The role of the BHLH038 transcription factor in the regulation of osmotic and drought stress responses in *Arabidopsis thaliana*

Maria Michailidi

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Abstract

The most important abiotic stress affecting agriculture worldwide is drought. In order to ensure food supply for the expanding world population two critical tasks must be undertaken; maintenance of crop yield as well as the increase of yield potential. Plants have built up a complex stress response system in order to survive under drought and other abiotic stresses. Analysis of transcriptomics data in studies, obtained from plants such as *Arabidopsis thaliana* exposed to drought stress have revealed a lot about plant responses to abiotic stresses. Genetic, molecular and biochemical studies have been conducted over the years in order to identify key regulators in plant drought response. Many regulators such as Transcription Factors (TFs) have been examined over the years in order to verify their roles in the enhancement of abiotic stress tolerance i.e.: drought in crops all over the world.

Recent work has revealed Gene Regulatory Networks using Variational Bayesian State Space Modelling, obtained from time-series slow drying microarray data. These Gene Regulatory Networks unveiled various Transcription Factors such as BHLH038 closely related to AGL22 a key hub gene for drought response in Arabidopsis as identified by Bechtold et al. (2016), indicating the significant role of TF genes in drought stress signaling.

In this review we focused on unveiling the role of BHLH038 TF gene in drought stress response of Arabidopsis plants. Loss-of function mutants were phenotyped under drought conditions both in soil and plate-based medium. Only *bhlh038-2* revealed a drought phenotype under plate-based assays in early developmental stages of Arabidopsis seedlings, whilst *bhlh038-4* revealed a drought phenotype in slow-drying soil experiments in late growth stages of Arabidopsis plants. Network connections between BHLH038 and other closely related TF genes were tested by qPCR. BHLH038 can be considered as a key regulatory gene in drought stress signaling, further investigation would be beneficial.

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List of abbreviations

µg – microgram
µl – microliter
µmol – micromoles
bp – base pair
cDNA – complementary DNA
mm – millimeter
Col-0 – Columbia-0
d^{-1} – per day
DNA – Deoxyribonucleic acid
g – gram
GRN – Gene Regulatory Network
H2O2 – Hydrogen Peroxide
KanR – Kanamycin resistance gene
LB – Left border of T-DNA
log – logarithm
ml – mililitre
mM – millimolar
mmol – millimoles
p – probability value
RB – Right border of T-DNA
RNA – Ribonucleic acid
rSWC – relative soil water content
s – second
Fv – Maximum variable fluorescence
Fm – Maximum fluorescence, when all PSII Rcs are closed
PSII – Photosystem II
RC – Reaction Center of PSII
T-DNA – Transfer DNA
U – units
VBSSM – Variational Bayesian State Space Modelling
WT – wild-type
℃ - Celsius
PCR – Polymerase Chain Reaction
qPCR – quantitative polymerase chain reaction
E.coli - *Escherichia coli*
RT – Room Temperature
rpm – revolutions per minute
UTR – untranslated region
Tris – HCl – Tris Hydrochloride
EDTA – Ethylenediaminetetraacetic acid
SDS – sodium dodecyl sulfate
dNTP – Deoxyribonucleotide triphosphate
TF – Transcription Factor
GRN – Gene Regulatory Network

INTRODUCTION

1.1. Abiotic stress

Once a plant is sown on soil it is exposed to numerous environmental factors, which are therefore crucial for its development and productivity. In general, plants are considered to be under stress when they experience a lack of an essential element or an abundance of potentially toxic or damaging substances. In nature plants usually face both the situations simultaneously. According to Cakmak (2005), the K-nutritional status of plants is of great importance for the survival of crops under environmental stress conditions. One of the problems of K+ deficient plants is the increase in NADPH oxidation. The severe degradation and intensive use of soils over the years has led to the poor productivity and fertility of the soil, conferring poor nutrient status to the plants; thus affecting crop yield at a great degree. Environmental factors can be of both abiotic and biotic nature. Abiotic factors involve extreme temperature (heat, freezing), salinity, light intensity, ion toxicity from heavy metals as well as the supply of water; All these potentially limit crop yield, they affect seed germination as well as many metabolic activities, as they are the primary resources required for plant growth and development (Cramer et al, 2011).

Climate change leads to abiotic stress conditions and as a consequence the plant is challenged to develop strategies in order to adapt to these changing conditions. Many studies have focused on the adaptation strategies involved in many crops around the world e.g.: maize, wheat, rice etc. (Halford et al., 2014). Climate change has already caused significant impacts on water resources, food security, hydropower and human health especially for African countries as well as to the whole world (Ziervogel et al., 2014)
Moreover crop efficiency and soil water equity have been studied with crop growth models. One of the most important limiting factors for crop production and thus food security is water availability (Li and Geng, 2013). Fujihara et al. (2008) specified that water shortage would not occur if water demand does not increase; in any case, if the irrigated zone is expanded under present irrigation efficiency rates, water shortage will occur. Therefore, it is urgent to ascertain the effects of climate change on crop production and water resources so as to develop potential adaptation systems. Correia et al (1995) demonstrated that plants' water demands differ diurnally, with the opening and shutting of stomata responses to light cues. Moreover, indicated by Chaves et al (2002), water availability may vary throughout days to years as a consequence of occasional drought or flooding. A literature survey on different abiotic stresses revealed how water shortage and osmotic stress via salinity has been shown to induce many physiological symptoms like decrease in photosynthetic activity via closure of stomata, growth inhibition, wilting and in severe cases even plant death (Chaves et al., 2004). In addition germination of seeds during growth season and vegetative growth are reduced by metabolic imbalance (Cramer et al., 2011). Therefore, understanding abiotic stress factors such as drought and osmotic stress tolerance traits along with how to maintain high crop yield is of central significance to mitigate the adverse impacts of climate change on the profitability of yields. (Mickelbart et al., 2015)

1.2 Drought responses

Drought stress is one of the principal limitations to agriculture worldwide and the main reasons for this, is the complexity of the water-limiting environment in which plants grow and develop as well as the changing climate.
Plants have evolved various mechanisms at the morphological, biochemical, cellular, molecular and physiological level to survive water shortage and drought stress conditions. Many of the key plant characteristics that have been used as indicators to valuate the degree of drought resistance of plants, involve root traits, leaf/shoot traits, osmotic adjustment capabilities, ABA content, water potential and others (Fang and Xiong, 2015). The drought resistance of plants can be classified into four groups, the drought tolerance, drought avoidance, drought escape and drought recovery.

Under drought, reduced dry matter accumulation takes place in all plant organs, although distinctive organs show shifting degrees of reduction. For example, drought induced an earlier response in roots of *Arabidopsis thaliana* plants than in shoots (Rasheed et al, 2016). Likewise, drought significantly reduced shoot and root dry weights in Asian red sage (Salvia miltiorrhiza L.), in spite of the fact that the greatest effect was on the roots rather than the shoots (Wei et al. 2016). Loss of turgor followed shortly after drought stress was induced and the leaf area as well as the number of the leaves was reduced (Farooq et al., 2017). Plants display stress tolerance or stress avoidance through acclimation and adaptation strategies that have evolved through natural selection. (Mickelbart et al., 2015)

Wang et al (2003) affirmed that abiotic factors, for example, drought, salinity, high light stress among others hugely affect world agriculture, and it has been suggested that they reduce average yields by >50% for most major crop plants. Additionally, according to Cramer et al (2011) stresses trigger plant responses that are complex and include versatile changes and/or deleterious effects. Another review by Chaves et al (2002), explored plant responses to water shortage and demonstrated that drought avoidance and tolerance strategies are involved, implying that early responses to water stress aid immediate survival whereas acclimation, leads to modifications in metabolism and structure of the plant in combination with altered gene expression, enhancing plant functioning under stress. According to Correia et al (1995) the stomatal conductance of plants was recorded and shown to decrease under drought/osmotic stress.

Abiotic stress tolerance has been assessed through the extensive study of molecular control mechanisms, which are based on the activation and regulation of various genes related to the stress experienced by the plant(s) (Wang et al., 2003). The ability of plants to endure low water content through adaptation strategies that lead to maintenance of cell turgor through osmotic adjustment and cellular elasticity, and increasing protoplasmic resistance (Basu et al. 2016).

The drought response *of A. thaliana* has been the subject of various recent publications (Kreps et al., 2002; Seki et al., 2002; Kawaguchi et al., 2004; Huang et al., 2008). A few of these reviews have been established on quickly initiated extreme water deficit conditions (Kreps et al., 2002; Seki et al., 2002b; Kilian et al., 2007). In these studies plant roots were exposed to high osmotic potential solutions (Kreps et al., 2002), dry air streams (Kilian et al., 2007) or blotting paper (Seki et al., 2002b) to initiate rapid water loss. While these methodologies involved exceptionally reproducible water loss and homogeneity

among the stress conditions, they did not involve water stress experienced by soil -grown plants in the environment. Other studies instigated drought stress in soil-grown plants by reducing water potential for varying periods of time to cause drought stress (Kawaguchi et al., 2004; Huang et al., 2008). Drought stress was alternatively demonstrated in soil grown Arabidopsis plants through watering with 400mM Mannitol solution for 10 days (Liu et al., 2015). A couple of these reviews have united multiple time intervals into their examinations. (Kawaguchi et al., 2004; Kilian et al., 2007); in any case, in each instance, their interest was transcriptome changes caused over the term of the stress and not in the impact of time of day on the drought response. (Kanchiswamy et al, 2015).

Combining high yield potential under normal conditions with great yield potential under normal conditions with good yield under drought stress is the perfect characteristic (Lukonge et al, 2007). Identification of mechanisms, traits, and genes directing yield under drought stress that are free from yield drag under normal conditions ought to be the main interest. There is considerable motivation to better comprehend how transcriptome-level changes shape drought responsiveness.

1.3 Transcriptome analysis of drought response

Transcriptome analysis of various molecular stress responses has been conducted in many studies, by exposing different groups of plants to either single or multiple stresses simultaneously, and their gene expression patterns are compared. As a result, overlapping sets of genes that are regulated by stresses are then identified and proposed to represent a generalized stress response or point of cross talk between signalling pathways (Guo Tao Huang et al 2012, Banerjee and Roychoundury, 2015). In response to drought stress, ABA stimulates a signaling pathway that leads to the production of reactive oxygen species (ROS), which results to an increase in cytosolic Ca^{2+}(Osakabe et al, 2014).

ABA (abscisic acid) is a phytohormone involved in signalling pathways during drought stress, which in turn causes stomatal closure and induces the expression of stress-related genes. Many conserved, as well as, species-specific regulatory and functional drought-responsive genes, including osmoprotectants, ROS-related, ABA biosynthesis, late embryogenesis abundant (LEA) and chaperone, ion homeostasis, and signalling genes, have been identified in various studies (Okasabe et al, 2014). Several drought-inducible genes are induced by exogenous ABA treatment, though others are not influenced. Both ABA-independent and ABA- dependent regulatory systems control drought-inducible gene expression and it is shown in many studies (Yamaguchi-Shinozaki and Shinozaki, 2005;Nakashima, Yamaguchi-Shinozaki and Shinozaki, 2014). The products of drought-inducible genes are split into two groups one of which includes regulatory proteins i.e.: protein factors involved in further regulation of signal transduction and stress- responsive gene expression. These involve various transcription factors such as bZip, MYB, NAC among others.

