  2     EFFTEYGEASQYQIQEVIGKGSYGVVAAAVDTRTGERVAIKKINDVFEHVSDATRILREI  61
             EFFTEYGEASQYQIQEV+GKGSYGVVAAA+DTRTGERVAIKKINDVFEHVSDATRILRE+
Sato   462   EFFTEYGEASQYQIQEVVGKGSYGVVAAAIDTRTGERVAIKKINDVFEHVSDATRILREV  641

Query  62    KLLRLLRHPDIVEIKHIMLPPSRREFQDIYVVFELMESDLHQVIRANDDLTPEHYQFFLY  121
             KLLRLLRHPD+VEIKHIMLPPSRREFQDIYVVFELMESDLHQVIRANDDLT EHYQFFLY
Sato   642   KLLRLLRHPDVVEIKHIMLPPSRREFQDIYVVFELMESDLHQVIRANDDLTAEHYQFFLY  821

Query  122   QLLRALKYIHAANVFHRDLKPKNILANSDCKLKICDFGLARASFNDAPSAIFWTDYVATR  181
             QLLRALKYIH ANVFHRDLKPKNILAN+DCKLKICDFGLAR SFNDAPSAIFWTDYVATR
Sato   822   QLLRALKYIHGANVFHRDLKPKNILANADCKLKICDFGLARVSFNDAPSAIFWTDYVATR  1001

Query  182   WYRAPELCGSFFSKYTPAIDIWSIGCIFAELLTGRPLFPGKNVVHQLDIITDLLGTPSSE  241
             WYRAPELCGSFFSKYTPAIDIWSIGCIFAELLTGRPLFPGKNVVHQLDIITDLLGTPSSE
Sato   1002  WYRAPELCGSFFSKYTPAIDIWSIGCIFAELLTGRPLFPGKNVVHQLDIITDLLGTPSSE  1181

Query  242   TLSRIRNEKARRYLSTMRKKHAVPFSQKFRNTDPLALRLLERLLAFDPKDRPSAEEALAD  301
             TLSRIRNEKARRYLS MRKKH VP +QKF N DPLA+RLL RLLAFDPKDRPSAEEALAD
Sato   1182  TLSRIRNEKARRYLSCMRKKHPVPLTQKFPNADPLAVRLLGRLLAFDPKDRPSAEEALAD  1361

Query  302   PYFASLANVEREPSRHPISKLEFEFERRKLTKDDVRELIYREILEYHPQMLQEYMKGGEQ  361
             PYFASLANVEREPSRHPISKLEFEFERRK+TKDDVRELIYREILEYHPQML+EYMKGG+Q
Sato   1362  PYFASLANVEREPSRHPISKLEFEFERRKVTKDDVRELIYREILEYHPQMLEEYMKGGDQ  1541

Query  362   ISFLYPSGVDRFKRQFAHLEENYSKGERGSPLQRKHASLPRERVGVSKDGYNQQNTNDQE  421
             ISFLYPSGVDRFKRQFAHLEE+YSKGERGSPLQRKHASLPR+RVG S DG N+Q+ +DQE
Sato   1542  ISFLYPSGVDRFKRQFAHLEEHYSKGERGSPLQRKHASLPRQRVGASNDGNNEQHISDQE  1721

Query  422   RSADSVARTTVSPPMSQDAQQHGSAGQNGVTSTDLSSRSYLKSASISASKCVAVKDNKEP  481
              SA+  AR  VSP   QDA      GQNG++ T LSSR+YLKSASISASKCV V  NK+P
Sato   1722  MSAEPDARGAVSPQKPQDAP---GVGQNGLSPTSLSSRTYLKSASISASKCVVVNPNKQP  1892

Query  482   E-DDYISEEMEGSVDGLSEQVSRMHS  506
             E DD ISEE EG+VDGLSE+VS+MH+
Sato   1893  EYDDAISEETEGAVDGLSEKVSKMHA  1970
```

Query: refers to amino acids of rice *OsBWMK1*.

Sato: refers to amino acids of barley cDNA (AK252439) donated by Dr. Sato.

Appendix 5.3: Alignment between two nucleotide sequences of *HvBWMK1*

```
Salem BWMK1.str from 1 to 1737
Alignment to
Sato BWMK1 ORF.str from 1 to 1737
Matches(|):1737
Mismatches(#):0

     1 ATGGGGGGAGGGAACGGCATCGTCGACGGCTTCCGCCGCTTGTTCCACCGCCGCACGCCCTCCGGCTCCGTGCTCGGCAG 80
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
     1 atggggggagggaacggcatcgtcgacggcttccgccgcttgttccaccgccgcacgccctccggctccgtgctcggcag 80

    81 CTCCAACCAGTCCTCCGCCGGCGAGGACTCCTCGGAGCTCGAGGCCGTCGAGGACCTGGATCTCGTGGGCCTCCGCCCCA 160
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    81 ctccaaccagtcctccgccggcgaggactcctcggagctcgaggccgtcgaggacctggatctcgtgggcctccgcccca 160

   161 TCCGCGTCCCCAAGCGCAAGATGCCGCTCCCCGTCGAGAGCCACAAGAAGAACATAATGGAGAAAGAATTCTTCACTGAG 240
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   161 tccgcgtccccaagcgcaagatgccgctccccgtcgagagccacaagaagaacataatggagaaagaattcttcactgag 240

   241 TACGGAGAGGCAAGCCAGTACCAAATCCAAGAAGTTGTTGGCAAAGGGAGTTATGGAGTGGTTGCTGCTGCAATAGATAC 320
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   241 tacggagaggcaagccagtaccaaatccaagaagttgttggcaaagggagttatggagtggttgctgctgcaatagatac 320

   321 CCGCACCGGCGAGCGGGTTGCGATTAAGAAGATCAATGATGTGTTTGAGCATGTCTCGGATGCCACACGCATCCTCCGTG 400
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   321 ccgcaccggcgagcgggttgcgattaagaagatcaatgatgtgtttgagcatgtctcggatgccacacgcatcctccgtg 400

   401 AGGTCAAGCTCCTTCGGCTGCTCCGTCATCCGGACGTGGTAGAGATCAAGCACATAATGCTCCCCCCTTCTCGGAGGGAA 480
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   401 aggtcaagctccttcggctgctccgtcatccggacgtggtagagatcaagcacataatgctccccccttctcggagggaa 480

   481 TTCCAAGATATATATGTTGTTTTCGAGCTCATGGAGTCGGATCTCCATCAGGTTATCAGAGCTAATGATGACCTCACGGC 560
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   481 ttccaagatatatatgttgttttcgagctcatggagtcggatctccatcaggttatcagagctaatgatgacctcacggc 560

   561 GGAGCATTACCAGTTTTTCCTTTACCAGCTTCTCCGCGCTCTCAAGTACATCCATGGGGCTAATGTATTTCATCGCGATC 640
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   561 ggagcattaccagtttttccttttaccagcttctccgcgctctcaagtacatccatggggctaatgtatttcatcgcgatc 640

   641 TGAAGCCCAAGAATATACTGGCCAACGCAGACTGCAAACTGAAAATTTGTGACTTTGGACTTGCGCGTGTATCATTTAAT 720
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   641 tgaagcccaagaatatactggccaacgcagactgcaaactgaaaatttgtgactttggacttgcgcgtgtatcatttaat 720

   721 GATGCACCTTCAGCTATATTTTGGACGGATTATGTAGCAACAAGGTGGTACAGAGCTCCTGAATTATGTGGCTCCTTTTT 800
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   721 gatgcaccttcagctatattttggacggattatgtagcaacaaggtggtacagagctcctgaattatgtggctcctttttt 800

   801 CTCGAAATACACTCCTGCTATTGATATTTGGAGTATTGGATGTATATTTGCCGAGCTTCTCACTGGACGACCACTTTTTC 880
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   801 ctcgaaatacactcctgctattgatatttggagtattggatgtatatttgccgagcttctcactggacgaccacttttttc 880

   881 CTGGGAAGAATGTTGTACACCAGTTAGATATAATAACAGATCTTCTTGGAACTCCATCATCAGAAACCTTATCTCGGATT 960
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   881 ctgggaagaatgttgtacaccagttagatataataacagatcttcttggaactccatcatcagaaaccttatctcggatt 960

   961 CGAAACGAGAAGGCCAGGAGGTACTTAAGTTGCATGCGAAAAAAACATCCTGTGCCCTTGACTCAGAAATTTCCTAATGC 1040
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   961 cgaaacgagaaggccaggaggtacttaagttgcatgcgaaaaaaacatcctgtgcccttgactcagaaatttcctaatgc 1040

  1041 TGATCCGTTGGCGGTTCGCCTACTGGGGCGTTTACTTGCATTTGATCCTAAAGACCGGCCTTCAGCTGAAGAGGCTTTGG 1120
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  1041 tgatccgttggcggttcgcctactggggcgtttacttgcatttgatcctaaagaccggccttcagctgaagaggctttgg 1120
```

```
1121 CAGACCCATATTTCGCATCTCTTGCTAATGTGGAACGTGAGCCTTCAAGGCATCCAATTTCGAAACTTGAGTTTGAGTTT 1200
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1121 cagacccatatttcgcatctcttgctaatgtggaacgtgagccttcaaggcatccaatttcgaaacttgagtttgagttt 1200

1201 GAGAGACGAAAGGTGACAAAAGATGATGTTAGAGAATTGATCTATCGAGAGATTTTGGAGTACCATCCACAAATGCTGGA 1280
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1201 gagagacgaaaggtgacaaaagatgatgttagagaattgatctatcgagagattttggagtaccatccacaaatgctgga 1280

1281 GGAGTACATGAAAGGGGGAGATCAGATTAGCTTCCTCTATCCAAGTGGGGTTGACCGCTTTAAGCGGCAGTTTGCGCACC 1360
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1281 ggagtacatgaaaggggggagatcagattagcttcctctatccaagtggggttgaccgctttaagcggcagtttgcgcacc 1360

1361 TGGAGGAGCATTACAGCAAAGGAGAACGAGGTTCTCCACTGCAAAGAAAGCATGCCTCTTTACCGAGGCAGAGAGTAGGT 1440
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1361 tggaggagcattacagcaaaggagaacgaggttctccactgcaaagaaagcatgcctctttaccgaggcagagagtaggt 1440

1441 GCATCGAACGACGGTAATAATGAACAGCATATTAGTGATCAGGAGATGAGTGCAGAGCCTGATGCCCGTGGTGCAGTGAG 1520
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1441 gcatcgaacgacggtaataatgaacagcatattagtgatcaggagatgagtgcagagcctgatgcccgtggtgcagtgag 1520

1521 CCCTCAAAAGCCACAGGATGCACCCGGTGTTGGCCAGAATGGTCTGAGCCCCACCAGCTTGAGCTCGCGGACCTACCTCA 1600
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1521 ccctcaaaagccacaggatgcacccggtgttggccagaatggtctgagccccaccagcttgagctcgcggacctacctca 1600

1601 AGAGCGCGAGCATTAGTGCTTCCAAGTGTGTCGTTGTCAACCCGAATAAACAGCCAGAGTATGACGATGCGATCTCTGAG 1680
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1601 agagcgcgagcattagtgcttccaagtgtgtcgttgtcaacccgaataaacagccagagtatgacgatgcgatctctgag 1680

1681 GAAACGGAAGGGGCCGTCGACGGACTATCCGAGAAGGTCTCCAAGATGCATGCCTAG 1737
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1681 gaaacggaaggggccgtcgacggactatccgagaaggtctccaagatgcatgcctag 1737
```

Salem: refers to the sequences of cloned *HvBWMK1*.

Sato: refers to the ORF sequences of unpublished barley cDNA donated by Dr. Sato.

Appendix 5.4: Multiple amino acids sequence alignment

```
CLUSTAL 2.0.10 multiple amino acids sequence alignment

Salem   MGGGNGIVDGFRRLFHRRTPSGSVLGSSNQSSAGEDSSELEAVEDLDLVGLRPIRVPKRK 60
Sato    MGGGNGIVDGFRRLFHRRTPSGSVLGSSNQSSAGEDSSELEAVEDLDLVGLRPIRVPKRK 60
Eckey   MGGGNGIVDGFRRLFHRRTPSGSVLGSSNQSSAGEDSSELEAVEDLDLVGLRPIRVPKRK 60

Salem   MPLPVESHKKNIMEKEFFTEYGEASQYQIQEVVGKGSYGVVAAAIDTRTGERVAIKKIND 120
Sato    MPLPVESHKKNIMEKEFFTEYGEASQYQIQEVVGKGSYGVVAAAIDTRTGERVAIKKIND 120
Eckey   MPLPVESHKKNIMEKEFFTEYGEASQYQIQEVVGKGSYGVVAAAIDTRTGERVAIKKIND 120

Salem   VFEHVSDATRILREVKLLRLLRHPDVVEIKHIMLPPSRREFQDIYVVFELMESDLHQVIR 180
Sato    VFEHVSDATRILREVKLLRLLRHPDVVEIKHIMLPPSRREFQDIYVVFELMESDLHQVIR 180
Eckey   VFEHVSDATRILREVKLLRLLRHPDVVEIKHIMLPPSRREFQDIYVVFELMESDLHQVIR 180

Salem   ANDDLTAEHYQFFLYQLLRALKYIHGANVFHRDLKPKNILANADCKLKICDFGLARVSFN 240
Sato    ANDDLTAEHYQFFLYQLLRALKYIHGANVFHRDLKPKNILANADCKLKICDFGLARVSFN 240
Eckey   ANDDLTAEHYQFFLYQLLRALKYIHGANVFHRDLKPKNILANADCKLKICDFGLARVSFN 240

Salem   DAPSAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSIGCIFAELLTGRPLFPGKNVVH 300
Sato    DAPSAIFWTDYVATRWYRAPELCGSFFSKYTPAIDIWSIGCIFAELLTGRPLFPGKNVVH 300
Eckey   DAPSAIFWTDYVATRWYRAPELCGSFFSKYTPAIEIWSIGCIFAELLTGRPLFPGKNVVH 300

Salem   QLDIITDLLGTPSSETLSRIRNEKARRYLSCMRKKHPVPLTQKFPNADPLAVRLLGRLLA 360
Sato    QLDIITDLLGTPSSETLSRIRNEKARRYLSCMRKKHPVPLTQKFPNADPLAVRLLGRLLA 360
Eckey   QLDIITDLLGTPSSETLSRIRNEKARRYLRYMRKKHPVPLTQKFPNADPLAVRLLGRLLA 360

Salem   FDPKDRPSAEEALADPYFASLANVEREPSRHPISKLEFEFERRKVTKDDVRELIYREILE 420
Sato    FDPKDRPSAEEALADPYFASLANVEREPSRHPISKLEFEFERRKVTKDDVRELIYREILE 420
Eckey   FDPKDRPSAEEALADPYFASLANVEREPSRHPISKLEFEFERRKVTKDDVRELIYREILE 420

Salem   YHPQMLEEYMKGGDQISFLYPSGVDRFKRQFAHLEEHYSKGERGSPLQRKHASLPRQRVG 480
Sato    YHPQMLEEYMKGGDQISFLYPSGVDRFKRQFAHLEEHYSKGERGSPLQRKHASLPRQRVG 480
Eckey   YRPQMLEEYMKGGDQISFLYPSGVDRFKRQFAHLEEHYSKGERGSPLQRKHASLPRQRVG 480

Salem   ASNDGNNEQHISDQEMSAEPDARGAVSPQKPQDAPGVGQNGLSPTSLSSRTYLKSASISA 540
Sato    ASNDGNNEQHISDQEMSAEPDARGAVSPQKPQDAPGVGQNGLSPTSLSSRTYLKSASISA 540
Eckey   ASNDSNNEQHISDQEMSAEPDAHGAVSPQKPQDAPGVGQNGLSPTSLSSRTYLKSASISA 540

Salem   SKCVVVNPNKQPEYDDAISEETEGAVDGLSEKVSKMHA 578
Sato    SKCVVVNPNKQPEYDDAISEETEGAVDGLSEKVSKMHA 578
Eckey   SKCVVVNPNKQPEYDDAISEETEGAVDGLSEKVSKMHA 578
```

Salem: refers to the amino acids of cloned *HvBWMK1*.

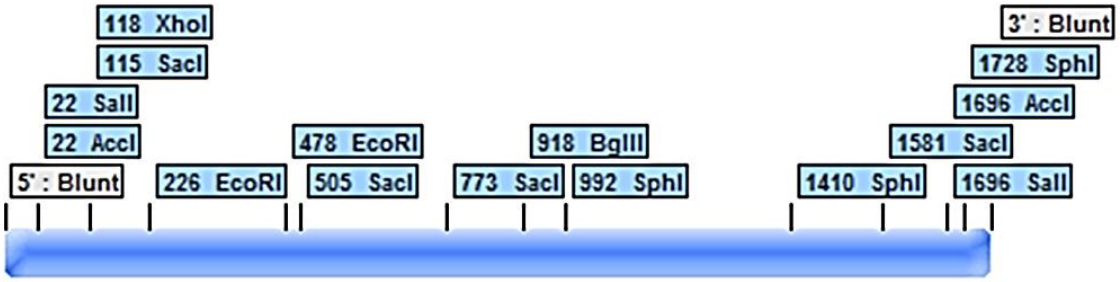
Sato: refers to the amino acids of unpublished barley cDNA (GenBank: AK252439.1) donated by Dr. Sato.

Eckey: refers to amino acids of unpublished powdery mildew infected barley cDNA (GenBank: AJ495775.1).

The highlighted letters refer to the phosphorylation motif site TDY.

The highlighted letters in bold refer to different amino acids.

Appendix 5.5: The restriction map of *HvBWMK1*.



The restriction map of *HvBWMK1* (1737 bp) created by Serial Cloner V2.5 software using selected enzymes that exist on pTopoActin-OCS plasmid, each enzyme has at least one restriction site on the plasmids' multiple cloning site and backbone.

Letters refer to the enzyme name, numbers refer to the nucleotide number where the restriction site, for example the enzyme *Xho*I cuts on the nucleotide number 118.

Appendix 5.6: Callus induction medium (CIM) (Harwood *et al*. 2009)

Callus induction medium Co-cultivation 3 days	Callus induction medium cultivation 4-6 weeks	Transition medium 2 weeks	Regeneration medium Full light	Callus induction medium
4.3 g MS	**4.3 g** MS	**2.7 g** MS	**2.7 g** MS	**4.3 g** MS
30 g Maltose	**30 g** Maltose	**20 g** Maltose	**20 g** Maltose	**30 g** Maltose
1g Casein hydrolysate	**1 g** Casein hydrolysate			**1 g** Caseine
350 mg Myo-inositol	**350 mg** Myo-inositol	**100 mg** Myo-inositol	**100 mg** Myo-inositol	**350 mg** Myo-inositol
690 mg Proline	**690 mg** Proline			
1 mg Thiamine	**1 mg** Thiamine	**0.4 mg** Thiamine	**0.4 mg** Thiamine	**1 mg** Thiamine
2.5 mg Dicamba	**2.5 mg** Dicamba			
3.5 g Phytagel	**3.5 g** Phytagel	**3.5 g** Phytagel	**3.5 g** Phytagel	**3.5 g** Phytagel
1.25 mg $CuSO_4$. $5H_2O$	**1.25 mg** $CuSO_4$. $5H_2O$	**1.25 mg** $CuSO_4$. $5H_2O$		
	50 mg Hygromycin	**50 mg** Hygromycin	**50 mg** Hygromycin	**50 mg** Hygromycin
	160 mg Timentin	**160 mg** Timentin	**160 mg** Timentin	**160 mg** Timentin
		165 mg NH_2 NO_3	**165 mg** NH_2 NO_3	
		750 mg Glutamine	**750 mg** Glutamine	
		0.1 mg 6-BAP		
		2.5 mg 2,4-D		
pH **5.8**	pH **5.8**	pH **5.8**	pH **5.8**	pH **5.8**