In the quest to find regulatory genes working in concert in abiotic signalling pathways, various transcription factors have been discovered. Transcription factors involved in these stress signal transduction pathways are of key importance in generating specificity in stress responses according to Atkinson

et al 2012. Some of the most important transcription factors responding to drought, low temperature, and high salinity stress belong to several classes, including the basic helix-loop-helix MYC, MYB (Abe et al 1997), bZIP (Hu et al, 2016), NAC, ABF/AREB, DREB/CBF and WRKY TFs (Ishida et al., 2012; Sakuma et al., 2006). The expression of many stress- inducible genes is regulated by Transcription Factors in either an independent manner or cooperatively and as a result establishes genes networks in Arabidopsis (Yamaguchi- Shinozaki and Shinozaki, 2006). Similar to the Arabidopsis findings, the products of stress-inducible genes identified in rice can also be classified into regulatory proteins i.e.: transcription factors.

1.3.1 Genetic modifications of TF genes

Genetic modification of regulatory genes that are involved in plant stress tolerance, leads to altered expression, this may have a great impact in the plants stress tolerance since it mimics or enhanced stress signals in order to regulate stress- responsive genes at a time (Century et al 2008, Yang et al 2011). Transgenic plants expressing OsNAC5, OsNAC9 and OsNAC10 TFs showed an increase in grain yield of 5 -26% under normal conditions. In many studies, transcription factor genes have played a significant role in regulating the expression of an array of functional downstream stress responsive genes by interacting with the specific cis-elements in their promoter region (Yamaguchi-Shinozaki and Shinozaki, 2006), therefore leading to stress tolerance/ resistance. For example, a recent study (Ye et al, 2017) showed that RD26 a drought-induced transcription factor has been identified as a negative regulator of the BR signalling pathway. Also DREB1 Transcription Factors involved in a cellular signal transduction pathway, were shown to be induced by dehydration and salt stress, bind to stress responsive cis-elements and therefore trigger the expression of stress responsive genes leading to stress tolerance (Khan, 2011). Another study presented a gene regulatory network where AGL22 in *Arabidopsis thaliana,* was found to be a master regulator of a transcriptional network during drought stress, linking changes in primary metabolism and the initiation of stress responses. (Bechtold et al, 2016).

1.4 Transcriptional Pathways Involved in Water Stress Responses

Gaining a better understanding on transcriptional regulation of drought is very important. Many reports have suggested the existence of crosstalk between TFs involved in drought stress. Therefore, studying the transcriptional pathways involved in water stress responses gives a better insight in the roles of these TFs in drought response. Several bZIP transcription factors from rice, maize and Arabidopsis plants respond to dehydration, cold and exogenous ABA treatment (Tang et al., 2012; Xiang et al., 2008). Several studies have reported these transcription factors to be involved in ABA-dependent pathways, exactly like MYC and MYB transcription factors. These transcription factors are considered to function in the regulation of ABA-inducible genes, which respond

to water stress rather slowly after the production of ABA-inducible transcription factors.

In one study, four bZIP genes displayed higher expression values in lead, flowers and root tissues in Cucumis sativus plants (Baloglu et al, 2014). In addition the expression of ten CsbZIP genes in root and leaf tissues of drought-stressed cucumber was analysed using RT-PCR, and all of the selected CsbZIP genes were measured as increased in root tissue at 24h upon PEG treatment, conferring water deficit. In contrast to leaf tissues whereas the down-regulation of all analysed CsbZIP genes was recorded. CsbZIP12 and CsbZIP44 genes showed gradual induction of expression in root tissues during time intervals (Baloglu et al., 2014). Another study provides evidence of bZIP transcription factors being involved in drought and salt tolerance in transgenic Arabidopsis. BnaABF2, a bZIP transcription factor from rapeseed (Brassica napus L.) was found to render drought and salt tolerance when overexpressed in Arabidopsis plants (Zhao et al, 2016).

Transgenic plants overexpressing BnaABF2, showed resistance to drought and salt stresses which was then proved to be due to reduced water-loss rate and expression of stress- responsive genes such as RD29B, RAB18 and KIN2. The expression of the above-mentioned genes, was regulated by BanABF2, is which involved in an ABA-dependent stress-signaling pathway, similar to MYB and MYC like transcription factors. (Zhao et al, 2016)

The involvement of WRKY transcription factors in drought stress has been reported recently (Rushton et al., 2010; Banerjee and Roychoudhury, 2015). Various WRKY transcription factors have been found to be involved in ABA signalling. WRKY63/ABO3 (ABA overly sensitive3) has been demonstrated to be involved in drought responses where the abo3 mutant exhibits hypersensitive response to ABA and reduced drought tolerance at the seedling stage (Ren et al, 2010). WRKY63 has been shown to bind to the promoter of AREB1/ABF2 and thereby positively regulating its expression (Ren et al., 2010). Many other genes have been reported to be involved in drought and salt stress responses (Bakshi et al., 2014; Banerjee and Roychoudhury, 2015).

As a result it is very important to produce transgenic crops and boost the responses of plant to stresses in order to prevent such dramatic reductions in crop yields around the world. Model organisms such as *Arabidopsis thaliana* have played a crucial role in the phenotyping of transgenic lines as well as the identification of complex gene networks along various important crops. In multiple cases, when the native orthologue of that gene is expressed in potato (Iwaki et al, 2013), soybean (Polizel et al 2011) among others, the transgenic crops enjoy the same quantitative improvement predicted from the Arabidopsis model.

1.5 MYC (bHLH) and MYB transcription factor genes Involved in Water Stress

Divergence within groups of transcription factors as well as gene or chromosomal duplication give rise to great diversity in morphological traits but also metabolic traits that characterize the higher plants (Feller et al 2011). Some of the biggest transcription factor groups that lead to diversity are MYB and bHLH families (Feller et al., 2011). MYC transcription factors belong to bHLH (basic-helix-loop-helix) transcription factor family of plants that have a

characteristic bHLH domain (Kazan and Manners, 2013). Ambawat et al. (2013) claimed that MYB proteins act as positive or negative regulators of Abscisic acid (ABA) and/ or abiotic stress responses. MYB proteins such as AtMYB2, AtMYB96, AtMYB15, and AtMYB44 are known to regulate ABA and abiotic stress responses.

Another study showed that AtMYB60 and AtMYB61 are two transcription factors that play a crucial role in modulating stomatal aperture for plant survival under diverse conditions and drought tolerance and they are expressed in guard cells (Cominelli et al., 2005).

AtMYB60 is a negative regulator of stomatal closure (Cominelli et al. 2005; Liang et al., 2005). The study (Park et al., 2011) focused on AtMYB52 gene, which was activated in one of the ABA response mutants, ahs1.

Evidence provided by this study, showed that overexpression lines of AtMYB52 were drought- tolerant and their seedlings were salt-sensitive. Knowing that ABA mediates various abiotic stress responses (Xiong et al 2002), especially drought stress response, Park et al (2011) investigated effect of drought stress in the growth and development of AtMYB52 overexpression transgenic plants, by withholding water for two weeks. Results showed that the survival rate of ATMYB52 OX lines #112 and #58 reached 81% and 75% whereas the wild-type rate was 3% and 10%. Additionally, salt sensitivity of the AtMYB52 OX lines was tested; the measure of the salt sensitivity was the shoot development of the transgenic plants, which was more extensively inhibited by the salt. The percentage of green cotyledons in wild-type seedlings was 95% whilst in transgenic OX lines #112 and #58 reached only 50% and 20% in 125mM NaCl. Thus, shoot development of the AtMYB52 OX lines was hypersensitive to salt. In conclusion, this study provides enough evidence that supports the statement on the MYB class transcription factors known to be involved in ABA and stress responses in Arabidopsis.

Regarding MYC class transcription factors, Tuteja and Gill (2013) confirmed that one of the major transcription factors involved in abiotic stress response is MYC-like bHLH protein. 162 genes in Arabidopsis encode proteins of the bHLH family (Feller et al 2011; Heim et al., 2003). According to Feller et al (2011), bHLH proteins function as transcriptional activators or repressors in transcriptional networks controlling a number of biological processes. The number of characterized plant bHLHs has increased in recent years, revealing the wide and diverse array of biological processes in which they are involved. Most bHLH proteins identified have been functionally characterized in Arabidopsis.

Based on the study of hormonal signalling, Abe et al (2003) investigated the role of a dehydration- responsive gene RD22, which was induced by abscisic acid (ABA). The regulation of expression of many genes under drought stress is the main role of ABA in plant response and drought stress tolerance (Zhu, 2011); Previous research has shown that the expression of rd22 is regulated by two cis-acting elements, MYC and MYB recognition elements found in the rd22 promoter region (Abe et al., 1997). MYC like transcription factor MYC2 and MYB like transcription factor MYB2, activate the transcription factors of the target gene i.e.: rd22 by binding to the two cis-elements MYC and MYB. Transgenic Arabidopsis plants, overexpressing the bHLH- related transcription factor rd22BP1 (also known as AtMYC2), interacting with MYC recognition sites, were highly sensitive to ABA. Not only that, transgenic plants overexpressing AtMYC2 and AtMYB2 simultaneously showed higher ABA sensitivity and osmotic tolerance (Abe et al 2003), by growth retardation when planted on soil. Over-expression and knockout mutants displayed contrasting phenotypes regarding ABA sensitivity, similar to AtMYB2 (Abe et al., 2003).

Thus this implied that the bHLH- related Transcription Factor rd22BP1, functions as a transcriptional activator in ABA-inducible gene expression under drought stress in plants. The upregulation of AtAIB transcription factor, known as a bHLH transcription factor, along with the overexpression of AtAIB in rice showed drought tolerance (Li et al., 2007; Park et al., 2011), once again suggesting that MYC (bHLH) transcription factor genes are of great importance in drought tolerance.

MYC2 protein has emerged as a master player in jasmonic acid signaling as well as cross talk between jasmonic acid and ABA signaling (Kazan and Manners, 2013). According to a recent review, the regulatory mechanisms of MYBs and bHLHs were proven to control plant metabolic pathways. MYBs and bHLH proteins were examined in various defense signalling pathways like jasmonate, flavonoid biosynthesis and cell death pathways.

Another study confirms one of the regulatory stress responsive functions of bHLH transcription factors involving hormonal signalling proving that *Arabidopsis thaliana* bHLH MYC3 and MYC4 (phylogenetically closely related to MYC2, known as a direct target of JAZ repressors), are activators of JA-regulated programs that act additively with MYC2 to regulate specifically different subsets of the JA- dependent transcriptional response. Evidence required the loss-of-function mutations of these two TFs, which impaired full responsiveness to JA and enhanced JA insensitivity of myc2 mutants. In addition, the overexpression of bHLHs confers tolerance to salt, osmotic, cold stress, and some bHLHs function as positive regulators of NaCl, drought and osmotic stress signalling (Liu et al 2014).