Appendix 5.7: Callus induction medium (CIM). Ms. Jill Middlefell-Williams.

Callus induction medium (co-cultivation) 2 days	Selection medium 4 weeks dark	Regeneration Medium 2 weeks full light	Rooting Medium
4.3 g MS	**4.3 g** MS	**4.3 g** MS	**4.3 g** MS
30 g Maltose	**30 g** Maltose	**30 g** Maltose	**30 g** Maltose
1 g Casein hydrolysate	**1 g** Casein hydrolysate	**1 g** Casein hydrolysate	**1 g** Casein hydrolysate
250 mg Myo-inositol	**250 mg** Myo-inositol	**250 mg** Myo-inositol	**250 mg** Myo-inositol
650 mg Proline	**650 mg** Proline	**650 mg** Proline	**650 mg** Proline
1 mg Thiamine	**1 mg** Thiamine	**1 mg** Thiamine	**1 mg** Thiamine
2.5 mg Dicamba	**2.5 mg** Dicamba		
3.9 g Phytagel	**3.9 g** Phytagel	**3.9 g** Phytagel	**3.9 g** Phytagel
	1.25 mg $CuSO_4.5H_2O$		
10 mM MES	**10 mM** MES	**10 mM** MES	**10 mM** MES
	50 mg Hygromycin	**50 mg** Hygromycin	**50 mg** Hygromycin
	160 mg Timentin	**160 mg** Timentin	**160 mg** Timentin
		0.1 mg 6BAP	
pH 5.8	**pH 5.8**	**pH 5.8**	**pH 5.8**

Appendix 5.8: Multiple comparisons of significant differences among the tested barley lines.

Metabolite name	Wild type		Empty vector		Antisense		Overexpression	
	C	S	C	S	C	S	C	S
2-Piperidinecarboxylic	a	c	a	b	a	c	a	c
Ethanolamine	abc	c	bc	c	a	bc	ab	bc
Fructose	ab	b	a	b	a	b	a	b
Galactose	a	b	a	b	a	b	a	b
Glucose	bcd	d	ab	cd	abc	d	a	cd
Glucose Gala glycerol	a	ab	cd	bc	d	bc	d	cd
Glycerol	a	abc	ab	c	a	bc	a	c
L-aspartic acid	ab	a	bc	a	c	a	ab	a
Leucine	a	bcd	bc	cd	b	d	b	d
L-isoleucine	a	c	b	c	b	c	ab	c
L-lysine	a	bc	abc	bc	a	bc	ab	c
L-methionine	a	bcd	abc	d	ab	c	ab	cd
L-proline	a	b	a	b	a	b	a	b
L-serine	a	c	a	b	a	b	a	bc
L-threonine	a	c	bc	bc	bc	c	ab	c
L-valine	a	b	a	b	a	b	a	b
n-docosanol	bc	abc	a	a	a	c	a	ab
n-heptadecanoic acid	d	abc	abc	bcd	abcd	cd	ab	a
n-hexacosanol	abc	a	ab	bc	ab	ab	c	ab
n-hexadecanoic acid	d	abc	abcd	ab	bcd	abc	cd	a
n-octacosanol	bc	abc	a	a	a	a	c	ab
n-octadecanoic acid	c	abc	bc	abc	ab	a	abc	a
Phosphate	abc	a	c	a	c	ab	bc	ab
Quinic acid	c	ab	b	a	b	a	b	a
Sucrose	d	d	a	c	a	bc	a	b
Threonic acid	bc	bc	ab	a	c	ab	ab	bc
Tryptophan	ab	abc	a	bc	a	abc	a	c
Unknown-7-36	c	bc	a	ab	a	ab	a	ab
Unknown-U2498	a	ab	a	b	a	b	a	b
Unknown-UA-10-0	c	abc	a	a	a	a	b	ab
α-Linolenic acid	c	abc	abc	a	bc	ab	bc	a
β-Sitosterol	c	a	a	a	ab	a	bc	a

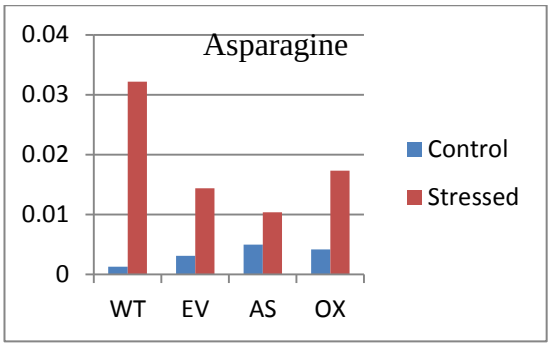
Different letters in each row show significant difference at p 0.05 by Fishers protected LSD's test. C = control treatment. S = stressed treatment.

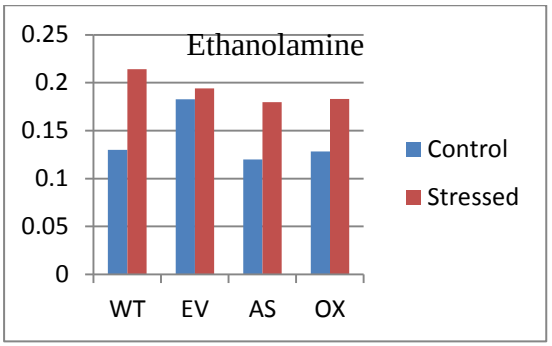
Appendix 5.9: The impact of salinity on metabolite compounds in barley freeze dried shoots.

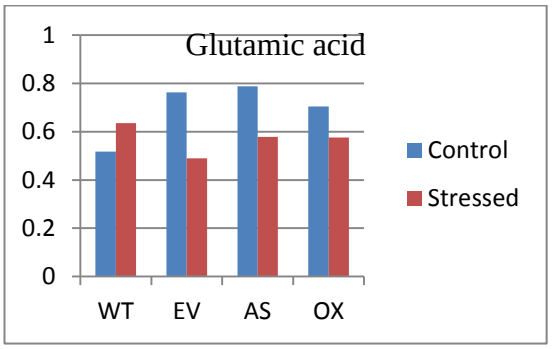
WT = Wild type. EV = Empty vector. AS = Antisense. OX = Overexpression.

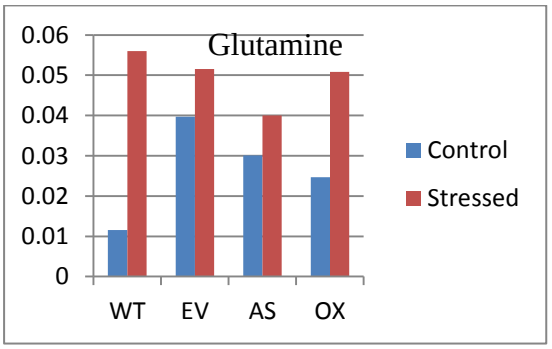
3 replicates for WT. 3 replicates for each transgenic line EV (line 1, 2 and 3), AS (line 3, 5 and 7) and OX (line 3, 4 and 5).

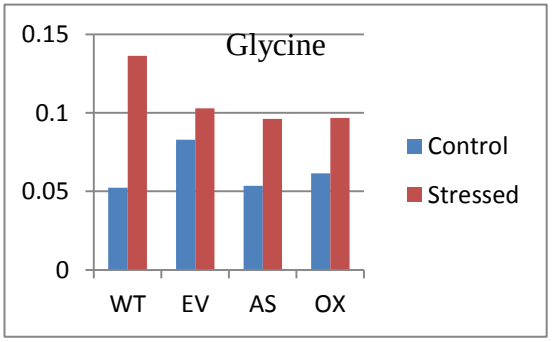
Amino acids and amines

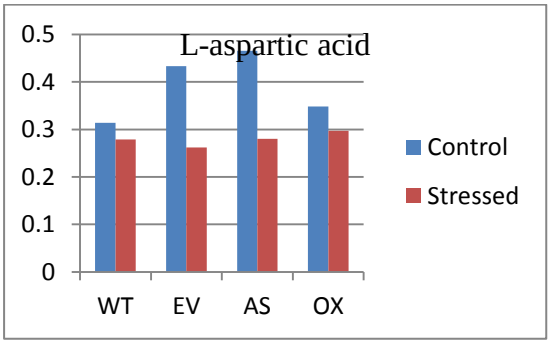


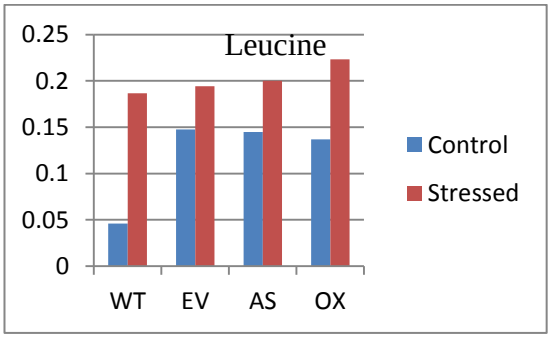


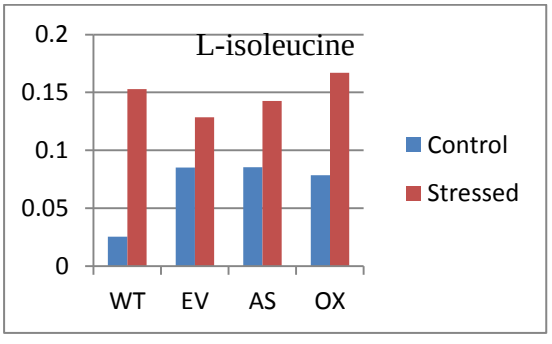


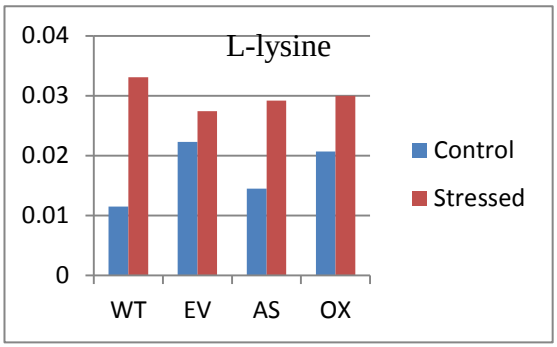


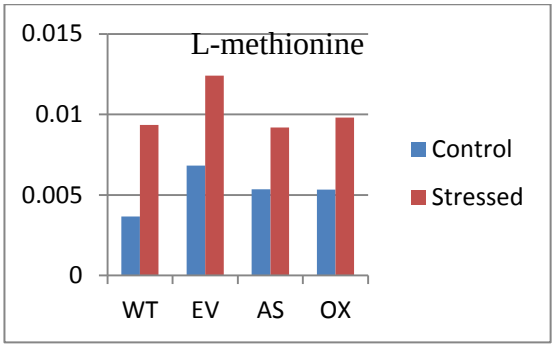









L-lysine
0.04
0.03
0.02
0.01
0
WT EV AS OX
Control
Stressed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0.01
0.005
0
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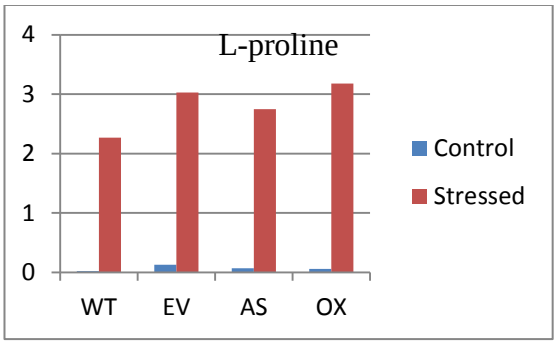
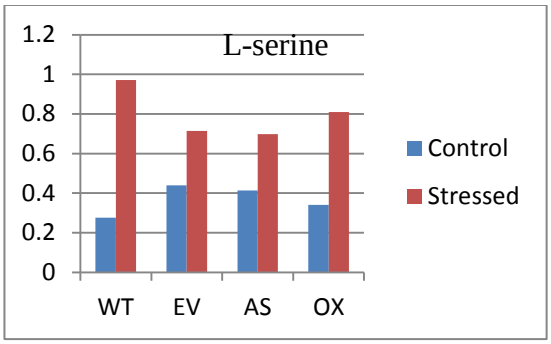
Control
Stressed


L-proline
4
3
2
1
0
WT EV AS OX
Control
Stressed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1
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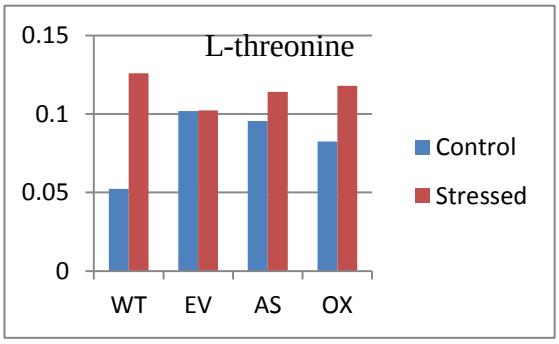
0.6
0.4
0.2
0
WT EV AS OX
Control
Stressed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0.1
0.05
0
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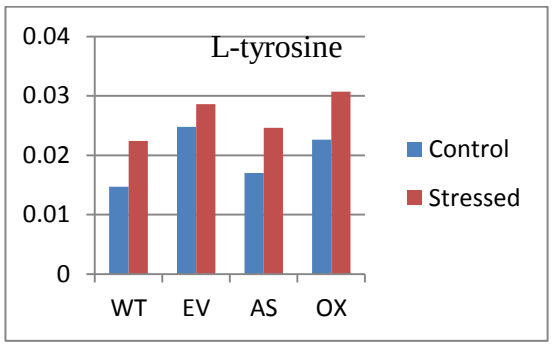
Control
Stressed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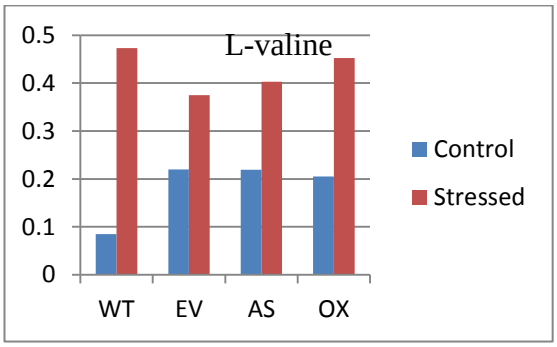
0.02
0.01
0
WT EV AS OX
Control
Stressed


L-valine
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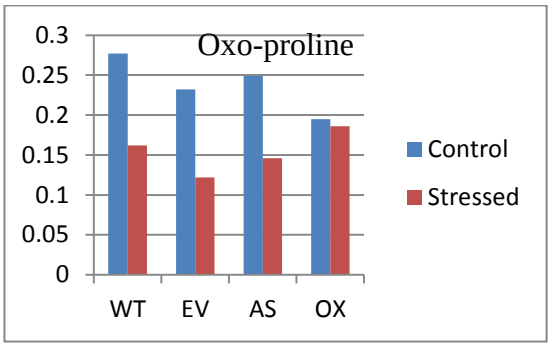
0.4
0.3
0.2
0.1
0
WT EV AS OX
Control
Stressed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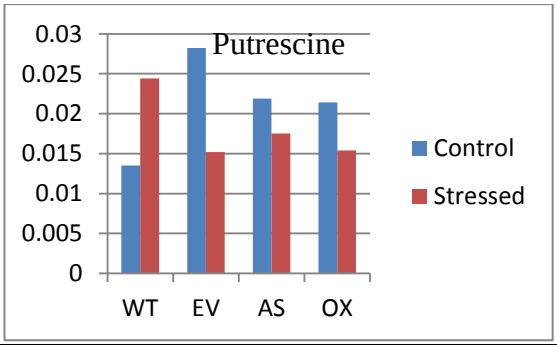
0.15
0.1
0.05
0
WT EV AS OX
Control
Stressed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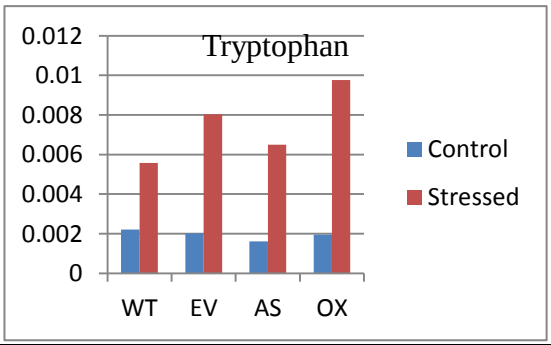
0.02
0.015
0.01
0.005
0
WT EV AS OX
Control
Stressed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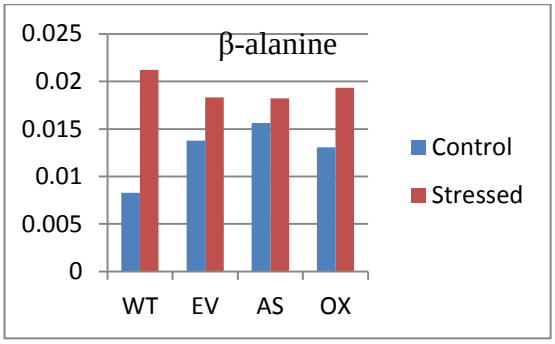
0.002
0
WT EV AS OX
Control
Stressed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EV
AS
OX
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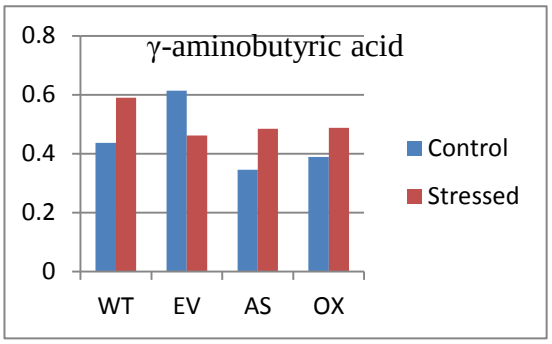
Stressed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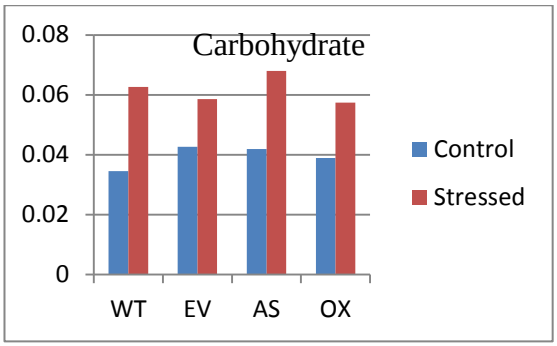
0.2
0
WT
EV
AS
OX
Control
Stressed

Sugars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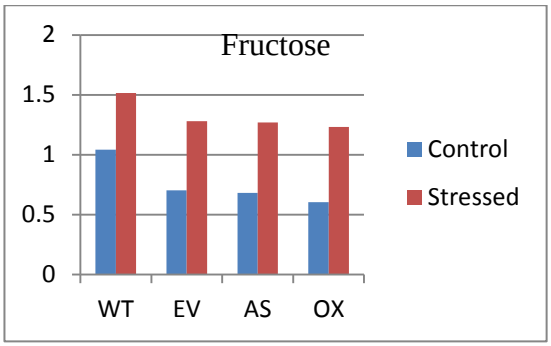
0.04
0.02
0
WT
EV
AS
OX
Control
Stressed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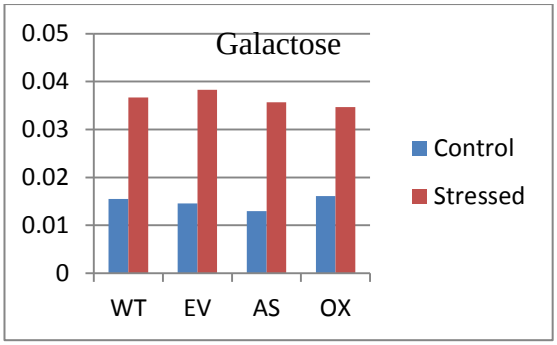
1.5
1
0.5
0
WT
EV
AS
OX
Control