Finally, some bHLHs are also involved in maintaining iron homeostasis (Long et al., 2010) and in the response to phosphate starvation (Yi et al., 2005). These two studies suggested that bHLH transcription factors are highly involved in biotic and abiotic stress responses via cellular transduction pathways. Lorenzo Carretero-Paulet et al 2010, carried out a genome-wide classification and evolutionary analysis of the bHLH family of Transcription Factors in plants and once again presented the role of bHLH TFs in biotic and abiotic stress responses regulated through signalling. This research has demonstrated that plant bHLHs serve as key regulatory components in transcriptional regulatory networks controlling a broad range of growth and developmental signaling pathways and abiotic stress responses (Zhang et al, 2009). Increasing evidence suggests that bHLHs play important roles in plants in response to abiotic stress. For example, Nakata et al. (2013) showed that a bHLH protein (JAM1) could negatively regulate JA-mediated plant stress responses. The response system of JA-regulated gene expression in drought tolerance mainly involves OsbHLH148 (Seo et al. 2011). Jiang et al (2009), revealed the importance of bHLH92 in plant's responses to osmotic stress. Recently, Tian et al (2015) investigated the phenotype of a bHLH family transcription factor gene, known as bHLH129; it seemed to be involved in an ABA response when overexpressed, regulating root elongation. The expression of bHLH129 was reduced in response to exogenously applied ABA, and elevated in the ABA biosynthesis mutant aba1-5. When expressed in Arabidopsis under the control of the 35S promoter, bHLH129 promoted root length growth and the transgenic plants were less sensitive to ABA in root elongation assays. To sum up, this study shows that bHLH129 negatively regulates ABA response in Arabidopsis, so it is considered to be a transcriptional repressor (Tian et al, 2015).

Another study carried out by Liu et al (2015), investigated the effect of AtbHLH112 in the expression of genes involved in abiotic stress tolerance. Once again, this key hub gene belongs to the plant family of basic helix-loop-helix (bHLH) transcription factors, which as mentioned above play essential

roles in abiotic stress tolerance. The expression profile of AtbHLH112 was studied in response to NaCl, mannitol and ABA and the expression of AtbHLH112 seemed to be highly induced in both roots and leaves in plants when under the stresses mentioned above; this thus confirmed that the AtbHLH112 plays a key role in the abiotic stress signalling pathway.

Moreover, mutant lines, RNAi-silenced AtbHLH112 transgenic plants (SE) as well as Arabidopsis plants overexpressing AtbHLH112 were also studied when exposed to salt, Mannitol and ABA stresses. The results from this study suggest that AtbHLH112 is a key regulator of abiotic stress tolerance, since plants overexpressing that gene, had reduced water loss and cell death, in response to salt, osmotic stress and ABA (Liu et al, 2015). In conclusion, plants overexpressing bHLHs display increased tolerance to salt, drought, and oxidative stress and freezing, demonstrating that bHLHs play pivotal roles in mediating abiotic stress responses (Xiaoyu Ji et al. 2016). Xiaoyu Ji et al (2016), showed that a bHLH gene (ThbHLH1), from Tamarix hispida, a woody halophyexhte known to be highly tolerant to salinity and drought (Pan et al. 2011), could improve the abiotic stress tolerance by increasing osmotic potential, improving reactive oxygen species (ROS) scavenging capability and enhancing second messenger in stress signalling when overexpressed. The data generated, suggested that ThbHLH1 induces the expression of stress tolerance-related genes.

More recently, Bechtold et al (2016) asserted that changes in metabolism and gene expression drive extensive drought tolerance in *Arabidopsis thaliana* and initiate diverse drought avoidance and escape responses. In the study conducted by Bechtold et al (2016), drought – responsive genes were identified through a high- resolution time series transcriptomics analysis as plants were subjected to a slow exposure from well watered to drought conditions. A gene regulatory network was generated and it indicated that AGAMOUS-LIKE22 (AGL22) is a key hub gene in a TF GNR. Many genes link abiotic stress responses such as drought responses to the growth and development of plants by regulating transcriptional networks (Bechtold et al., 2016). Bayesian State space modelling helps to identify and verify some of these genes and the study conducted by Bechtold et al., (2016), revealed that AGL22 is one of the most highly linked genes to early and late drought responses. Not only AGL22 was absent in the gene regulatory network of well-watered Arabidopsis plants but also when two independent T-DNA insertion lines were isolated, knockout mutants for AGL22 were confirmed some of which were differentially expressed under drought conditions in at least one of the agl22 mutants compared with the wild type implying that ~50% of the network connections were regulated at least partially through AGL22.To sum up, AGL22 is considered a key hub gene up regulating and/or down regulating many TFs under drought stress.

The Gene Regulatory Network based on the time-series microarray data of the slow-drying experiment, which was generated using Variational Bayesian State Space modeling, revealed another TF gene linked to the key hub gene AGL22 that could be a potential key regulatory TF gene in drought stress signaling. This particular gene is known as BHLH038, seemed to up-regulate and down-regulate many drought-responsive genes involved in this network but at the same time the gene was shown to be independent of all other TFs including AGL22 regarding its own expression (Bechtold et al., 2016). The role of BHLH038 gene in this study is vague and mainly unknown, however this study (Bechtold et al., 2016) seems to imply the gene's significant role in drought response.

1.6 Conclusion

Extremes of environmental conditions, such as drought, cold, and high salinity, induce stress in plants and decrease growth and productivity. Photosynthesis and the related metabolism are among the processes most strongly affected by these abiotic stresses. Interestingly, both stomatal and non-stomatal responses to abiotic stress involve transcriptional regulation and consequently the involvement of many transcription factors. From the literature cited above, it is clear that phenomenal progress has been made towards the identification and verification of stress responsive genes, regulatory gene elements, signal molecules and their network, as part of global responses of plants to various abiotic stress conditions. Overall, abiotic stress signaling is an important area with respect to increase in crop yield under sub-optimal conditions.

Adjustment to stress conditions may be improved by modifying the transcription factors in plants. Many transgenic plants over-expressing Transcription factors show improved abiotic stress tolerance related to enhance growth parameters. However, knowledge about the MYC transcription factor genes involved in the regulation of growth-related genes by the different abiotic stresses is still limited. The overall progress of research on bHLH (MYC)-like ABA related stress responsive genes and their products reflect their central role in plant growth and development under stress conditions.

A lot of effort is still required to uncover in detail of the bHLH (MYC) transcriptional factor gene family. In order to comprehend better the role of transcription factor genes in their contribution to stress tolerance in dry land crops, the present investigation is undertaken with Arabidopsis thaliana, a dry-land drought tolerant plant, with a main objective to verify and identify phenotypes of bHLH038 TF gene since enough evidence is provided (Bechtold et al., 2016), suggesting that it is involved in the regulation of drought stress responsive genes.

1.7 Aims and Objectives

(i) To verify the transcription networks regulated by bHLH038 Transcription Factor during early and late drought responses by assessing gene expression of various genes in mutant lines.

(ii) To analyze knockout and over-expressing mutants of hub genes for altered drought and other abiotic and biotic stress phenotypes

(iii) To study the molecular mechanism and signaling networks cantered around other hub genes.

(iv) To assess *Arabidopsis* development and biomass of three different genotypes

(v) To phenotype homozygous mutants *bhlh038-2* and *bhlh038-4* under drought stress, osmotic stress.

(vi) To apply and maintain drought stress in pots containing 5 week old Arabidopsis thaliana seedlings of three different genotypes in order to determine the survival rate and as well as the rSWC.

(vii) To assess quality and yield of DNA extracted from dried leaves

MATERIALS AND METHODS

2.1. Plant material, growth conditions and stress treatments

Plants were grown in soil (Levington F2+S, The Scotts Company, Ipswich, UK) and placed in a growth chamber with controlled conditions – 8/16-hour light (~120umol $m^{-2}s^{-1}$)/ dark cycle at 23℃ +/- 1 ℃ and 60% relative humidity.
Regarding the progressive drought experiment, small pots (7x7x9 cm) were filled with identical amount of soil (4.5 gr approx.). In order to determine the 100% SWC and the 0% SWC, control pots were set up simultaneously containing the same amount of soil (4.5 gr approx.) The plants were kept well watered for 5 weeks. Half of the plants of each genotype (Col-0, *bhlh038-2* and *bhlh038-4*) were regularly watered while the other half underwent drought, where water was withdrawn and the weight of each pot was recorded on a daily basis. All plants were saturated in water with 95% rSWC, and then the drought season begun
The rSWC (relative soil water content) was calculated daily until the pots reached 20% rSWC. The drying rate was obtained as the slope of decline in rSWC was measured on a daily basis during the drying season.
For gene expression, the rosettes were harvested, frozen with liquid N_2 (Nitrogen) and then water supply was resumed. For mannitol treatment, 4-week-old plants were water supplemented (15ml to each pot) with 300mM Mannitol every two days for a period of 5 days. The rosettes were further examined using the Fluorimager in order to detect the green areas of the inflorescence base. In all soil treatments a negative control was used.

2.2 Molecular Biology Techniques

2.2.1 Primer design

All primers used in this project were designed using the website Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Ye et al., 2012).

Table 1: Primer Sequences used

Primers	**Sequence**
qPCR_PIL2_F	TTGGGGCCGAACTACTCTCA
qPCR_PIL2_R	GCAAGCCAGCTCCTAGAACA
qPCR_DEL2_F	TCTCGCTCCCCAGGTTTACA
qPCR_DEL2_R	CGGCATCATCGAGCCCAAAT
qPCR_BZO2H1_F	CTGCTCCCATGACGACGAAG
qPCR_BZO2H1_R	TCACAGACCCAACCCGAAGA
qPCR_BPC7_F	ACCCACTACCCATGAGCACA
qPCR_BPC7_R	GTCATAGCCTTCGTCCGCAA
AGL22_F (qPCR)	CTCTCCGTTCTCTGCGACG
AGL22_R (qPCR)	GGGCGTGATCACTGTTCTCA
BHLH39 LB	CAATCCGCATGATAAATCACC
BHLH39 RB	TTGGTGGCTGCTTAACGTAAC
BHLH038_5UTRclo	AATATATGAGATCGAATAAGGATATGAATTTAC
BHLH038_3UTR	AACAAGTCTCTTCTGAAACTGGCTTTCACA
BHLH039 F qPCR	TCATGTCTTCCTGCCTCTGG

BHLH039 R qPCR	CTTGCTCTTGCAGCTCTGGT
ACTIN F qPCR	ACCTTGCTGGACGGACCTTACTGAT
ACTIN R qPCR	GTTGTCTCGTGGATTCCAGCAGCTT

2.2.2 Identification of knockout mutants from T-DNA insertion lines

2.2.2.1 T-DNA insertion lines were obtained from Dr. Subramaniam

Two independent T-DNA insertion mutants (*bhlh038-2* and *bhlh038-4*) for BHLH038 gene were obtained from Dr. Subramaniam (Subramaniam, 2016), genotyping and verification of homozygosity did not take place in this study. SALK_025676 (*bhlh039*) mutants were obtained from the Nottingham Arabidopsis Stock Centre (Arabidopsis.info, 2018, http://www.arabidopsis.info/) in order to screen for T-DNA insertion lines. SALK (Alonso et al., 2003) and SAIL (Sessions et al., 2002) lines compared with Col-0 (wild-type) Arabidopsis *thaliana* seedlings were used in this project.