Stressed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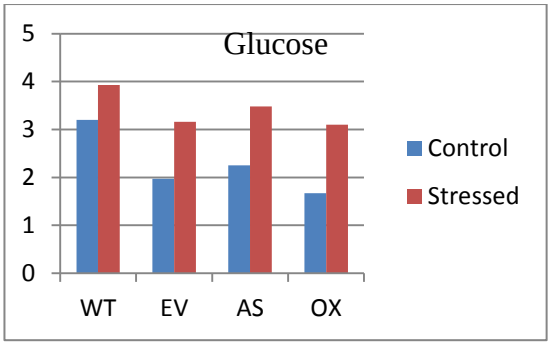
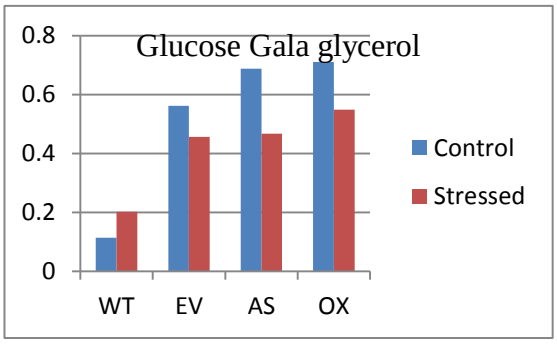
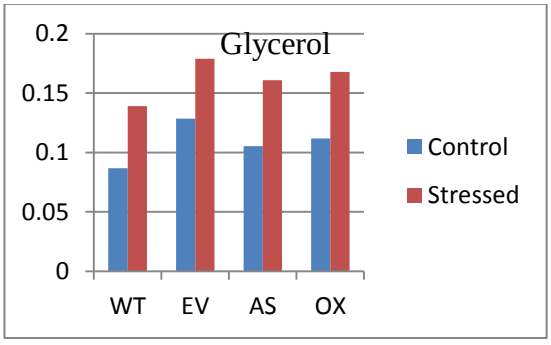
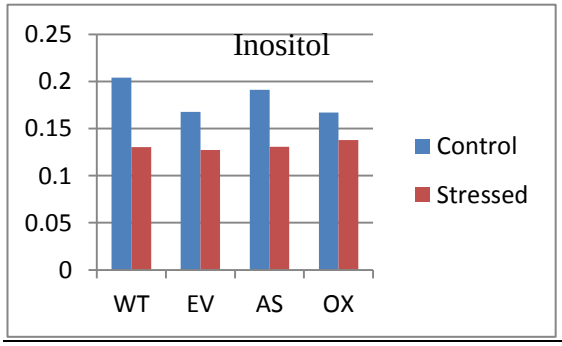
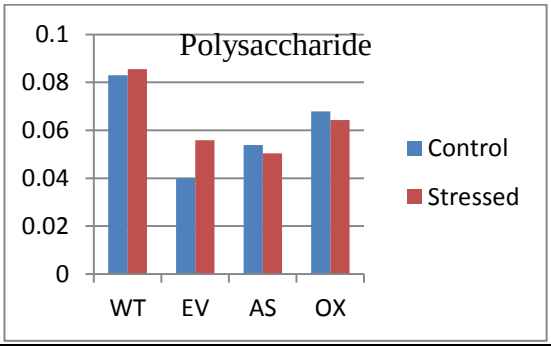
0.04
0.03
0.02
0.01
0
WT
EV
AS
OX
Control
Stressed

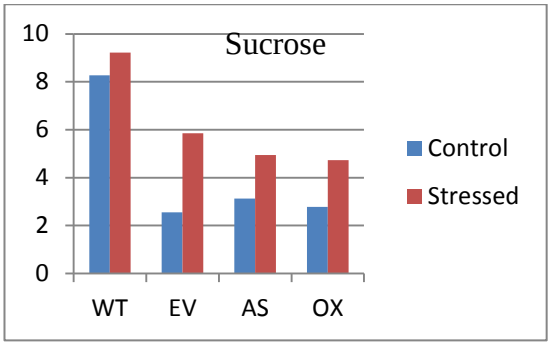

Glucose
5
4
3
2
1
0
WT
EV
AS
OX
Control
Stressed

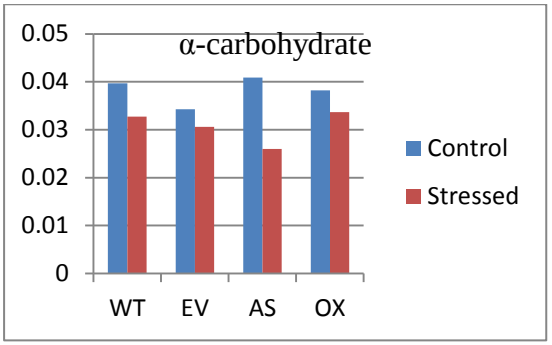

Glucose Gala glycerol
0.8
0.6
0.4
0.2
0
WT
EV
AS
OX
Control
Stressed


Glycerol
0.2
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

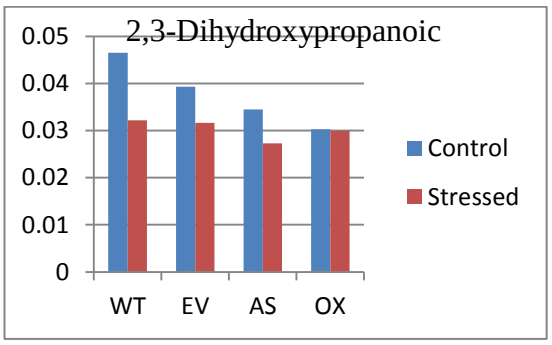

Inositol
0.25
0.2
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

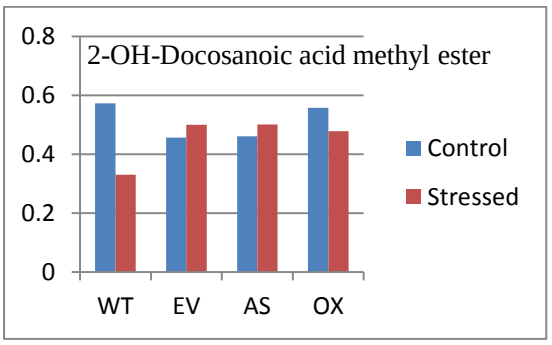

Polysaccharide
0.1
0.08
0.06
0.04
0.02
0
WT
EV
AS
OX
Control
Stressed

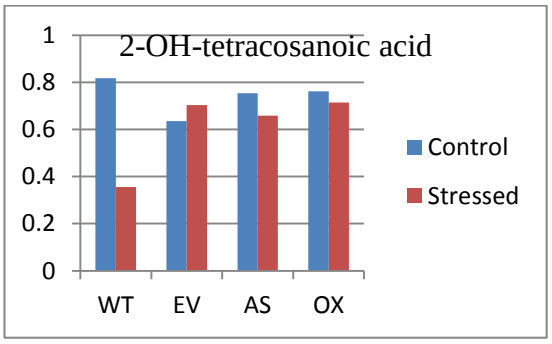


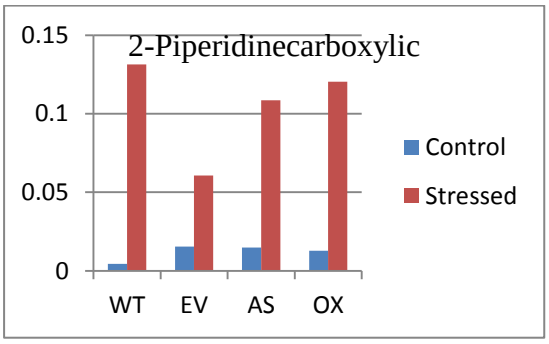


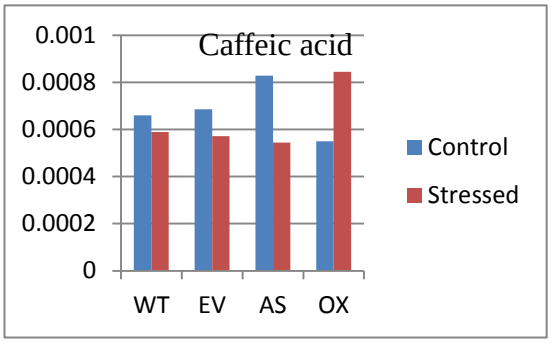
Organic and fatty acids

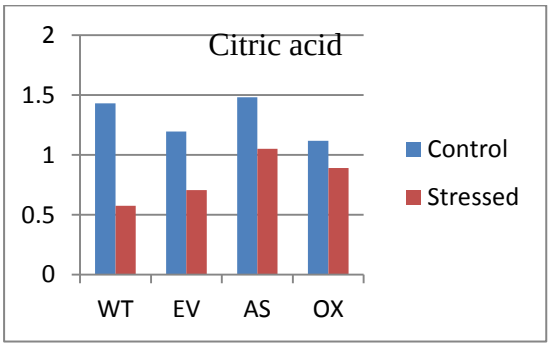


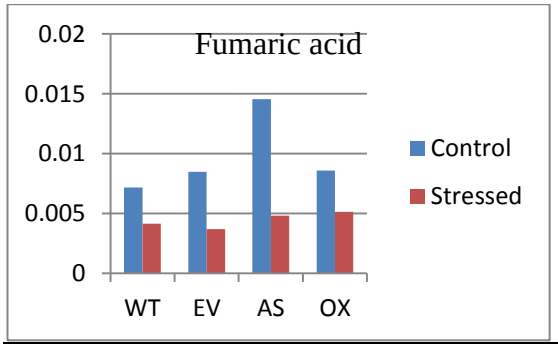


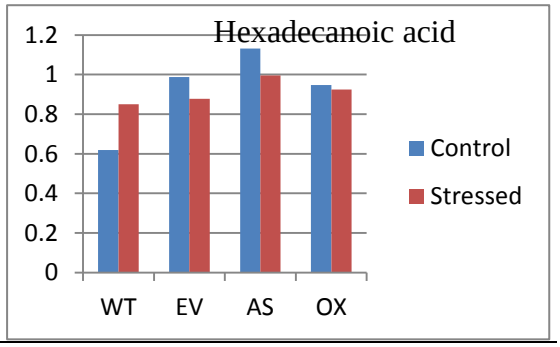


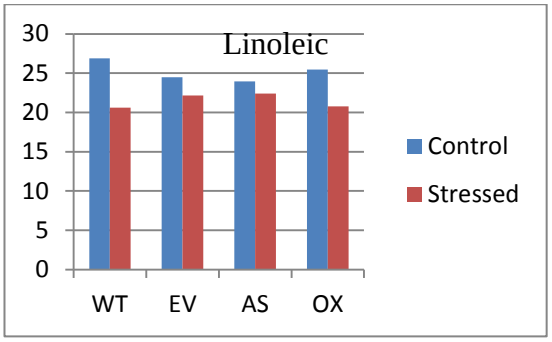


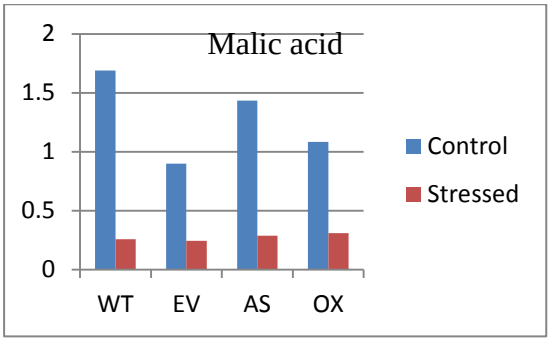


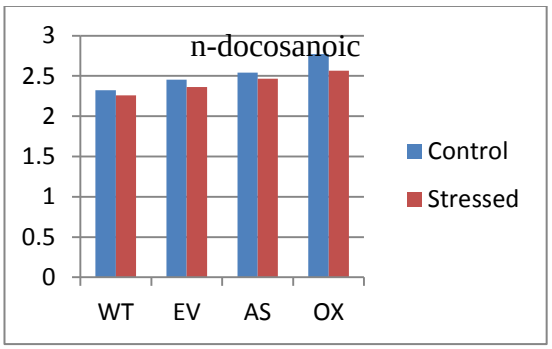


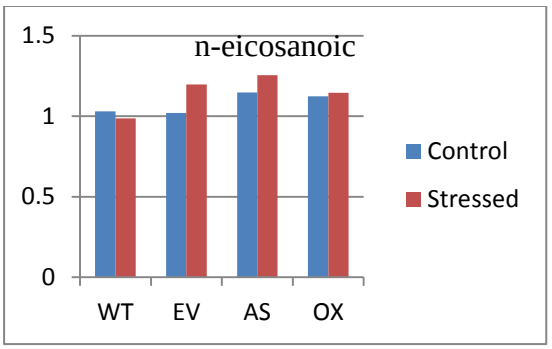


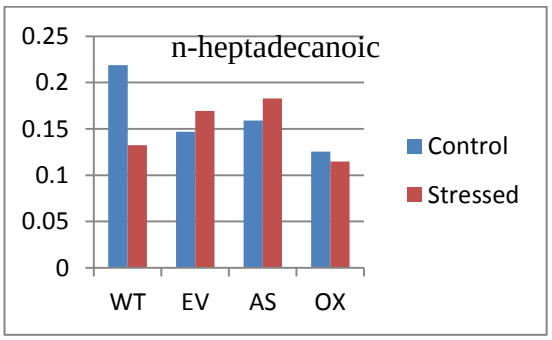


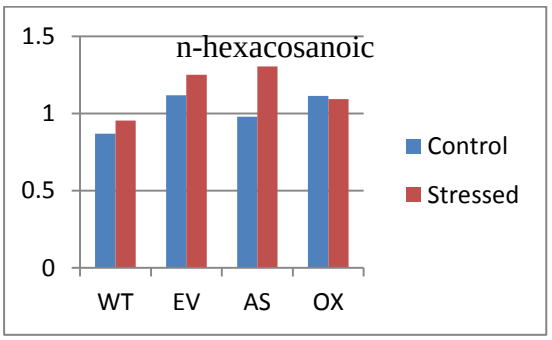

Linoleic
30
25
20
15
10
5
0
WT
EV
AS
OX
Control
Stressed

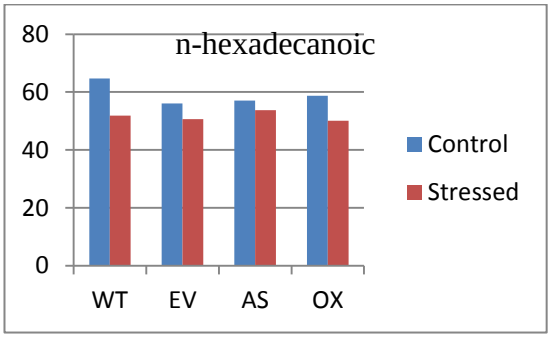

Malic acid
2
1.5
1
0.5
0
WT
EV
AS
OX
Control
Stressed

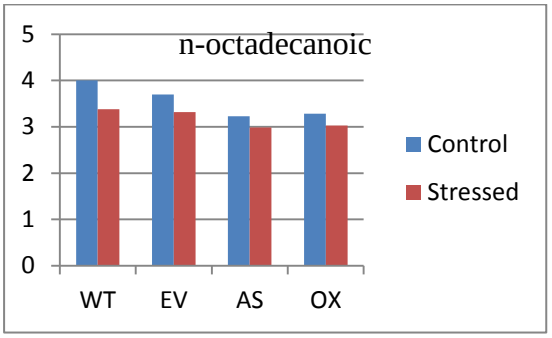

n-docosanoic
3
2.5
2
1.5
1
0.5
0
WT
EV
AS
OX
Control
Stressed

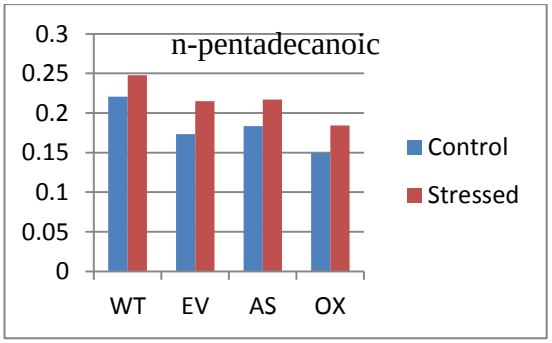

n-eicosanoic
1.5
1
0.5
0
WT
EV
AS
OX
Control
Stressed

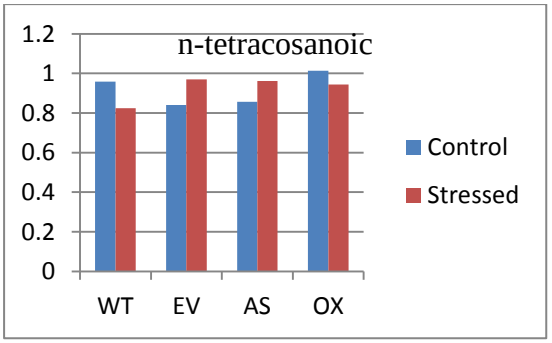

n-heptadecanoic
0.25
0.2
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

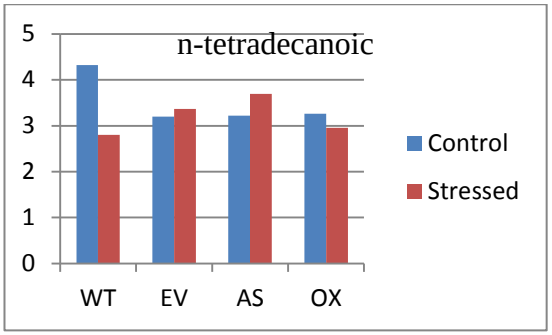

n-hexacosanoic
1.5
1
0.5
0
WT
EV
AS
OX
Control
Stressed

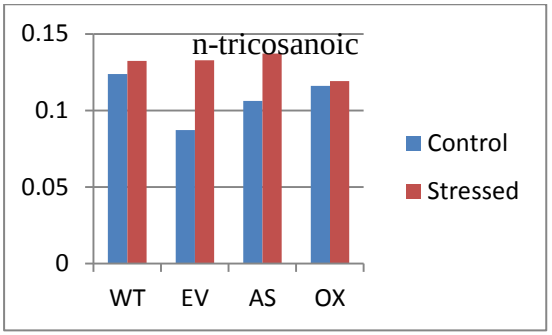

n-hexadecanoic
80
60
40
20
0
WT
EV
AS
OX
Control
Stressed

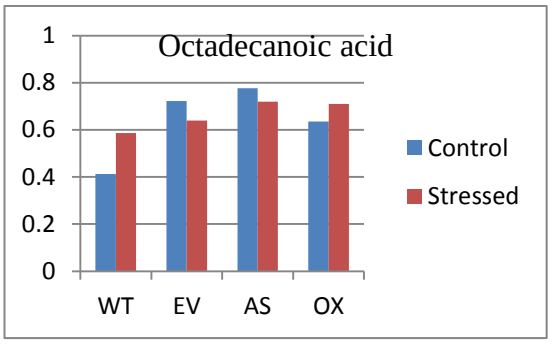

n-octadecanoic
5
4
3
2
1
0
WT
EV
AS
OX
Control
Stressed

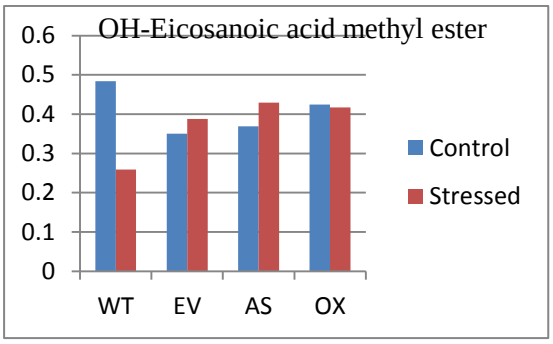

n-pentadecanoic
0.3
0.25
0.2
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

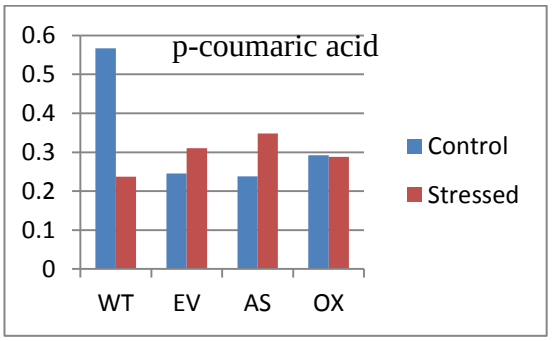

n-tetracosanoic
1.2
1
0.8
0.6
0.4
0.2
0
WT
EV
AS
OX
Control
Stressed

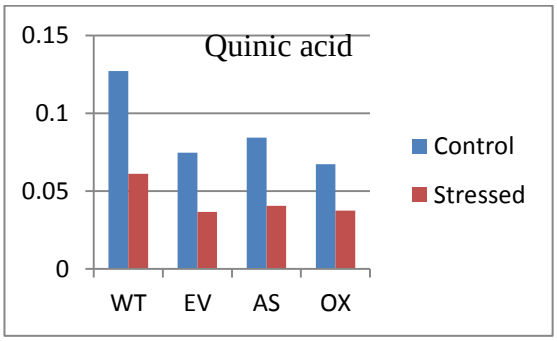

n-tetradecanoic
5
4
3
2
1
0
WT
EV
AS
OX
Control
Stressed

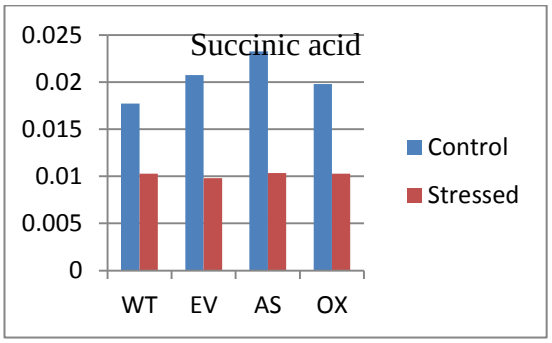

n-tricosanoic
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

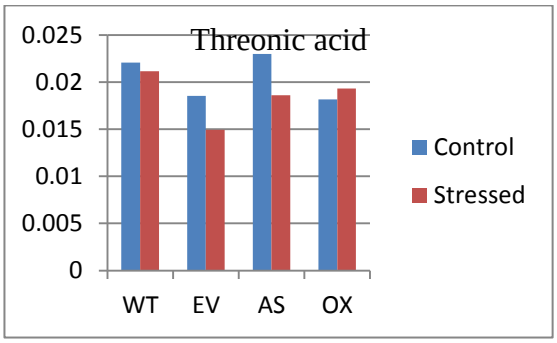

Octadecanoic acid
1
0.8
0.6
0.4
0.2
0
WT
EV
AS
OX
Control
Stressed

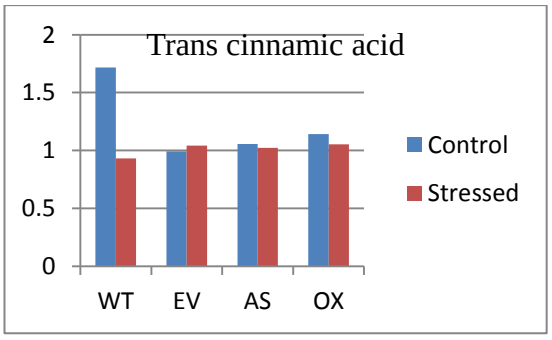

OH-Eicosanoic acid methyl ester
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
EV
AS
OX
Control
Stressed

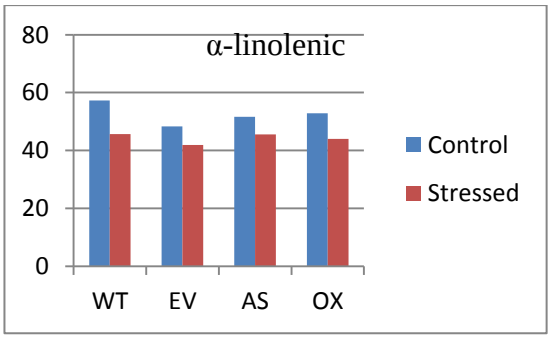

p-coumaric acid
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
EV
AS
OX
Control
Stressed


Quinic acid
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

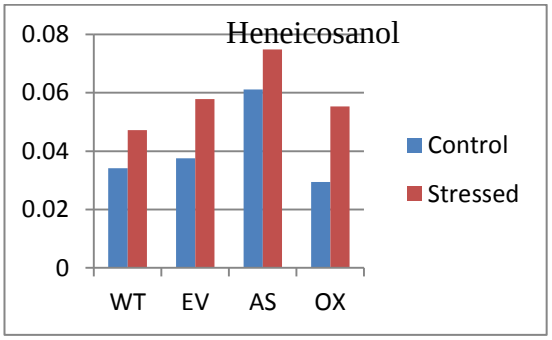

Succinic acid
0.025
0.02
0.015
0.01
0.005
0
WT
EV
AS
OX
Control
Stressed

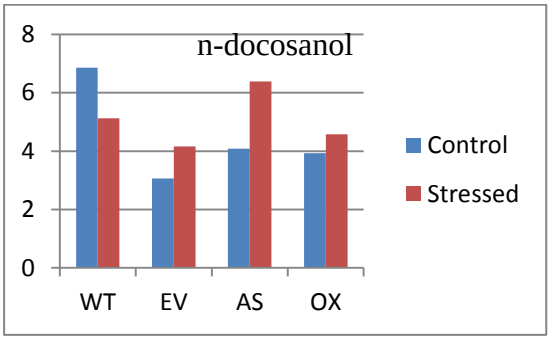

Threonic acid
0.025
0.02
0.015
0.01
0.005
0
WT
EV
AS
OX
Control
Stressed

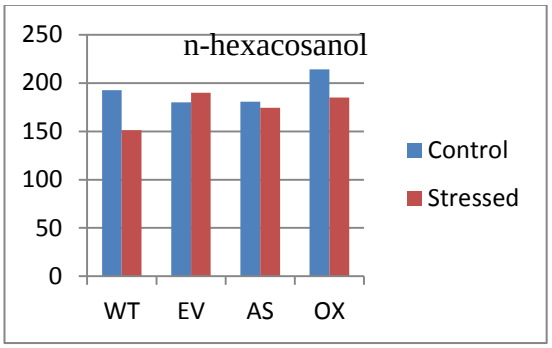

Trans cinnamic acid
2
1.5
1
0.5
0
WT
EV
AS
OX
Control
Stressed

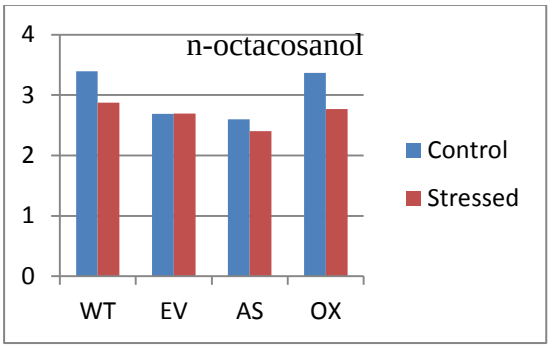

α-linolenic
80
60
40
20
0
WT
EV
AS
OX
Control
Stressed

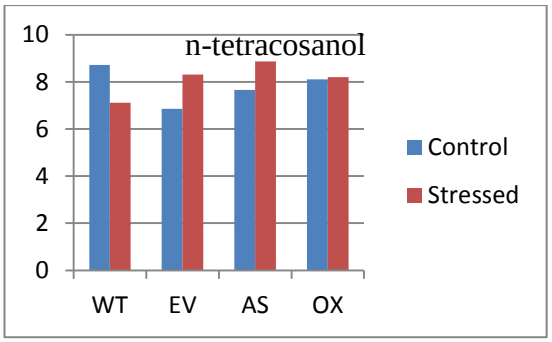
Others

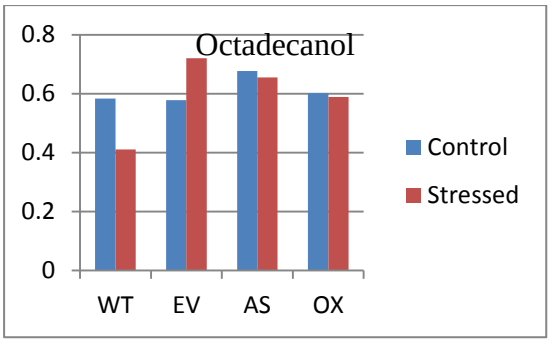

Heneicosanol
0.08
0.06
0.04
0.02
0
WT EV AS OX
Control
Stressed

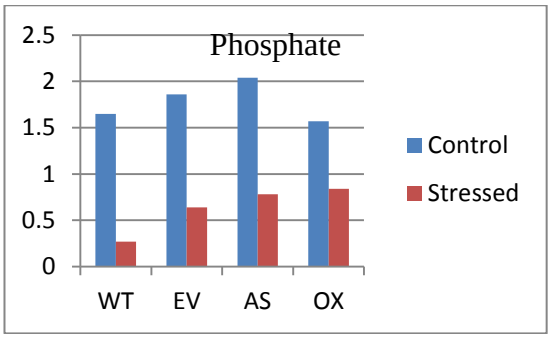

n-docosanol
8
6
4
2
0
WT EV AS OX
Control
Stressed

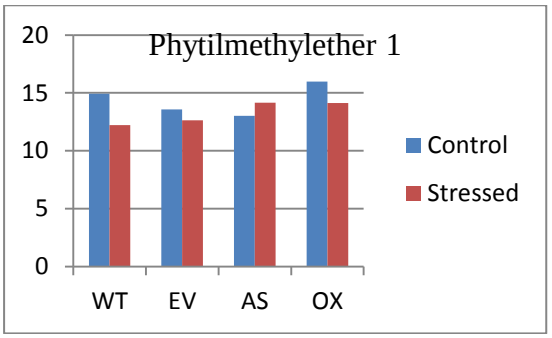

n-hexacosanol
250
200
150
100
50
0
WT EV AS OX
Control
Stressed

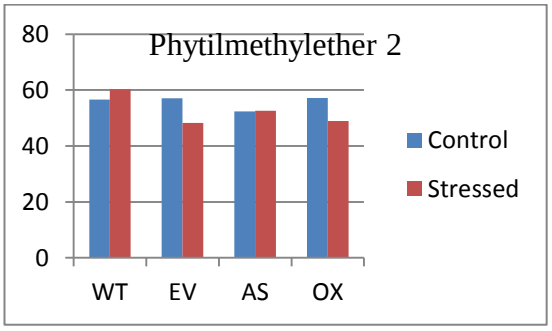

n-octacosanol
4
3
2
1
0
WT EV AS OX
Control
Stressed

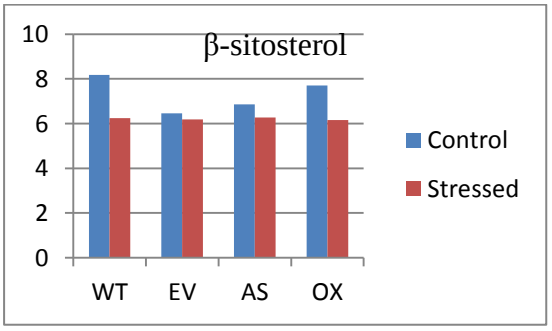

n-tetracosanol
10
8
6
4
2
0
WT EV AS OX
Control
Stressed


Octadecanol
0.8
0.6
0.4
0.2
0
WT EV AS OX
Control
Stressed

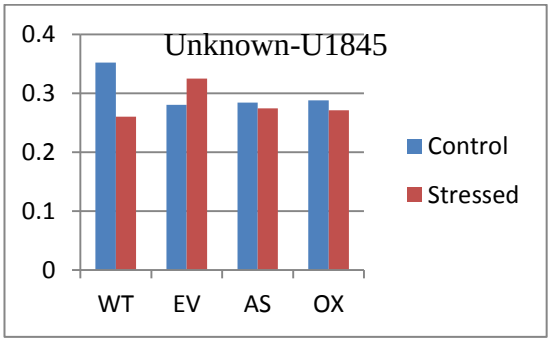

Phosphate
2.5
2
1.5
1
0.5
0
WT EV AS OX
Control
Stressed

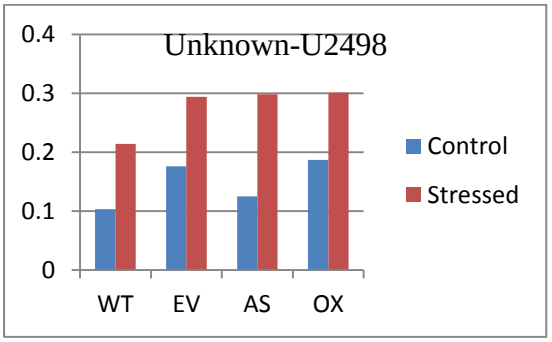

Phytilmethylether 1
20
15
10
5
0
WT EV AS OX
Control
Stressed

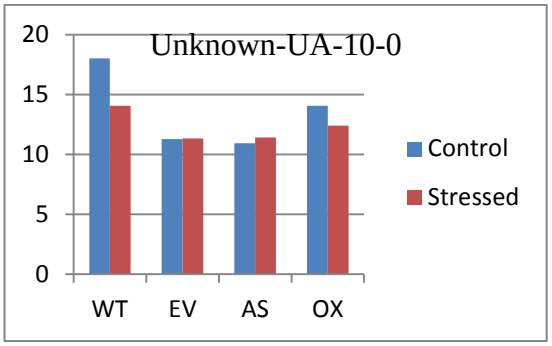

Phytilmethylether 2
80
60
40
20
0
WT EV AS OX
Control
Stressed

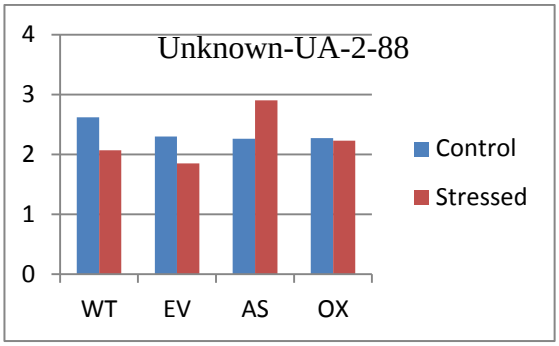

β-sitosterol
10
8
6
4
2
0
WT EV AS OX
Control
Stressed

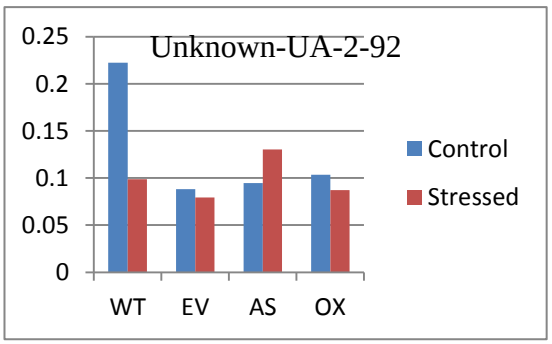
Unidentified compounds

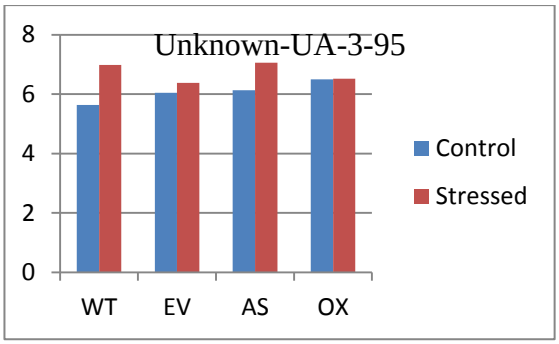


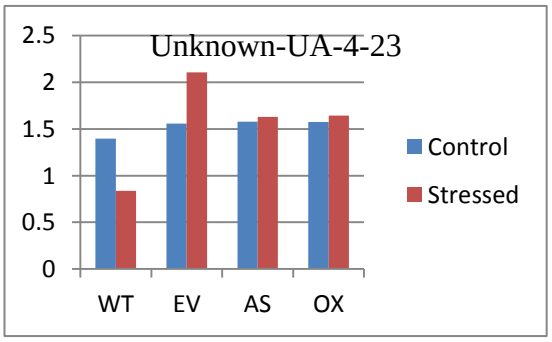


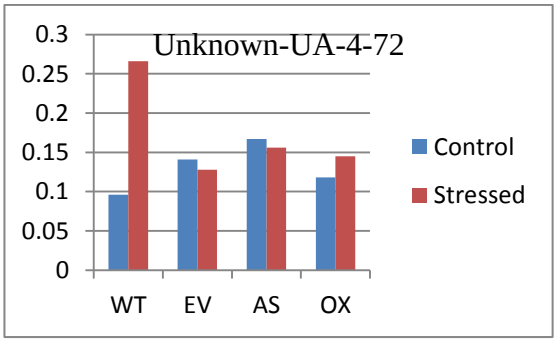


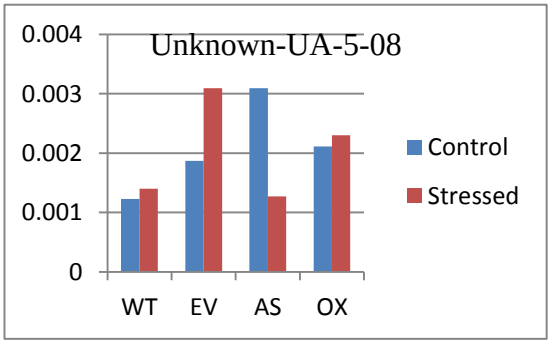


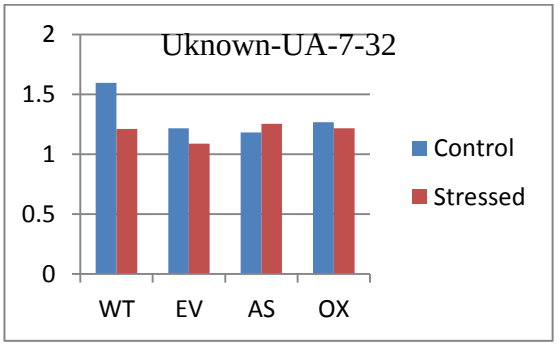


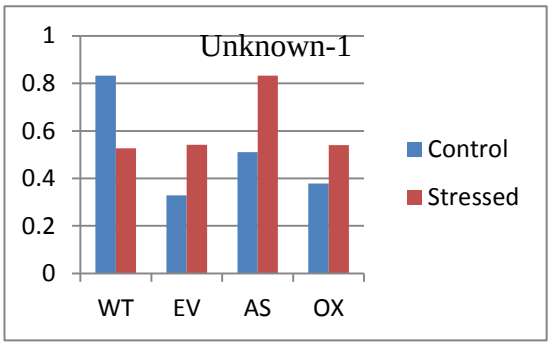


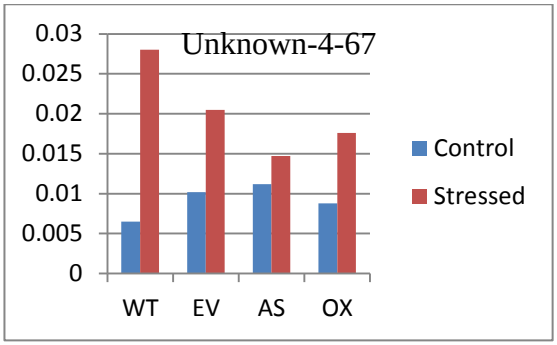









Unknown-1
1
0.8
0.6
0.4
0.2
0
WT
EV
AS
OX
Control
Stressed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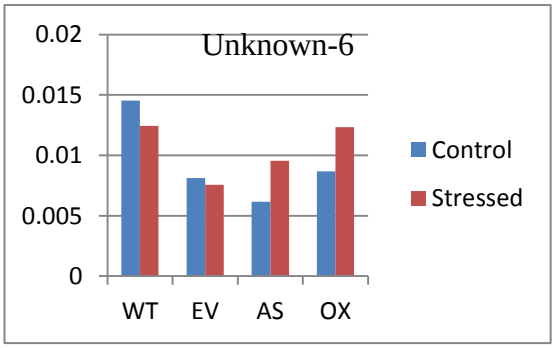
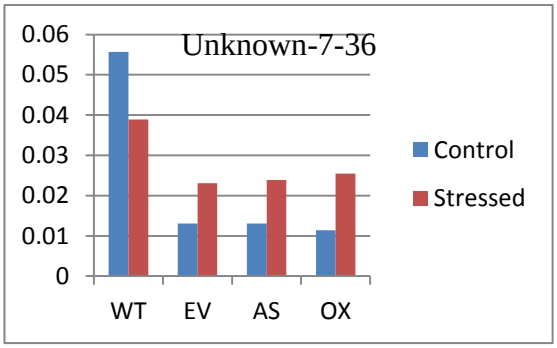
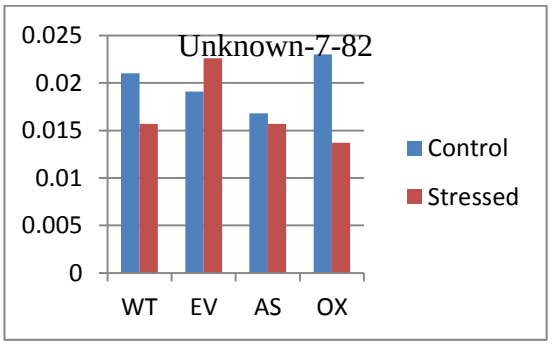