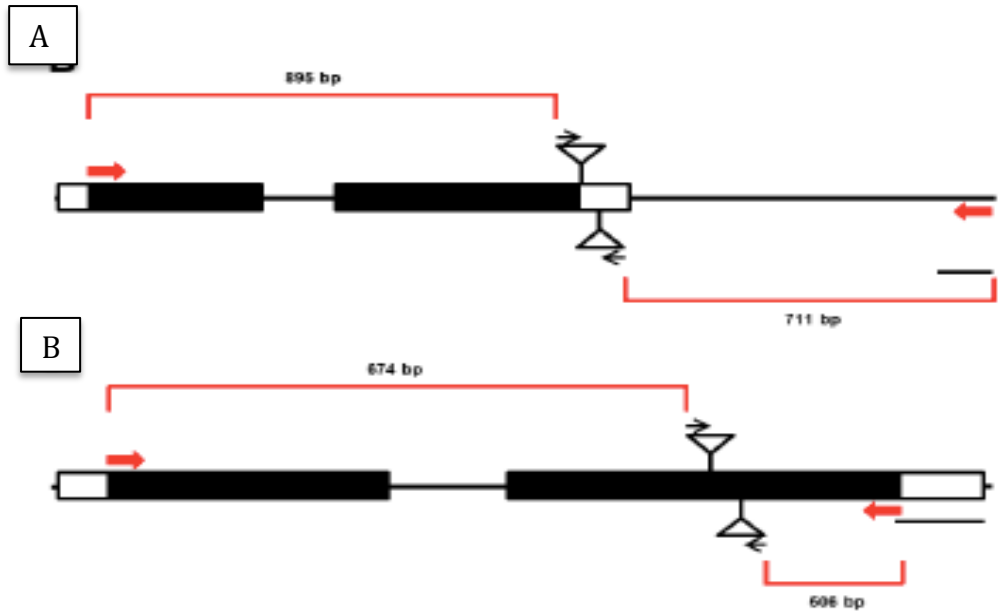

Figure 1: Representation of T-DNA

Position of T-DNA insertions, is illustrated by schematic diagrams, the binding sites of primers are displayed, the black arrows indicating the orientation of the insertions in the gene, the untranslated regions (UTRs – open rectangles), the exons (black rectangles) and the introns (lines). (A) T- DNA insertion in gene for *bhlh038 -2.* (B) T-DNA insertion in gene for *bhlh038-4* (Subramaniam, 2016).

2.2.4 Genomic DNA extraction

DNA extraction was carried based on the protocol made by Edwards et al. (1991). Micropestles were used in order to grind one or two leaves taken from each plant (Col-0/ *bhlh039*) in 500ul of DNA extraction buffer (200mM Tris-HCl, pH 7.5, 250 mM NaCl, 25mm EDTA, 0.5% SDS). The samples were then spun in a microcentrifuge at 13000rpm for 10 minutes, and the supernatant was mixed with isopropanol. Once again the sample was centrifuged at the same speed for 10 minutes, before the pellet was washed with 70% ethanol. Finally the dried pellet was resuspended in 50ul of sterile Reverse Osmosis (RO) water.

2.2.5 PCR for T-DNA insertion in *bhlh039* mutant screening

In order to verify the existence if the T-DNA insertion line within the gene of interest Polymerase Chain Reaction was carried out. DNA polymerase (#EP0402, ThermoFisher Scientific, Loughborough, UK) of 1U/50ul concentration was used for the PCR; in addition 5x HF Buffer of 1X concentration was used. A mix of 10mM (dNTPs; #R0191, ThermoFisher Scientific) deoxyribonucleotide triphosphates was used whose final concentration was 200uM per reaction, 0.4ul in 20ul reaction mix. Forward and reverse primers (10uM each) were further diluted to a final concentration of 0.5uM in the final reaction mix (20ul), which corresponds to 1ul of each primer (Table 1).
The PCR involved the following conditions; 3 minutes at 95°C for the initial denaturation, then 35 cycles of denaturation at 94°C for 30 seconds, 30 seconds at 58-60°C for primer annealing and 2.5 minutes at 72°C for extension; final extension lasted 10 minutes at 72°C.

2.2.6 Agarose Gel Electrophoresis

6x DNA loading dye (10mM Tris-HCl, 0.03% Bromophenol Blue, 0.03% Xylene Cyanol FF, 60% Glycerol, 60mM EDTA) was added to the PCR products. Agarose gels of a final concentration of 1% were then prepared by dissolving 0.8g of agarose in 80ml of 1x TBE (Tris/Borret/EDTA) buffer, using brief heating in a microwave oven. The PCR products were run alongside the GeneRulerTM DNA Ladder Mix at 110V for 30-35 minutes. SafeView (#NBS-SV1, NBS Biologicals Ltd., Huntingdon, UK) was used in order to stain the gel, in a 1:10,000 dilution and the DNA was made visible using the GeneGenius Bioimaging System (SYNGENE, SYNOPTICS Ltd, Cambridge, UK).

2.2.7 RNA extraction from Arabidopsis plants

Total RNA from plant tissue of T-DNA insertional mutants (*bhlh038-2/ bhlh038-4/* 4.3.6ox*)* as well as from Col-0 plants was extracted. The RNA was extracted using TRI Reagent solution (#AM9738, ThermoFisher Scientific). Approximately 100mg of leaf tissue was isolated in an Eppendorf tube and frozen using liquid Nitrogen. A mortar and a pestle were used to finely grind the leaf tissue once frozen, without letting it defrost. 1ml of TRI Reagent was added to the sample. Then the sample was incubated for 5 minutes at room temperature (RT). 200ul of Chloroform was added and the solution was mixed well using a vortex before 5 minutes of incubation at RT. The samples were then centrifuged at 13,000rpm for 20 minutes at 4°C. The aqueous phase was transferred to a new Eppendorf tube and mixed with isopropanol, before centrifuging again at 13,000rpm for another 20 minutes at 4°C to isolate the RNA in a pellet. The RNA pellet was washed with 1ml of ice-cold 70% Ethanol at 13,000rpm for 5 minutes at 4°C and then air-dried in a fume hood to remove any excess Ethanol. 26ul of freshly autoclaved RO water was then added to the DNase-treated and dry RNA pellet in the Eppendorf tube.

2.2.8 DNaseI treatment of RNA

DNaseI treatment followed right after the RNA extraction to make sure that the sample did not contain any DNA. Recombinant DNase I (rDNase I; #AM2235, ThermoFisher Scientific) was used. 10X DNase I buffer was added to the RNA sample along with the rDNase I, and then incubation followed at 37°C for 30 minutes. EDTA (2.4mM) was added and the mix was placed in a water bath at 75℃ for 5min in order to inactivate the rDNase I.

2.2.9 cDNA preparation from RNA samples

1µg of RNA was used together with 1ul of random hexamer primers to make up a volume of 12µl, which was then incubated for 10 min at 65°C. The samples were placed on ice and 2ul 10 x RT buffer (Invitrogen), 1ul dNTP mix (1mM final concentration), 0.2 to 2µl of RT enzyme (10U/µl) and H20 to a total volume of 20ul were added and mixed. The samples were placed in the PCR machine

programmed as follows: 42°C for one hour and 70°C for 5 min. The samples were then stored in -20°C.

2.2.10 Quantitative real-time PCR

The reaction volume of the mixture used for the qRT-PCR was 20µl. The mixture contained, SYBR Green I, AmpliTaq DNA Polymerase (Applied Biosystems, Foster City CA) and H20. 2µl of each 10µM primer was used in a 20µl reaction mix. A 1 in 5 dilution of cDNA took place. Once the samples were placed in the PCR machine, the following protocol was performed; 6min at 95°C, followed by 40 cycles of 15s at 95°C, 30s at 60 °C and 30s at 72°C. Negative controls, lacking cDNA, were included each time. In addition, data was collected at each extension phase. For each of the cDNA samples, measurements of the gene expression were obtained in triplicate, and the mean of these values was used for further analysis. The reference control gene used for gene expression normalization was actin.

2.3 Data Analysis for gene expression

MS Excel spreadsheet was used as a tool to normalize gene expression of genes based on raw Ct values obtained. Statistical significance was calculated using t-test. For the qPCR, the standard error of the calculated ratios of fold differences for gene expression data, errors of individual means were combined "in quadrature" as the final ratio was a combination of the error of the two different means of the control and stress treated samples. The stable reference gene used was Actin. Graphic representation of bHLH038 absolute gene expression in Arabidopsis obtained using eFP Browser (Winter D. et al, 2007).

2.4 Seed Sterilization and ½ MS agar media preparation

Arabidopsis thaliana seeds (Col-0, *bhlh038-2, bhlh038-4* and 4.3.6ox) were treated with 75% ethanol for 3 min. the seeds were washed with 75% ethanol and 0.1 Tween 20% for 1 min three more times before placed to be grown vertically on nutrient-agar media in 10cm square Petri dish plates. The medium comprised: 2.25g Murashige and Skoog salts and 0.9% Agar. To minimize evaporation but still permit gas exchange plates were wrapped with one layer of bandage tape (Micropore, 3M Company, St Paul MN). For osmotic stress two types of plate treatments were used, one involved ½ MS agar supplemented with NaCl (75mM) and the other supplemented with (200mM) Mannitol. The positive control was ½ MS agar medium. For each experiment, a minimum of 4 plates with 10-12 seedlings each was prepared.

Once the seeds were plated on the media, they were placed in a growth room under constant growth conditions (23℃ +/- 1 ℃, ~120umol $m^{-2}s^{-1}$). Root Growth was recorded every other day starting from day 7 with a meter rule. The developmental stage of the seedlings on the plate was recorded based on Boyes et al (2002) protocol, using a magnifying glass. Repeated measures ANOVA was conducted using SPSS (version 21.0;IBM Corp., Armonk, NY, USA) in order to determine significance in average root growth and the average time to reach a developmental stage between different genotypes.

2.5 Selection of BASTA resistant Arabidopsis transformants

In order to select an overexpressing line of bHLH038, six lines of bHLH038 overexpressor seedlings obtained from Subramaniam, were selected on BASTA medium. When the *bar* gene is successfully transferred to embryonic cultures of *Arabidopsis thaliana* by particle bombardment transformants are selected on BASTA medium and they survive. The transgenic plants of *Arabidopsis thaliana* from six transformed sublines were analysed for continued tolerance to BASTA and one of these lines was selected for further research. A stock solution of BASTA at 50mg/ml was prepared and filter sterilized. 500uL were added to 500mL-cooled ½ MS medium just before pouring plates in sterile tissue culture hood. Overexpressor seeds that were previously stratified for 3 days and sterilized were plated onto ½ MS plates supplemented with BASTA in order to identify the successful overexpressing lines of bHLH038.

2.6 Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence parameters were measured using a Fluorimager chlorophyll fluorescent imaging system (Technologica). The protocol run, involved dark adaptation for 20 minutes, 20 sec in darkness followed by a saturating light pulse at 4000umol $m^{-2}s^{-1}$). The calculation and imaging of the parameter Fv/Fm (Baker, 2008) were carried out automatically by the Fluorimager's software in order to examine the osmotic stress applied to plants via 300mM Mannitol (Fluorimager, Barbagallo et al., 2003)

2.7 Complementation

2.7.1 Phusion PCR for gene of interest with native promoter amplification

In order to amplify the gene of interest with the native promoter in wild type Arabidopsis, Phusion Polymerase Chain Reaction was carried out. 0.2ul of Phusion High Fidelity DNA polymerase (ThermoFisher Scientific, Loughborough, UK) of 1U/50ul concentration was used for the PCR; Furthermore 4ul 5x Phusion HF Buffer of 1X concentration was used. A mix of 10mM (dNTPs; #R0191, ThermoFisher Scientific) deoxyribonucleotide triphosphates were used whose final concentration per reaction was 200uM. 1ul of 5' –end primer and 3' –end primer (0.5uM each) was added to the final reaction mix, along with DNA and sterile water making the reaction mixture up to 20ul (Table 1).

The PCR involved the following conditions; 3 minutes at 95℃ for the initial denaturation, then 35 cycles of denaturation at 98℃ for 5-10 seconds, 30 seconds at 52-60℃ (depending on the annealing temperature of each primer designed) for primer annealing and 2.5 minutes at 72℃ for extension; final extension lasted 10 minutes at 72℃.