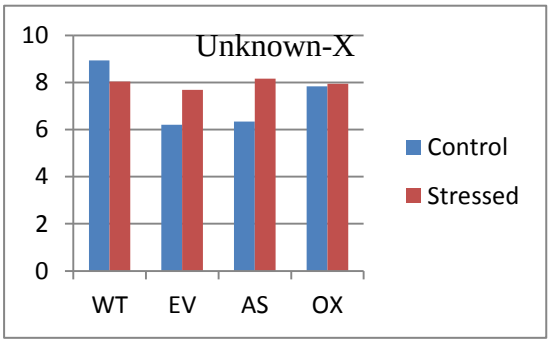
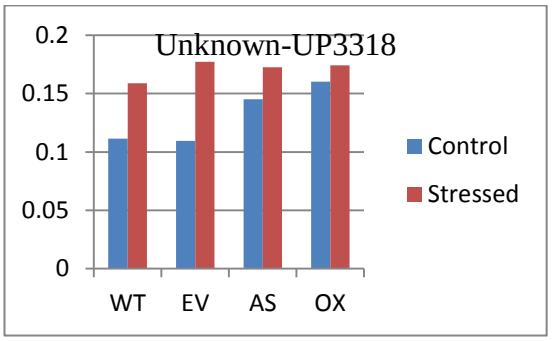
0.03
0.025
0.02
0.015
0.01
0.005
0
WT
EV
AS
OX
Control
Stressed


Unknown-6
0.02
0.015
0.01
0.005
0
WT
EV
AS
OX
Control
Stressed

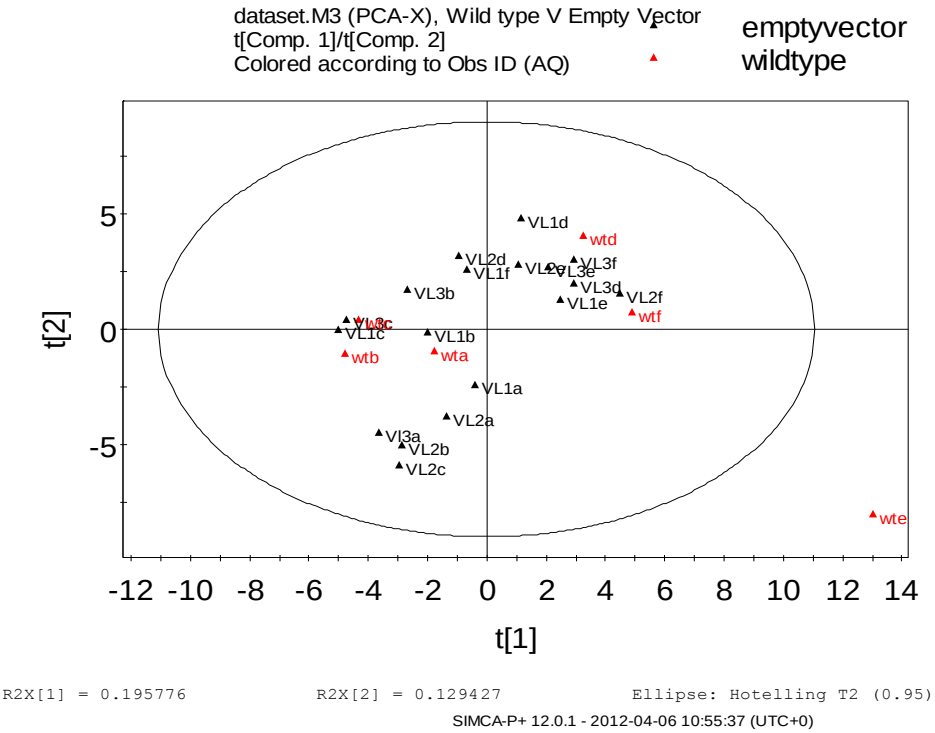

Unknown-7-36
0.06
0.05
0.04
0.03
0.02
0.01
0
WT
EV
AS
OX
Control
Stressed


Unknown-7-82
0.025
0.02
0.015
0.01
0.005
0
WT
EV
AS
OX
Control
Stressed


Unknown-X
10
8
6
4
2
0
WT
EV
AS
OX
Control
Stressed

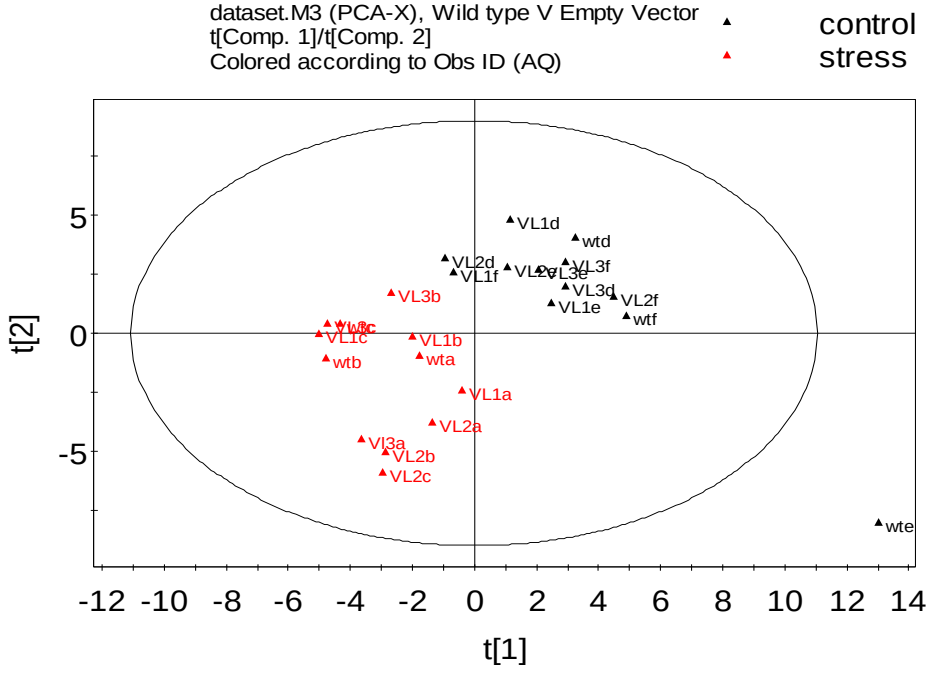

Unknown-UP3318
0.2
0.15
0.1
0.05
0
WT
EV
AS
OX
Control
Stressed

Appendix 5.10: Principle component analyses (PCA) scores and loading plots.



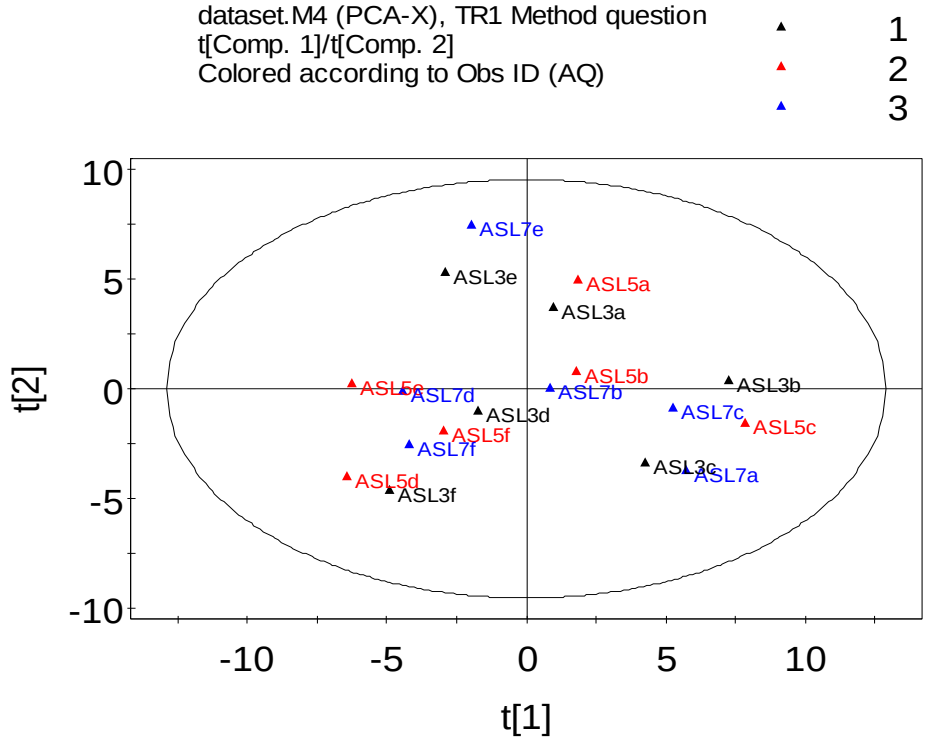
R2X[1] = 0.195776 R2X[2] = 0.129427 Ellipse: Hotelling T2 (0.95)

SIMCA-P+ 12.0.1 - 2012-04-06 10:55:37 (UTC+0)

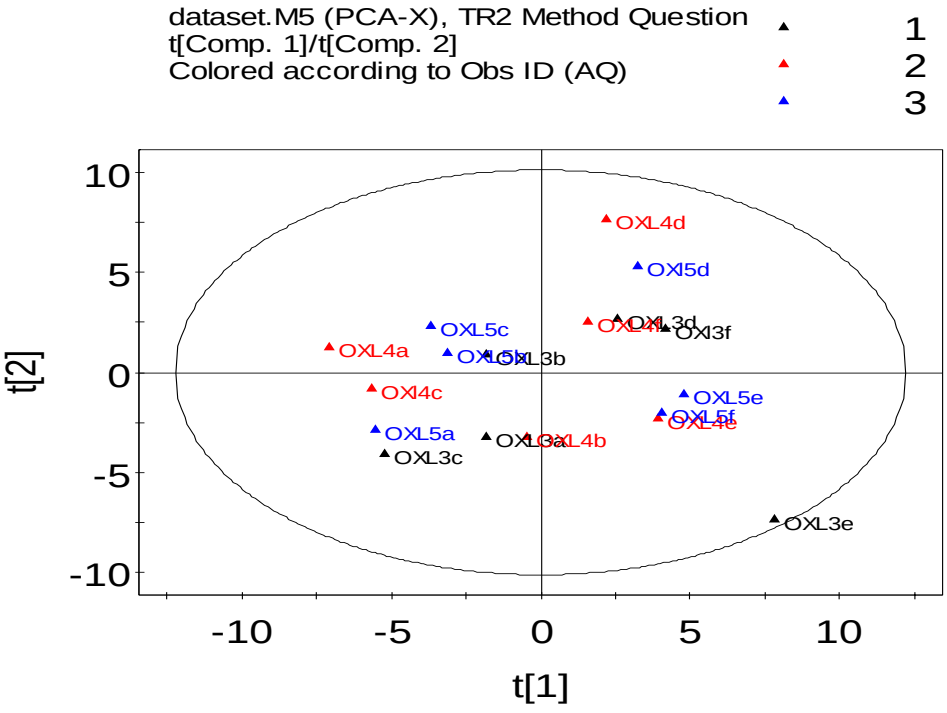


R2X[1] = 0.195776 R2X[2] = 0.129427

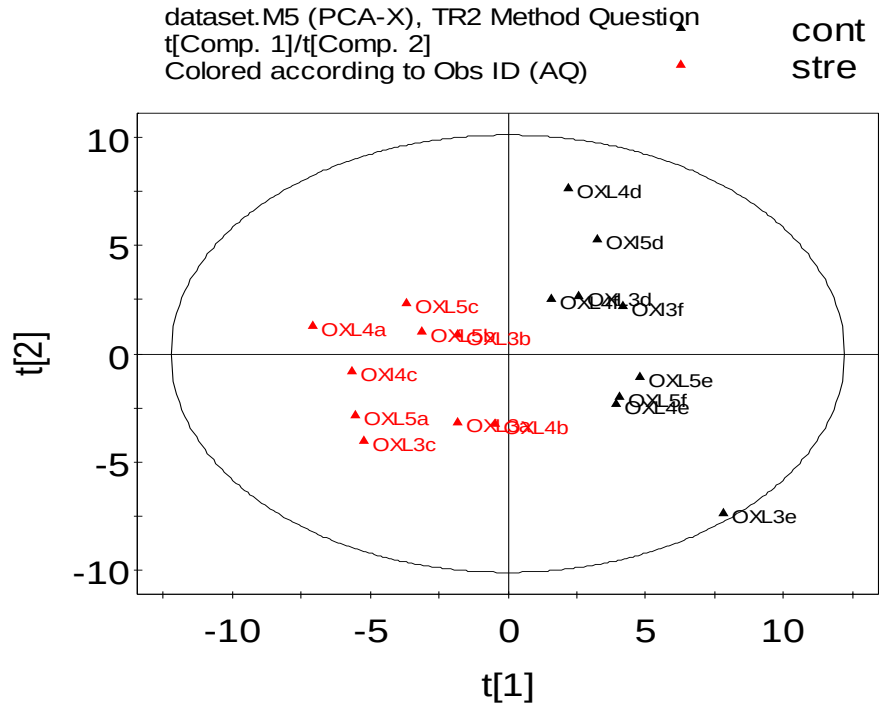
Ellipse: Hotelling T2 (0.95) SIMCA-P+ 12.0.1 - 2012-04-06 10:56:26 (UTC+0)



R2X[1] = 0.247705 R2X[2] = 0.135492
Ellipse: Hotelling T2 SIMCA-P+ 12.0.1 - 2012-04-06 11:06:50 (UTC+0)

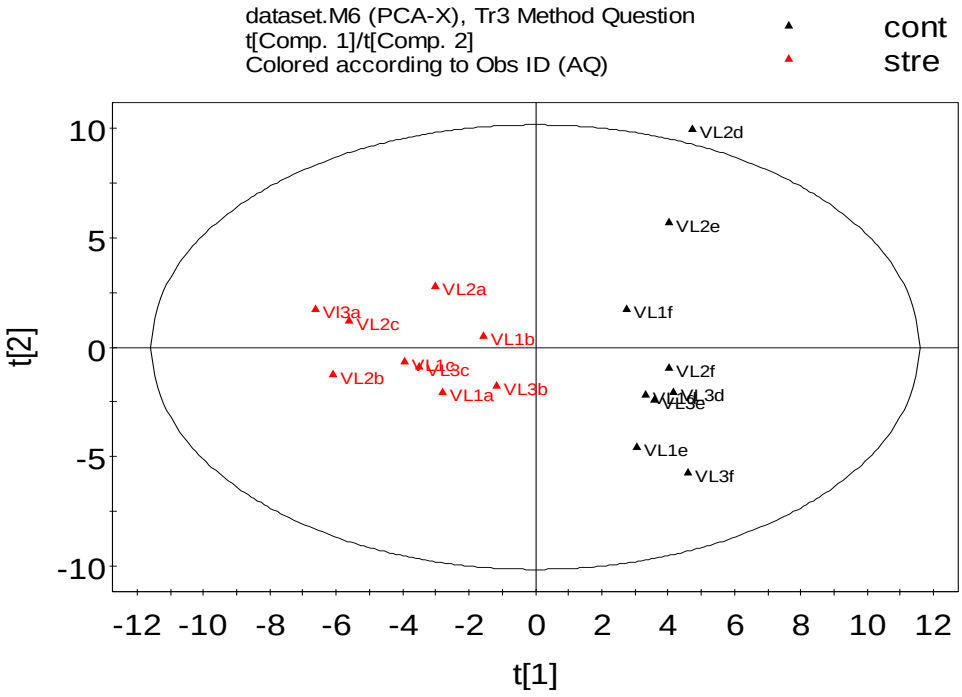


R2X[1] = 0.2227 R2X[2] = 0.152287
Ellipse: Hotelling SIMCA-P+ 12.0.1 - 2012-04-06 11:10:06 (UTC+0)



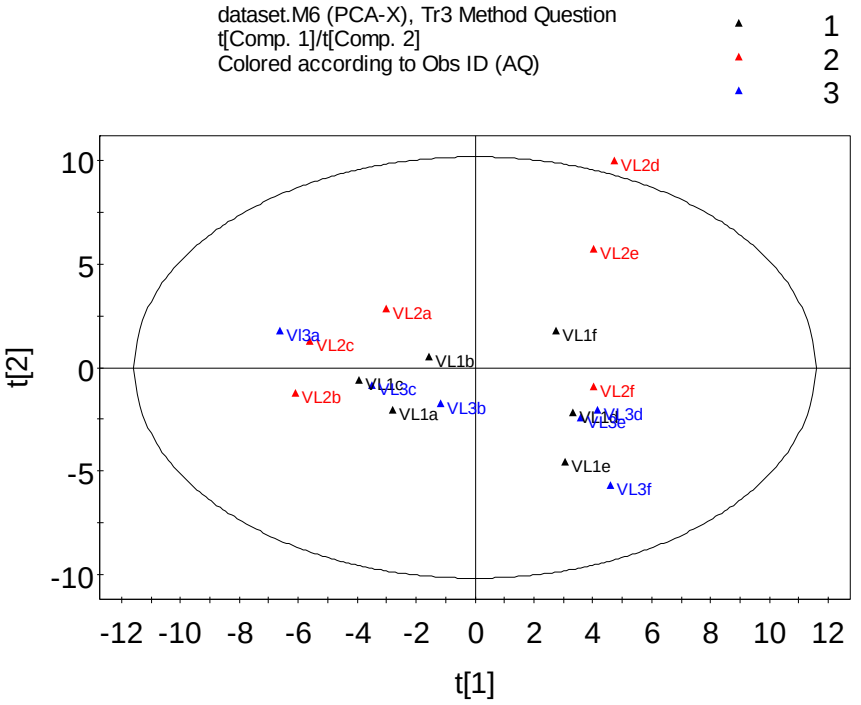
R2X[1] = 0.2227 R2X[2] = 0.152287 Ellipse: Hotelling T2 (0.95)

SIMCA-P+ 12.0.1 - 2012-04-06 11:09:39 (UTC+0)



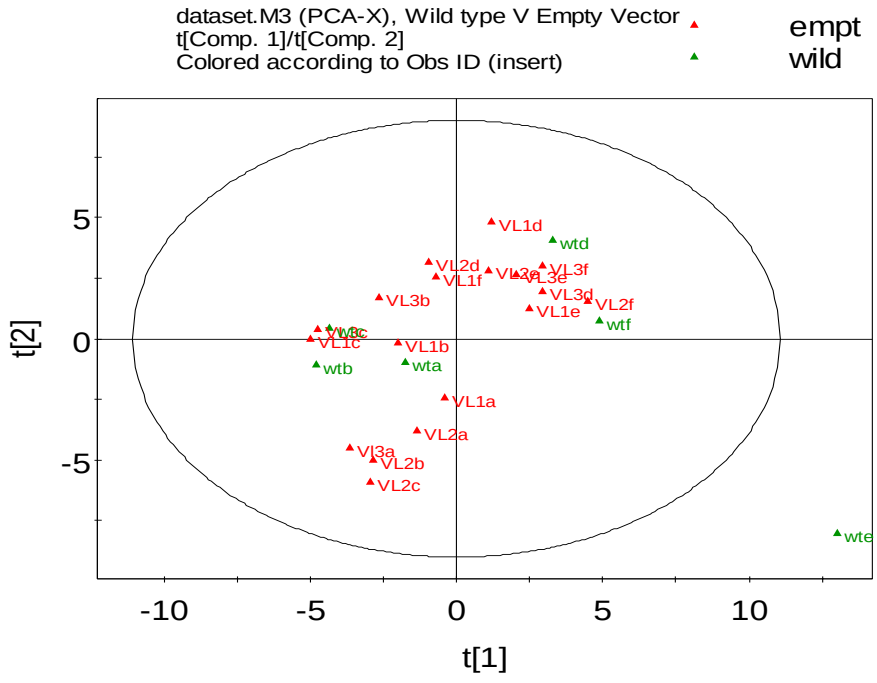
R2X[1] = 0.19962 R2X[2] = 0.15336

Ellipse: Hotelling T2 (0.95) SIMCA-P+ 12.0.1 - 2012-04-06 11:17:21 (UTC+0)



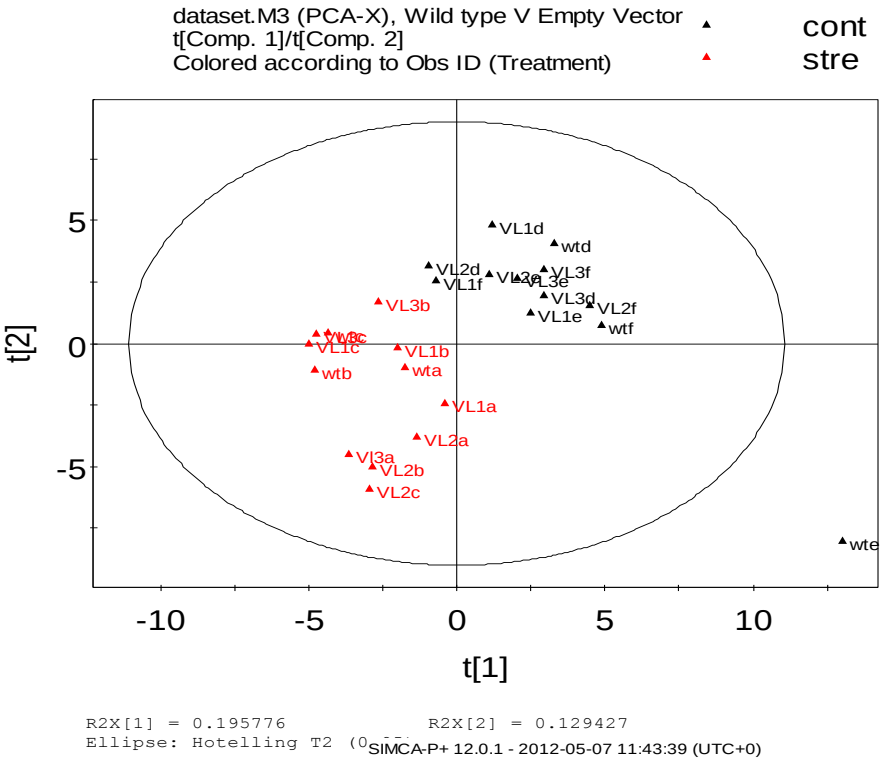
R2X[1] = 0.19962 R2X[2] = 0.15336 Ellipse: Hotelling T2 (0.95)

SIMCA-P+ 12.0.1 - 2012-04-06 11:16:01 (UTC+0)

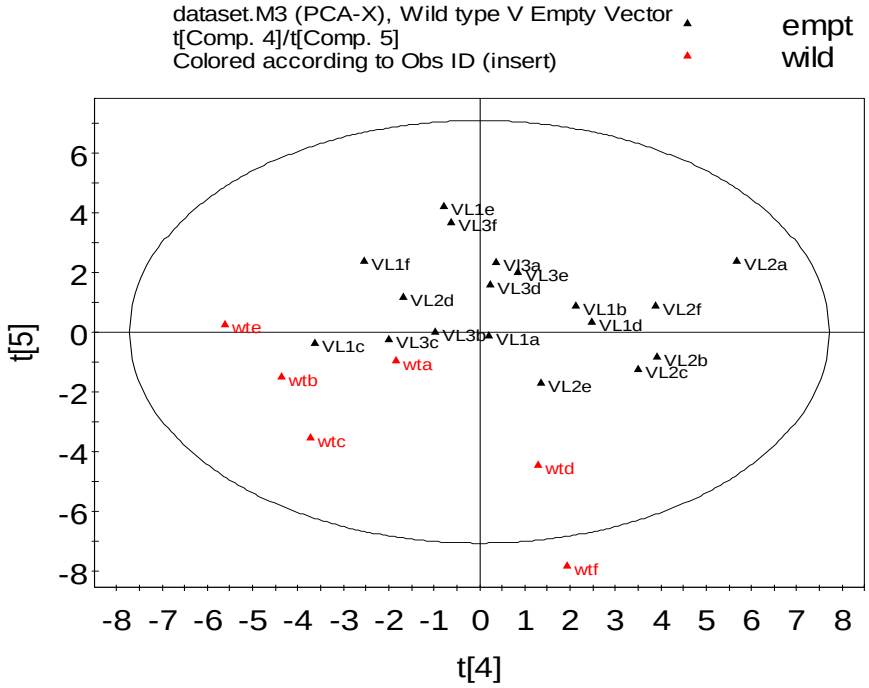


R2X[1] = 0.195776 R2X[2] = 0.129427 Ellipse: Hotelling T2 (0.95)

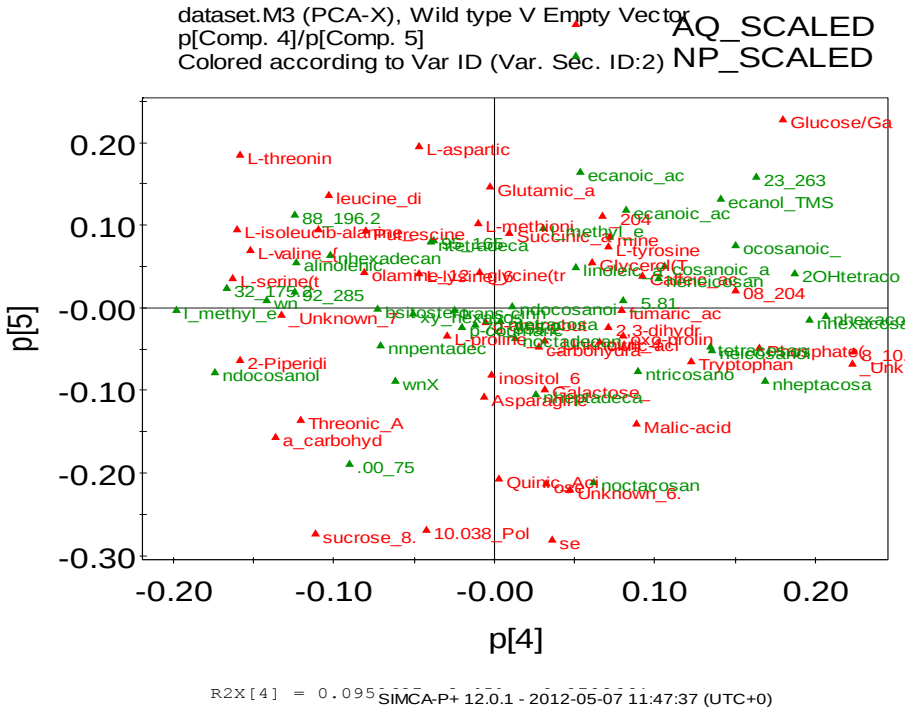
SIMCA-P+ 12.0.1 - 2012-05-07 11:42:04 (UTC+0)

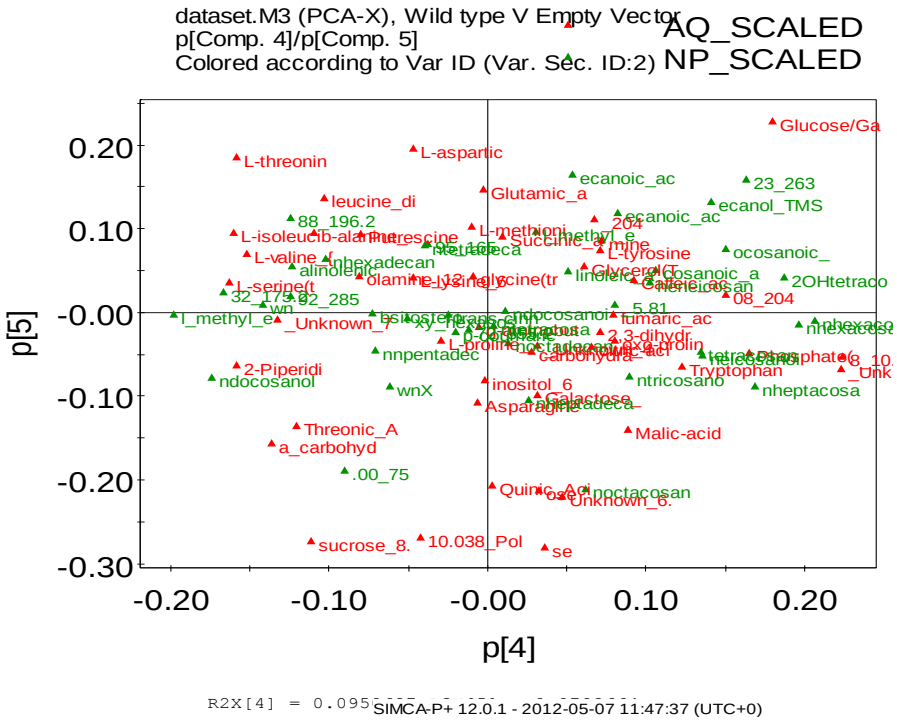


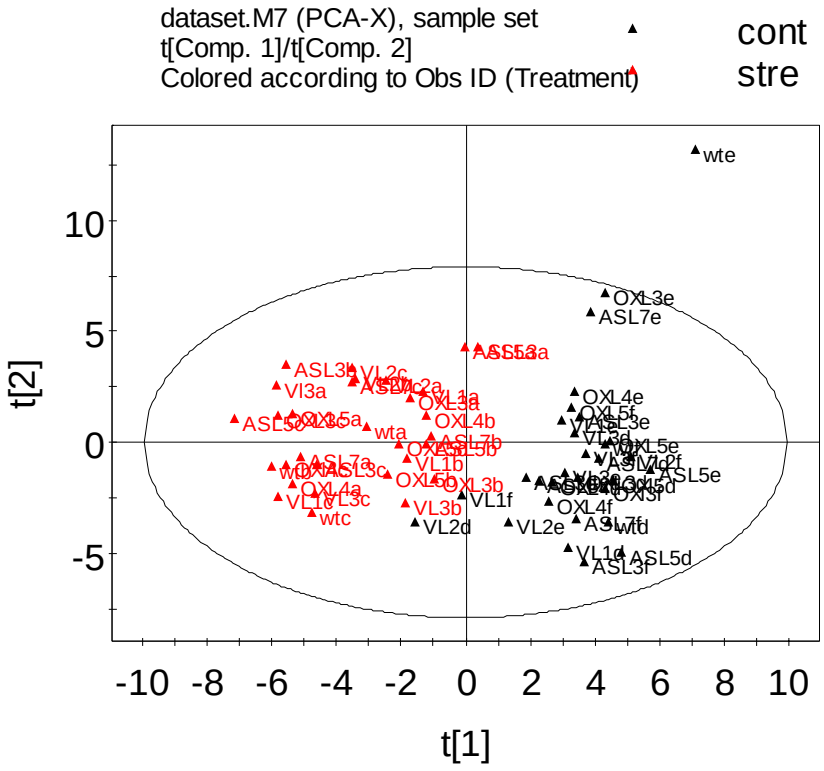
R2X[1] = 0.195776 R2X[2] = 0.129427
Ellipse: Hotelling T2 (0
SIMCA-P+ 12.0.1 - 2012-05-07 11:43:39 (UTC+0)



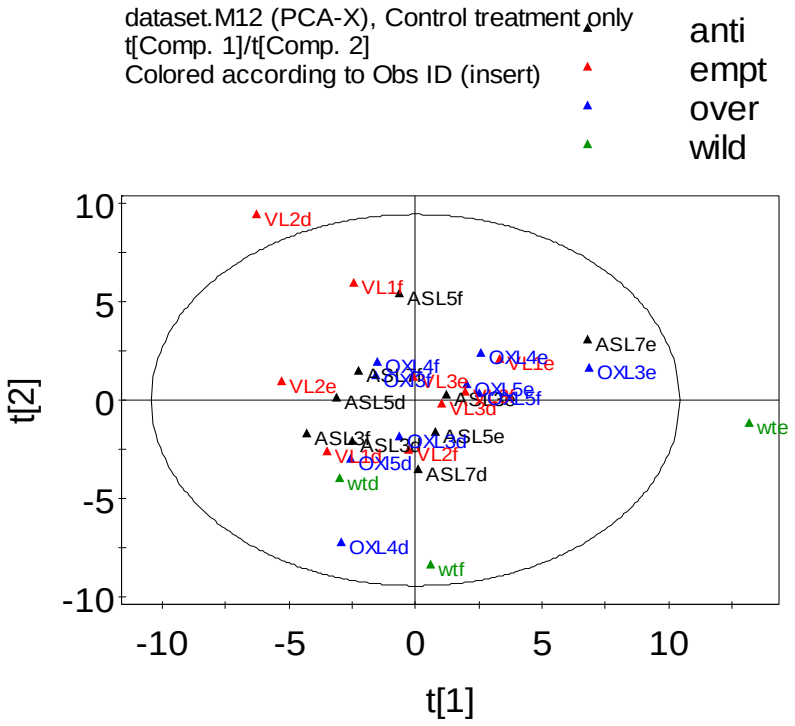
R2X[4] = 0.0950687 R2X[5] = 0.0798664
Ellipse: Hotelling T2 (0
SIMCA-P+ 12.0.1 - 2012-05-07 11:47:08 (UTC+0)


dataset.M3 (PCA-X), Wild type V Empty Vector
p[Comp. 4]/p[Comp. 5]
Colored according to Var ID (Var. Sec. ID:2)
AQ_SCALED
NP_SCALED
p[5]
p[4]
0.20
0.10
-0.00
-0.10
-0.20
-0.30
-0.20
-0.10
-0.00
0.10
0.20
Glucose/Ga
L-threonin
L-aspartic
ecanoic_ac
23_263
Glutamic_a
leucine_di