2.7.2 Extraction of PCR product

Gel electrophoresis was carried out in order to make the PCR product visible and ready for extraction. Once the gel was run, it was moved to an open UV box, with a clean, sterile razor blade the desired DNA fragment was sliced from the gel. It was then placed on a labeled microfuge tube, and the PCR product was extracted using a QIA Quick Gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.7.3 Digestion of vector DNA

The vector chosen for the cloning was pGreenII, an Agrobacterium binary vector with a gene that confers kanamycin - resistance (Hellens et al., 2000). 10X restriction enzyme buffer (Tango) was mixed with 4ul vector DNA (pGReenII) in -20°C and 0.5-2ul of Restriction enzyme SmaI. Moreover, in a final volume of 20ul, the restriction enzyme stored in -20°C was added last. Gentle mixing took place by tapping the tube or pipetting the solution up and down for a few seconds. Then it was incubated at 30°C for 1-16 hours.

2.7.4 Purification of digested vector DNA

Agarose gel electrophoresis (see 2.2.6) was used in order to purify the entire sample and eventually separate the digested vector from the small DNA fragment that was removed by the digestion. The process of extraction of the vector DNA from the agarose gel was completed through a commercial gel extraction kit (QIAquick Gel Extraction kit, QIAGEN)

2.7.5 Ligation of DNA fragments with blunt ends

For the standard ligation reaction of DNA fragment with 2-4 bases blunt ends the following protocol was used. Approximately 0.1- 1ug of digested vector DNA was used. The digested insert to vector molar ratio was 3:1. The reaction mix contained 5X ligase Reaction buffer, 1 unit T4 DNA ligase and H_2O to make up a total volume of 20ul. T4 DNA ligase is supplied by most manufacturers in concentrated solutions (e.g. 400,000 units/ ml from NEB). The ligase enzyme was added last to the reaction mix. Pipetting the solution up and down took place. The reaction mixture was then incubated at 16°C for 2h to overnight.

2.7.6 Competent cells Standard Transformation Protocol

Competent cells (provided by Subramaniam) were removed from -70°C and placed on ice for 5 minutes or until just thawed. Flicking the tube gently mixed the thawed Competent Cells, and 100ul was transferred to each chilled Eppendorf tube. 1- 50ng of DNA per 100ul of Competent cells was added. Pipetting up and down while dispensing mixed the solution. The tubes were immediately returned to ice for 15 minutes. Cells were heat-shocked for 30 seconds in a water bath at exactly 42°C. The tubes were then immediately placed on ice for 5 minutes. 900ul of room temperature LB broth was added to each transformation reaction, and incubated for 60 minutes at 37°C with shaking (approximately 225rpm). For the best transformation efficiency, the tubes were laid on their sides and taped to the platform. Cells were then spun down for exactly 15 seconds at 13,000rpm. 900ul of supernatant were removed from the tube and discarded. The cells were then resuspended in remaining liquid by pipetting up and down very gently. Cells were transferred into kanamycin plates, which were left overnight at 37°C, in order for colonies to grow.

RESULTS

Phenotyping mutant lines of hub gene BHLH038 under osmotic and salt stress.

3.1.1 Introduction

BHLH038 (BASIC HELIX-LOOP-HELIX 38) is involved in maintaining iron homeostasis and uptake (Yuan et al., 2008). The two closely related bHLH factors BHLH038 and BHLH039 (BASIC HELIX-LOOP-HELIX 39) have been suggested to act in concert with FIT (FER-LIKE IRON DEFICIENCY- INDUCED) transcription factor to allow the expression of FRO2 (FERRIC REDUCTION OXIDASE 2) and IRT1 (IRON-REGULATED TRANSPORTER 1). The overexpression of FIT with either AtBHLH038 or AtBHLH039 in plants lead to the constitutive expression of FRO2 and IRT1.This resulted in tolerance to iron deficiency as well as a greater amount of iron accumulated in the shoots of *Arabidopsis* plants (Yuan et al., 2008). Iron deficiency is a major constraint for crop yield and quality, to cope with Fe deficiency, plants have developed sophisticated mechanisms to keep cellular Fe homeostasis via various physiological, morphological, metabolic and gene expression changes to facilitate the availability of Fe (Li and Lan, 2017). There is increasing evidence showing that phytohormones play vital roles in the Fe deficiency response of plants. Expression of FRO2 and IRT1, mentioned above, is positively or negatively affected by several hormones, such as auxin, ethylene, cytokinins, jasmonic acid among other signalling molecules which have been extensively explored in the involvement of Fe deficiency response or other abiotic stress responses by hormone precursors or inhibitors or hormone related mutants (Li and Lan, 2017). Studies have shown that genes involved in response mechanisms in an abiotic stress such as Fe deficiency have led to the identification of some novel regulators, such as FIT, MYB72, MYB10 which are also involved in mechanisms of other abiotic stresses (Abe et al., 1997). From networks perspective, the regulatory interactions between TFs (Transcription factors) and their TGs (Target genes) involved in various abiotic stresses can be explored, and presented as directed, bipartite graph depicting transcriptional activation or repression. In general such transcriptional stress regulatory networks in plants can be used for predicting global and stress-specific transcriptional regulators (Barah et al, 2016).

Moreover, Bechtold et al (2016) suggested that AGL22 (AGAMOUS-LIKE 22) regulates a transcriptional network during drought stress (Fig.2), linking changes in primary metabolism with the induction of plant stress responses. Based on that study, BHLH038 gene was involved in a gene regulatory network, where AGL22 was the key hub gene. However BHLH038 and AGL22 appeared to share the regulation of a great number of genes during drought stress (Fig. 2). The fact that BHLH038 either activates or represses the transcription of many drought response genes also regulated by AGL22, implies that BHLH038 could play a significant role as a key regulatory gene in drought response of *Arabidopsis thaliana* plants. Also, BHLH038 seemed to regulate itself and was the only TF gene in the GRN generated by Bechtold et al. (2016) that was not regulated by AGL22. However it is still unknown whether BHLH038 regulates AGL22 in drought response. Thus the above hypothesis still remains to be answered. Whether BHLH038 plays a significant role in drought response was one of the main aims and objectives in this study and was investigated by phenotyping two independent mutant lines *bhlh038-2* and *bhlh038-4 Arabidopsis thaliana* against Col-0 plants under osmotic and salt stress.

This chapter depicts the growth and developmental phenotyping of the two-*bhlh038* mutants lines under low osmotic potential.

The average time taken for seedlings to achieve a specific developmental stage as developed by Boyes et al (2001) was used as a measure of the development of seedlings, while average root length was used as a measure of growth. Both general growth and development parameters were used to determine the impact of osmotic stress on plant performance. The study, observation and quantification of plant root growth and root systems has been and remains an important area of research in all disciplines of plant science (Judd, Jackson and Fonteno, 2015). Root growth is a critical component in overall plant performance during production in containers and thus it is important to understand the factors that influence and/ or possible enhance it (Judd, Jackson and Fonteno, 2015).

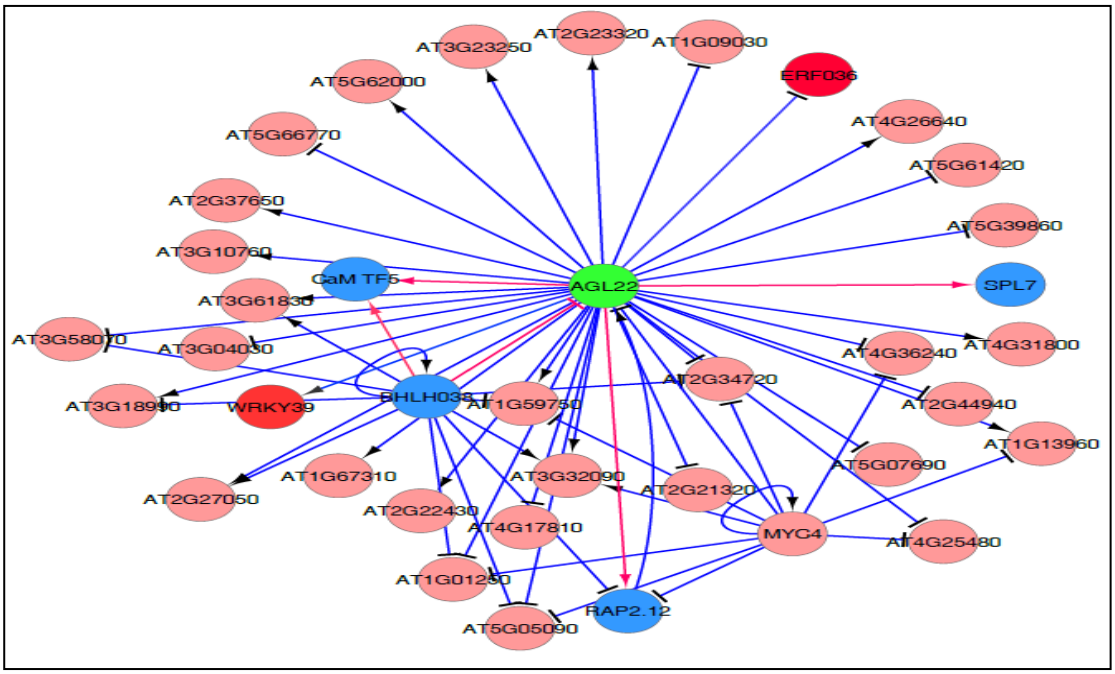

Figure 2: GRN of early and late drought response time series data (Bechtold et al. 2016)

The central hub gene of the GRN is AGL22 (Fig.2). Transcription factor genes in red were upregulated during drought stress; the ones in green were downregulated in drought stress; genes highlighted in blue were not regulated by the central hub gene (AGL22) at all. Interaction of BHLH038 TF is also revealed.

3.1.2 Phenotyping of knockouts of BHLH038

3.1.2.1 Average time of Arabidopsis seedlings to reach a Developmental Stage under osmotic stress

In order to investigate the role of *BHLH038* in osmotic stress response, two knockouts of *BHLH038* (*bhlh038-2* and *bhlh038-4*) were subjected to low osmotic potential achieved via NaCl and Mannitol treatments. As mentioned before genotyping of the two independent mutant lines (*bhlh038-4* and *bhlh038-2*) was not conducted in this study, the lines were previously genotyped by Dr. Subramaniam

(2016). Col-0 wild type seedlings were used as a control for both knockouts. Seedlings were grown in plates containing ½ MS agar medium supplemented with either NaCl (75mM) or Mannitol (200mM). The control treatment was represented by ½ MS agar medium without supplementation. The time taken for each seedling in the plate to reach a specific developmental stage was recorded, based on Boyes et al., (2001; Table 2). Figure 3 represents the average time taken for all the lines to reach these developmental stages under control conditions.

Table 2: Developmental Stage measurements performed during Plate-Based Phenotypic Analysis (Boyes et al. 2011)

First – Phase Measurements	
Measurement/ Observation	**Growth Stage Defined**
Radicle emergence reached/ passed	Stage 0.5
Hypocotyl and cotyledons visible	Stage 0.7
Cotyledons fully opened	Stage 1.0
Number of rosette leaves > 1mm	Principal growth stage 1

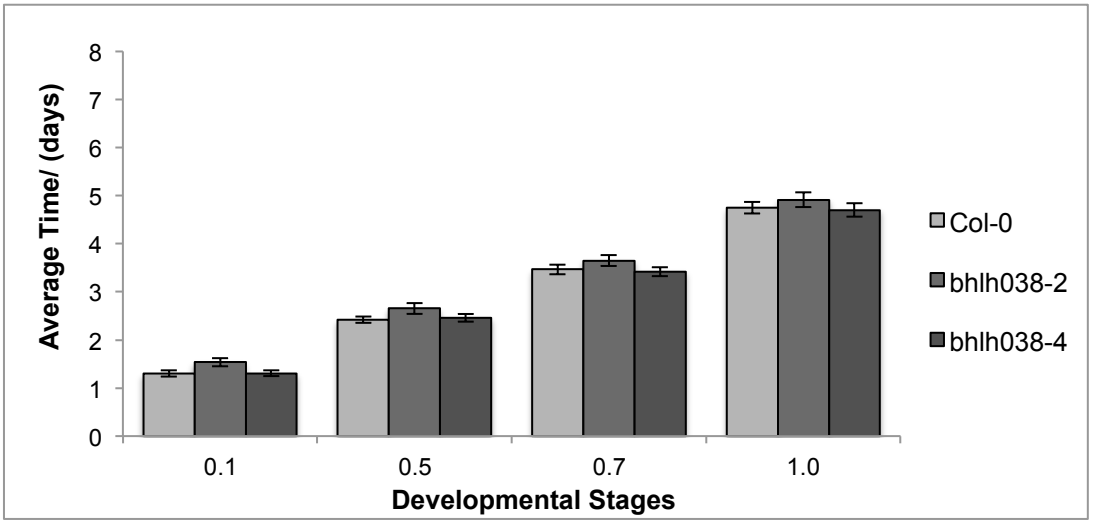

Figure 3: Average time taken to reach early developmental stages for knockouts and Col-0, grown in control conditions (½ MS media).

Data represents means (n≥ 100) ± SE. One-way ANOVA test followed by post-hoc Tukey test resulted in a p-value >0.05, indicated no statistically significant difference between the three genotypes in plant development under normal conditions.

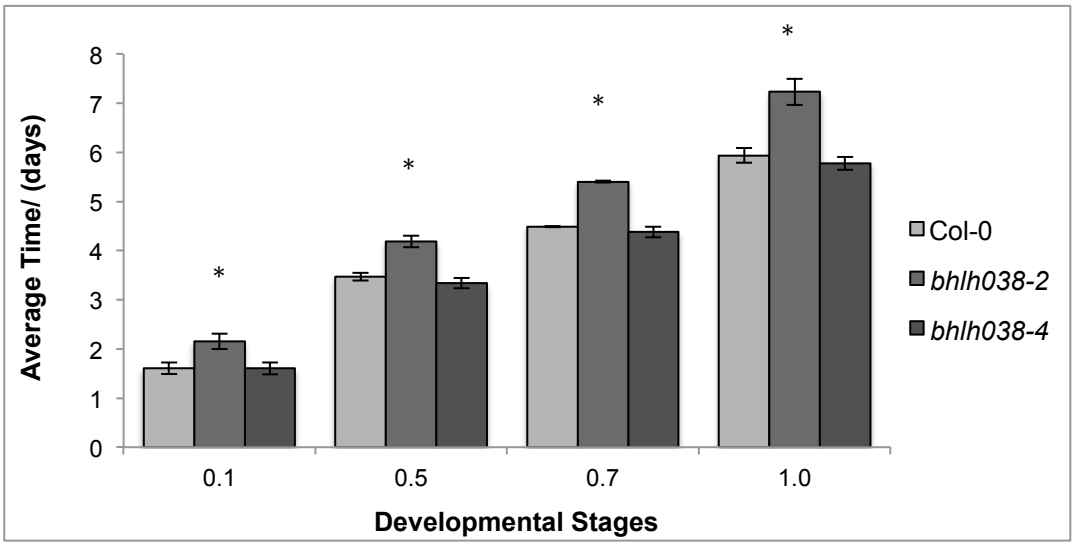

Figure 4: Growth Stage progression for Wild Type (Col-0) and two knockouts when exposed to osmotic stress (200mM Mannitol).

Data represents means (n≥ 100) ± SE. One-way ANOVA test followed by post-hoc Tukey resulted in a p-value <0.05 between *bhlh038-2* and Col-0 (see *), indicating a statistically significant difference in plant development performance under osmotic stress.

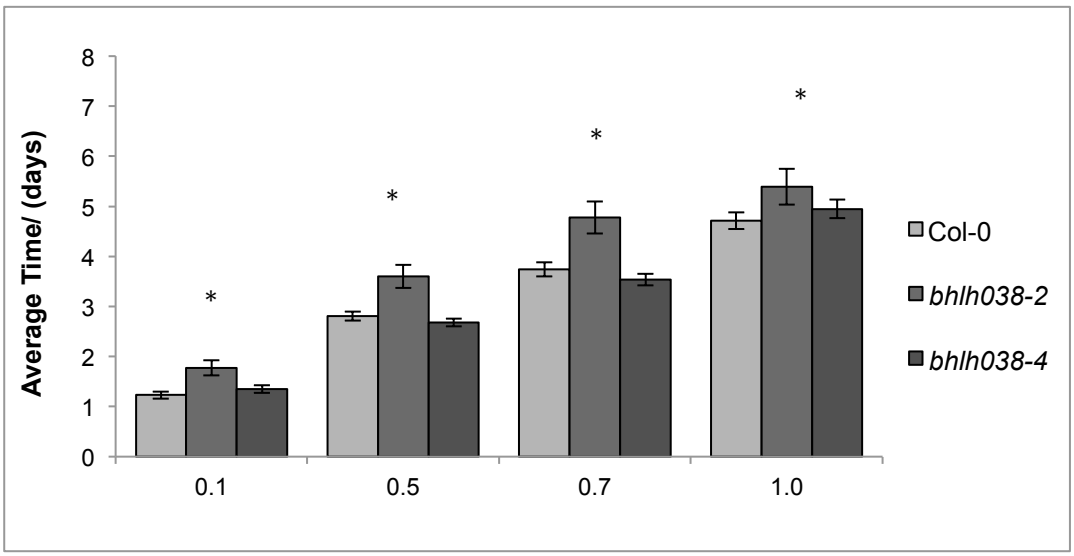

Figure 5: Growth Stage progression for Wild Type (Col-0) and two knockouts when exposed to salt stress (75mM NaCl).

Data represents means (n≥ 100) ± SE. One-way ANOVA test followed by post-hoc Tukey resulted in a p-value <0.05 between *bhlh038-2* and Col-0, indicated a statistically significant difference in plant development performance under salt stress (see *).

The *bhlh038-2* mutant showed a significantly slower development than the Col-0 wild-type seedlings under salt stress (Fig.5). The growth stages, which showed most

significant difference, were 0.7 and 1.0 (see Table 2, Boyes et al, 2001). No significant difference in growth stage development was observed between the *bhlh038-4* mutant and the Col-0 (Fig.5).
Similarly, a significantly slower early stage development was observed in the *bhlh038-2* mutant, compared to Col-0 wild type seedlings under 200mM mannitol treatment (Fig.4). Overall, the two independent mutant lines reveal a significant difference in early stages development i.e. 0.1 and 0.5 developmental stages, under both osmotic and salt stress. Mutant *bhlh038-4* seems to behave identical to Col-0 (Fig.4 and 5).
Apart from the phenotyping of the two independent lines of bHLH038, screening took place in order to identify T-DNA insertions within the bHLH039 gene, a closely related gene to bHLH038, using the appropriate primers (Table 1, Materials and Methods). Unfortunately, no mutant alleles with T-DNA insertions inside bHLH039 were present (Appendix, Fig.2). As a result no phenotyping of bHLH039 took place in plate-based assays.

To further characterize the function of *BHLH038* in abiotic stress resistance, a constitutive overexpressor line was selected after BASTA treatment (see Appendix, Fig.1). The early stage developmental performance of overexpressor 4.3.6ox was assessed in plates along with *bhlh038-2* mutant and Col-0. No significant difference in development of knockout seedlings in comparison to Col-0 seedlings was observed in control ½ MS media (Fig.6). Overall, *bhlh038-2* was more sensitive to both stress treatments (Fig 7 and 8), while 4.3.6ox seemed to be significantly tolerant to salt stress (100mM NaCl) (Fig.8).

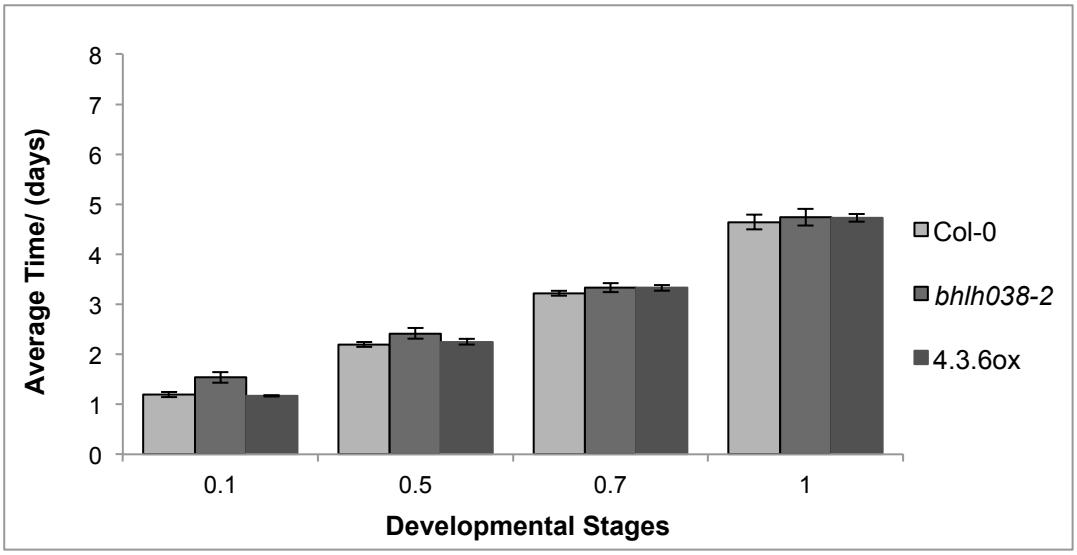

Figure 6: Growth Stage progression for Wild Type (Col-0), *bhlh038-2* mutant and a BHLH038 overexpressor (4.3.6ox) in control conditions ($^1/_2$MS media).

Data represents means (n≥ 100) ± SE. One-way ANOVA test followed by post-hoc Tukey test resulted in a p-value >0.05 between *bhlh038-2*, Col-0 and 4.3.6ox meaning that there was no statistically significant difference in plant development performance under control conditions.