ecanol_TMS
88_196.2
L-methioni
L-tyrosine
ocosanoic_
L-valine
2OHtetraco
08_204
5.81
fumaric_ac
2-Piperidi
ndocosanol
wnX
inositol_6
Tryptophan
nheptacosa
Threonic_A
a_carbohyd
Malic-acid
.00_75
sucrose_8.
10.038_Pol
se
Unknown_6.
R2X[4] = 0.095
SIMCA-P+ 12.0.1 - 2012-05-07 11:47:37 (UTC+0)

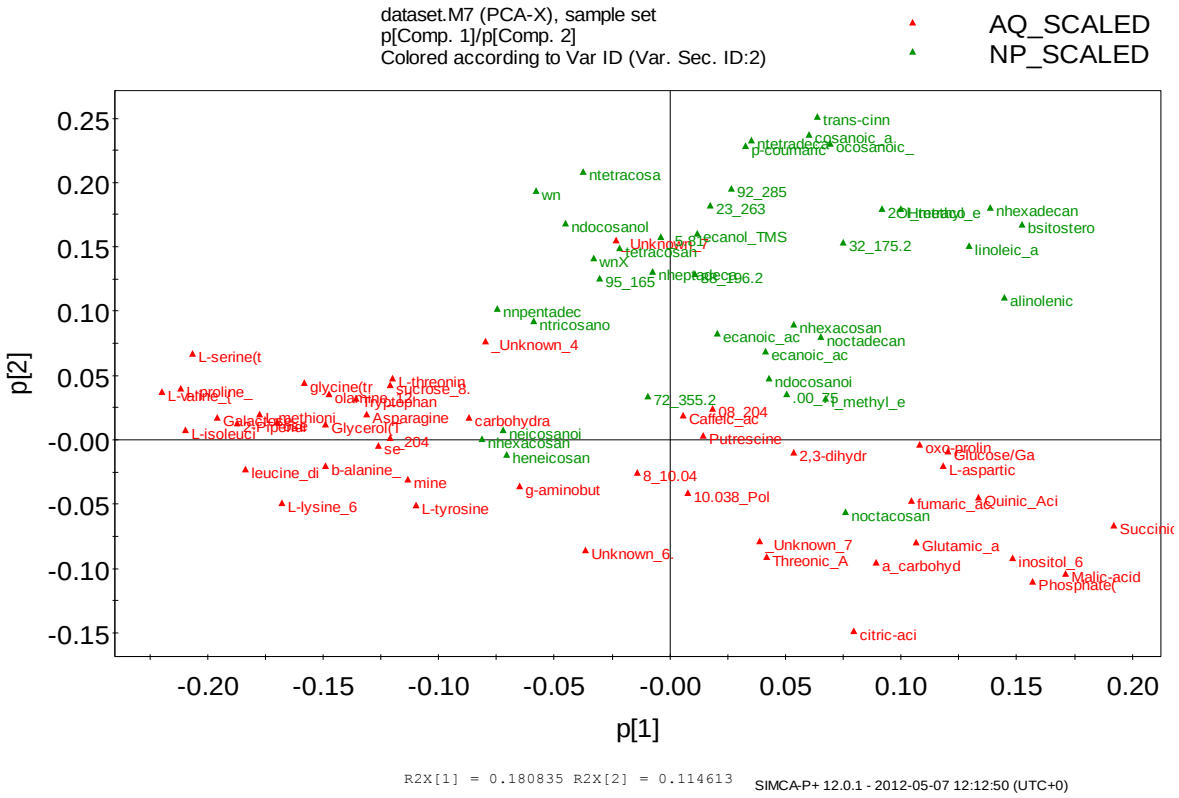

dataset.M3 (PCA-X), Wild type V Empty Vector
p[Comp. 4]/p[Comp. 5]
Colored according to Var ID (Var. Sec. ID:2)
AQ_SCALED
NP_SCALED
p[5]
p[4]
0.20
0.10
-0.00
-0.10
-0.20
-0.30
-0.20
-0.10
-0.00
0.10
0.20
Glucose/Ga
L-threonin
L-aspartic
ecanoic_ac
23_263
Glutamic_a
leucine_di
ecanol_TMS
88_196.2
L-methioni
L-tyrosine
ocosanoic_
L-valine
2OHtetraco
08_204
5.81
fumaric_ac
2-Piperidi
ndocosanol
wnX
inositol_6
Tryptophan
nheptacosa
Threonic_A
a_carbohyd
Malic-acid
.00_75
sucrose_8.
10.038_Pol
se
Unknown_6.
R2X[4] = 0.095
SIMCA-P+ 12.0.1 - 2012-05-07 11:47:37 (UTC+0)



R2X[1] = 0.180835 R2X[2] = 0.114613
Ellipse: Hotelling
SIMCA-P+ 12.0.1 - 2012-05-07 12:12:02 (UTC+0)

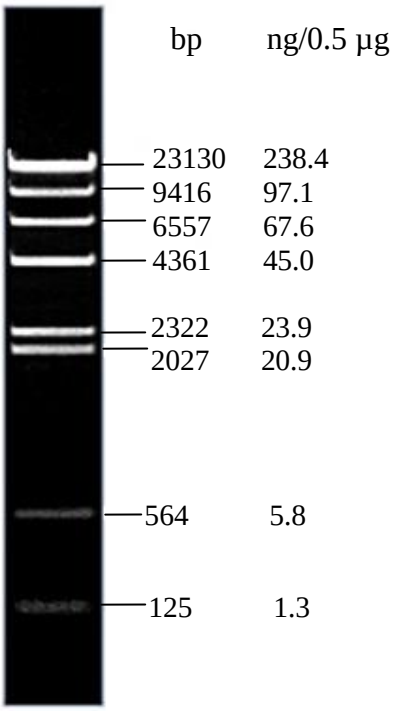


R2X[1] = 0.184944 R2X[2] = 0.1506
Ellipse: Hotelling
SIMCA-P+ 12.0.1 - 2012-05-07 13:55:37 (UTC+0)


dataset.M7 (PCA-X), sample set
p[Comp. 1]/p[Comp. 2]
Colored according to Var ID (Var. Sec. ID:2)
AQ_SCALED
NP_SCALED
p[2]
p[1]
0.25
0.20
0.15
0.10
0.05
-0.00
-0.05
-0.10
-0.15
-0.20
-0.15
-0.10
-0.05
-0.00
0.05
0.10
0.15
0.20
trans-cinn
p-coumaric
ntetracosa
wn
92_285
23_263
nhexadecan
bsitostero
ndocosanol
32_175.2
linoleic_a
wnX
95_165
alinolenic
nnpentadec
ntricosano
nhexacosan
ecanoic_ac
noctadecan
_Unknown_4
L-serine(t
ndocosanoi
72_355.2
P_methyl_e
Caffeic_ac
Putrescine
carbohydra
Asparagine
nhexacosan
heneicosan
2,3-dihydr
Glucose/Ga
L-aspartic
leucine_di
b-alanine_
mine
8_10.04
g-aminobut
10.038_Pol
fumaric_ac
noctacosan
L-lysine_6
L-tyrosine
Succinic
_Unknown_7
Glutamic_a
Unknown_6
Threonic_A
a_carbohyd
inositol_6
Malic-acid
Phosphate(
citric-aci
R2X[1] = 0.180835 R2X[2] = 0.114613
SIMCA-P+ 12.0.1 - 2012-05-07 12:12:50 (UTC+0)

Appendix 5.11: DNA and RNA ladders.

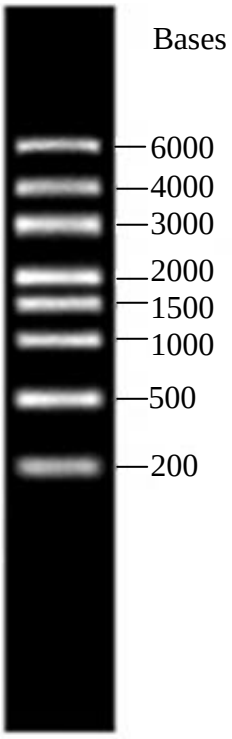
A



A: Lambda DNA/*Hind*III marker (Fermentas).

0.5 µg/ lane, 8 cm length gel, 1% agarose, 1X TAE.

B



B: RiboRuler high range RNA ladder (Fermentas).

2 µl/ lane, 8 cm length gel, 1% formaldehyde agarose, 1X MOPS.

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Agrawal, G. K., Iwahashi, H. and **Rakwal, R. (2003b).** Rice MAPKs. Biochemical and Biophysical Research Communications, **302,** 171-180.

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