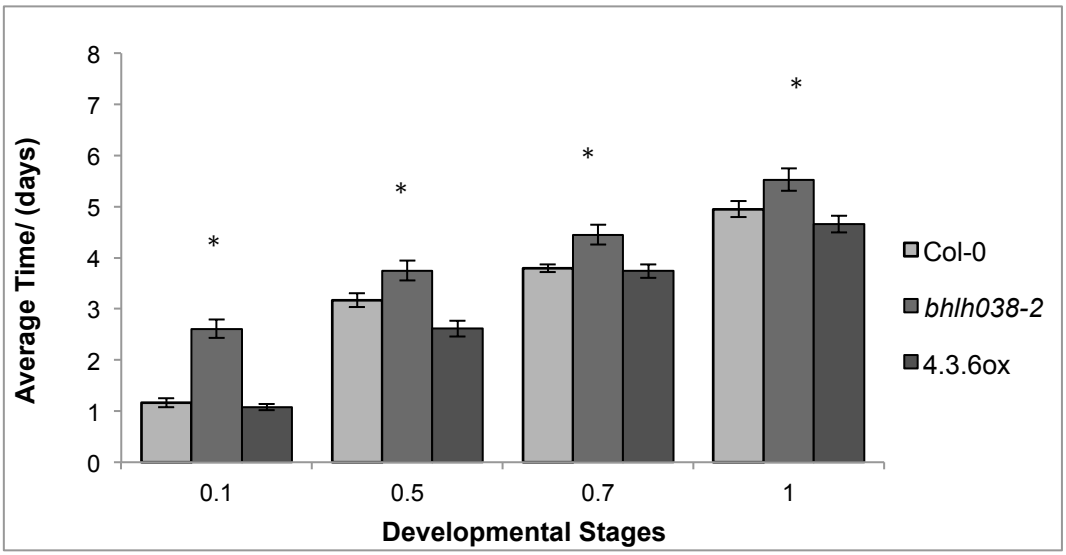

Figure 7: Growth Stage progression for Wild Type (Col-0), *bhlh038-2* mutant and 4.3.6ox when exposed to osmotic stress (200mM Mannitol).

Data represents means (n≥ 100) ± SE. One-way ANOVA test followed by post-hoc Tukey presented a p-value <0.05 between *bhlh038-2* and Col-0 (see *), indicated a statistically significant difference in plant development performance under osmotic stress.

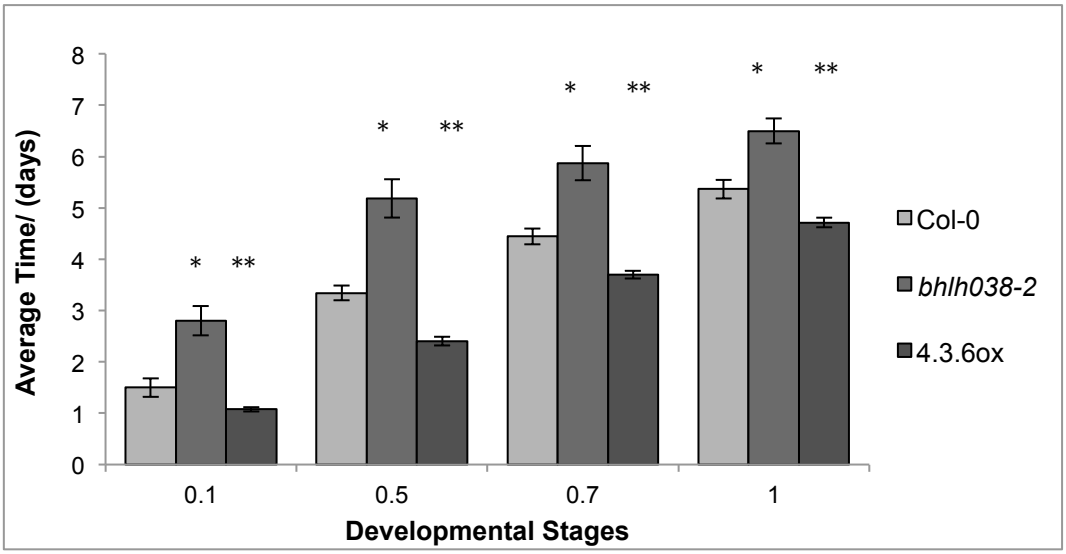

Figure 8: Growth Stage progression for Wild Type (Col-0), bhlh038-2 mutant and 4.3.6ox under salt stress (100mM NaCl).

Data represents means (n≥ 100) ± SE. One-way ANOVA test followed by post-hoc Tukey presented a p-value <0.05 between *bhlh038-2*, Col-0 (see *) and 4.3.6ox (see **) meaning that there was statistically significant difference in plant development performance under salt stress**.**

3.1.2.2 Average root length of *Arabidopsis* seedlings under osmotic stress

The same osmotic stress treatments of 75mM NaCl and 200mM mannitol were used to examine the effects of osmotic stress on primary root growth of the two mutant lines and 4.3.6ox line.
Under osmotic stress, we observed a reduction in primary root length, which indicated a reduction in growth (Fig 10 and 11).

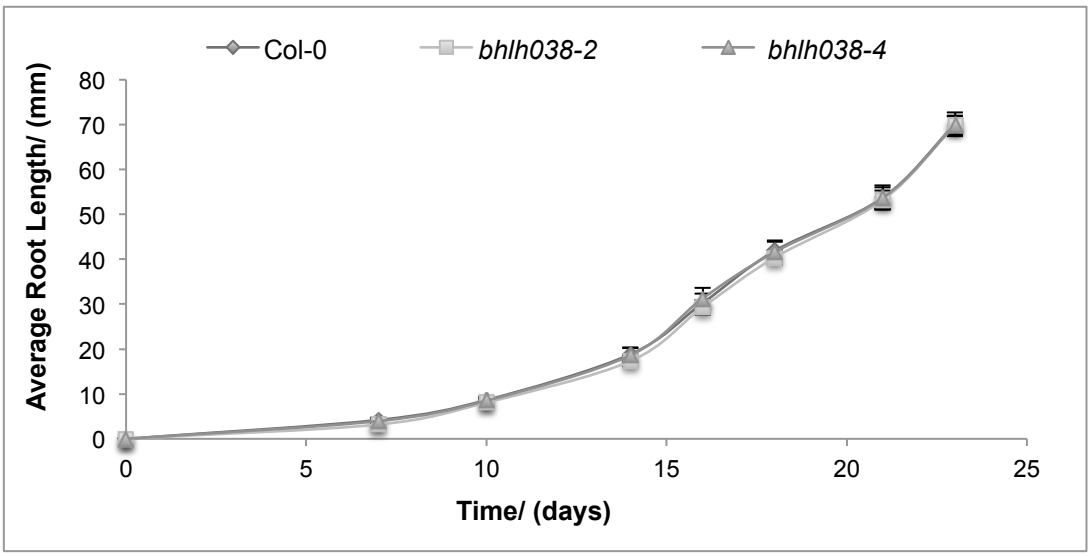

Figure 9: Primary root growth of seedlings of the three different genotypes grown in control (1/2 MS media) for 23 days.

Data represents means (n≥ 100)

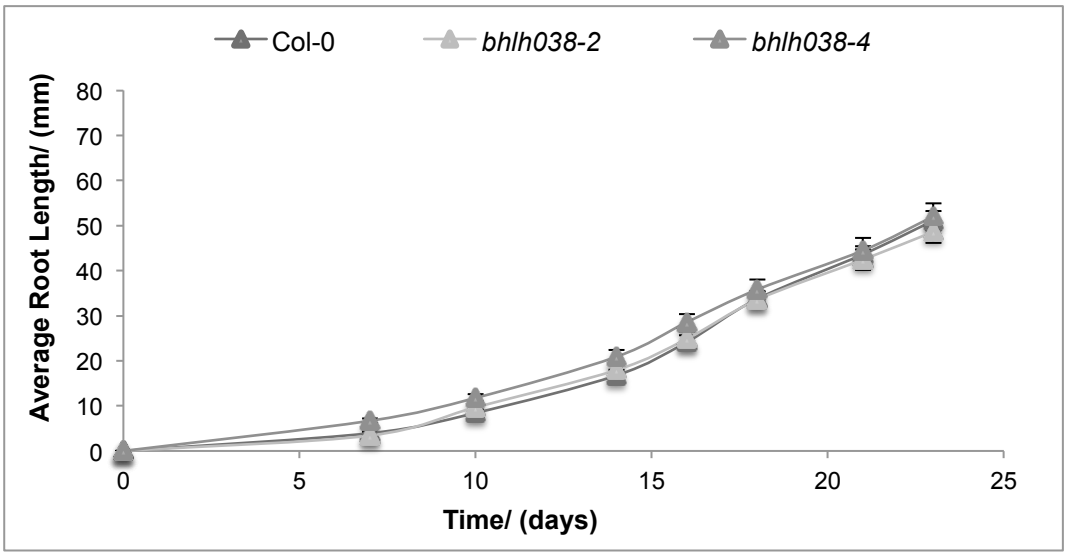

Figure 10: Salt response of mutants of Arabidopsis thaliana seedlings during primary root growth. Osmotic stress (75mM NaCl).

Data represents means (n≥ 100).

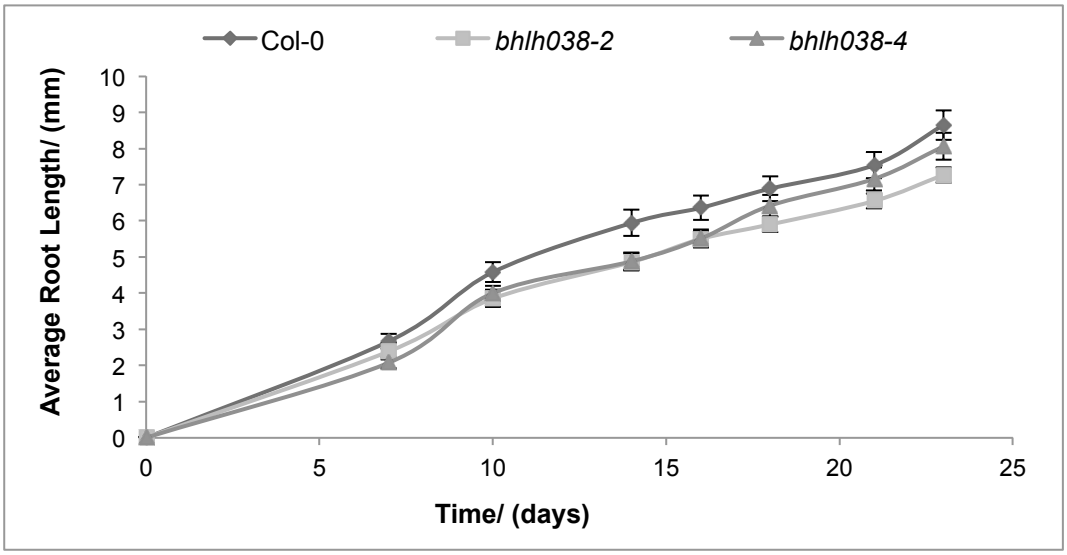

Figure 11: Osmotic response of mutants of *Arabidopsis thaliana* seedlings during primary root growth.

Osmotic stress (200mM Mannitol). Repeated measures ANOVA p-valye <0.05 indicating a statistically significant difference between Col-0 and *bhlh038-2* mutant in root growth. Data represents means (n≥ 100).

Root elongation of seedlings grown in control [½ MS media] showed no significant difference (Fig.9). Overall, osmotic stress had an impact on root growth of seedlings of all genotypes, since the average root growth was reduced significantly compared to control [½ MS media](Fig.9 and 11). Moreover the average root elongation of seedlings of the three genotypes

decreased by ~87% under osmotic stress on day 23 (Fig. 11), whilst the average root length of seedlings of each genotype was reduced by ~25-30% in salt stress compared to control [½ MS media] on day 23 (Fig.10). The three genotypes appeared to respond in a similar way in salt stress and control. Under osmotic stress (200mM Mannitol), the mutant *bhlh038-2* showed significantly greater sensitivity regarding root elongation compared to WT, on day 23 the average root length of *bhlh038-2* mutants was 13% lower than Col-0 seedlings.
The two independent mutant lines behaved differently under osmotic stress (200mM Mannitol), since *bhlh038-4* showed similar sensitivity to Mannitol as WT, while *bhlh038-2* showed much greater sensitivity (Fig.11). On the other hand, in salt stress, the two mutant lines respond in a similar way, indicating no significant difference compared to WT (Fig.10).

When 4.3.6ox seedlings were plated on the MS medium, the treatments were modified. The bHLH038 overexpressing plants showed obvious root growth advantages compared to wild type (WT) and *bhlh038-2* seedlings grown in MS medium supplemented with 100mM NaCl, a higher concentration than the one previously used to assess the phenotype of the two knockouts (Fig.13). Statistical analysis revealed a significantly enhanced root growth of the 4.3.6ox plants compared to WT by 40.5% in salt stress on day 23 (Fig.13). In addition the *bhlh038-2* knockout showed significant sensitivity under salt stress compared to WT (Fig.13), displayed by 31.5% decrease in average root length on day 23. On the other hand, the 4.3.6ox seemed to respond similarly to wild type plants under osmotic stress (200mM Mannitol), whilst once again statistical analysis indicated significant inhibition of root growth in *bhlh038-2* mutant compared to wild type (WT) seedlings, showing a decrease of ~17% in average root growth of *bhlh038-2* mutants on day 23 (Fig.14).

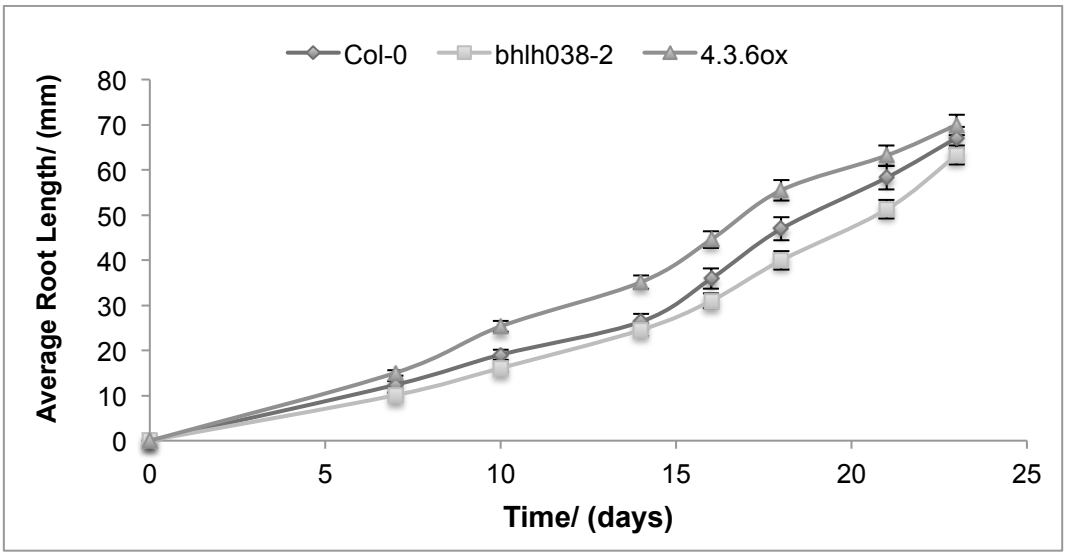

Figure 12: Primary root growth of 4.3.6ox and *bhlh038-2 Arabidopsis thaliana* seedlings grown in control (1/2 MS media) for 23 days.

Data represents means (n≥ 100).

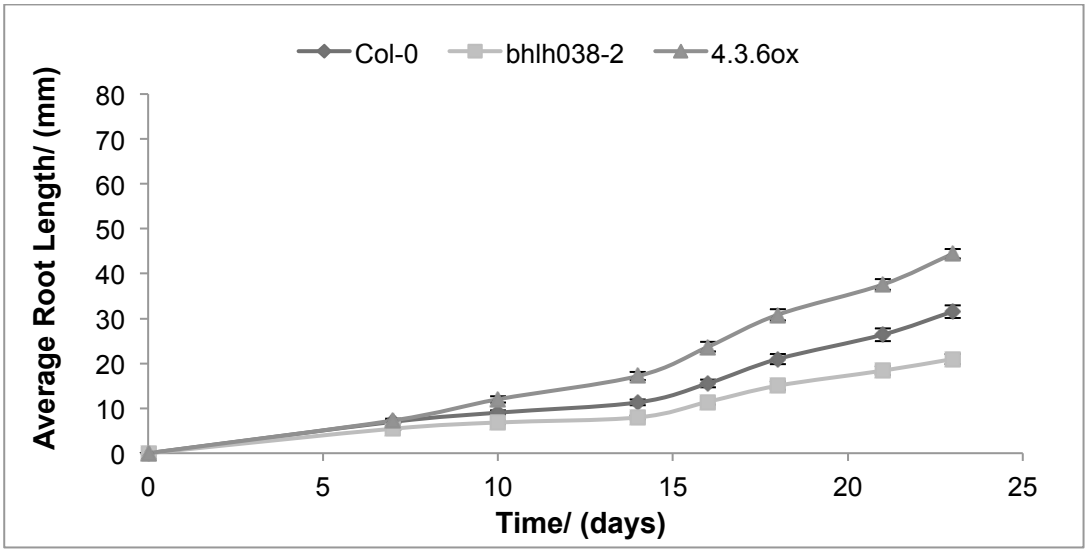

Figure 13: Salt response of mutants of Arabidopsis thaliana seedlings during primary root growth.

Osmotic effects (100mM NaCl supplemented media). Repeated measures ANOVA p-value <0.05 indicates significant difference between *bhlh038-2* and Col-0 and 4.3.6ox. Data represents means (n≥ 100), error bars are standard errors +/- SE.

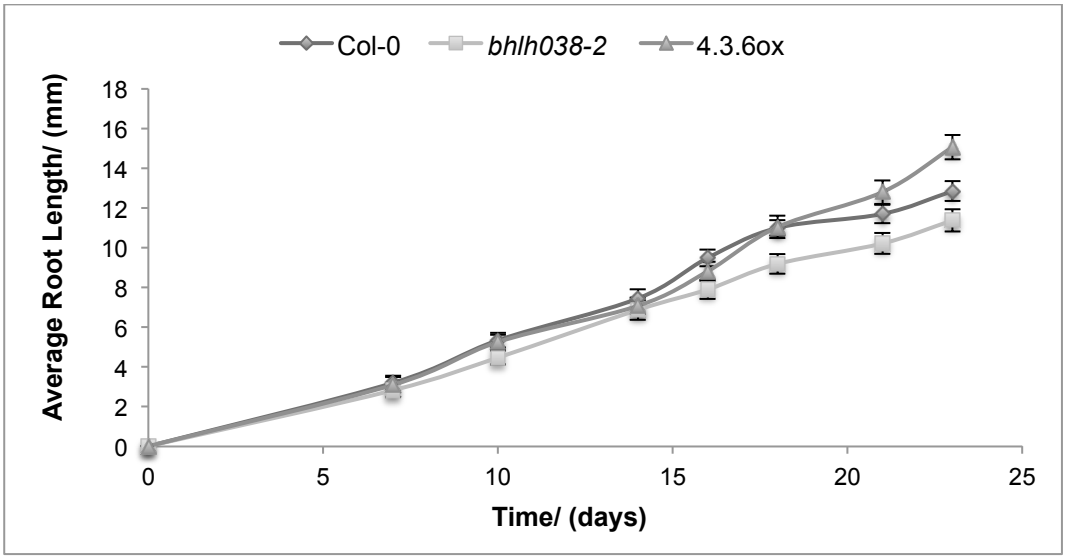

Figure 14: Primary root growth in osmotic stress (200mM Mannitol) for 23 days.

Repeated measures ANOVA p-value <0.05, indicating a statistically significant difference between Col-0 and *bhlh038-2* mutant in root growth. Data represents means (n≥ 100).

In conclusion, the results suggested that the T-DNA insertion in *bhlh038-2* mutants specifically enhances the sensitivity of seedling development and root growth to osmotic and salt stress. Overall the results imply that BHLH038 may act as a key regulator in drought stress response, since its presence in 4.3.6ox plants seems to enhance the tolerance to salt stress. Although salt and osmotic stress induced by mannitol treatment confer the same low water potential stress to the roots, seedling

development and productivity of crops, the signaling pathways that give rise to a response has been shown to differ (Jian-Kang Zhu, 2002). Furthermore, the levels to which plants are stressed by an exogenous supply of salt or mannitol varies significantly with the concentrations used (Shavrukov, 2012). On the other hand, it is unknown why *bhlh038-4* mutants did not display a phenotype under stress. Taking into account the fact that genotyping did not take place before phenotyping the two mutant lines, thus not knowing whether the mutant seedlings are homozygous or not it is difficult to draw any conclusions. Further research needs to be conducted in order to confirm that a single T-DNA insertion in found in *bhlh038-4* mutants; It is still unclear whether phenotypes observed in the mutant, are exclusively due to knockout BHLH038. Further investigation via the genotyping of mutants as well as the complementation of the mutants would be beneficial. Finally, it is clear that any significance in the growth and development of plants is due to differences in osmotic stress and not because plants of different genotypes develop differently anyway.

3.2 Phenotyping of mutant lines of key hub gene BHLH038 under progressive drought

3.2.1 Introduction

The approach of osmotica has been beneficial in many studies regarding the reduction of water potential in plant growth media. The water potential can be controlled more precisely and reproducibly when this approach is used, while also a larger number of treatments can be performed quickly. In comparison to progressive soil drying, osmotic stress seems to have its own set of potential problems.

In most cases, as the soil water content decreases water is withdrawn from both the cell wall and the protoplast resulting in cytorrhysis, a process where both the cell wall and the protoplast shrink (Verslues et al., 2006). However, this contrasts with the stress response to low molecular weight solutes such as mannitol that are often used to lower the water potential (Verslues et al., 2006). In this case the solute freely penetrates the pores of the cell wall and causes plasmolysis; a loss of water from and decrease in volume of the protoplast while the volume of the cell wall remains unchanged (Verslues, 2006). Plasmolysis is not part of the typical soil drying response and may cause cellular damage that is perceived and responded to differently from water loss cause by soil drying (Verslues et al., 2006).

On contrary we need to take into account that plate-based assays facilitate *in vitro* study and analysis. In nature, roots are plant organs that typically lie below the soil and grow in darkness (Xu et al., 2013). A plant raised in agar culture has its roots illuminated and exposed to nutrients in the medium (Xu et al., 2013). However, light affects root growth and response to stress (Xu et al., 2013). Thus such unnatural treatments to roots can cause substantial differences in terms of root growth and responses to environmental signals (Xu et al., 2013). Therefore, a soil experiment using small pots to assess the response of different genotypes to drought stress facilitated an *in vivo* investigation and analysis.

To study the response of Arabidopsi*s* to reduced soil water deficit drought, mutants and Col-0 plants were grown under well-watered conditions, and drought stress was applied by withholding water 5 weeks after sowing. This enabled the identification of genes whose expression was regulated by the gene of interest BHLH038, and thus the verification of BHLH038 to act as a key regulator of drought response. This was

achieved through the phenotyping of the two -*bhlh038* mutant lines under soil water deficit drought.

3.2.2 Water use under progressive drought stress.

A progressive slow soil dehydration experiment was conducted, starting at 95% relative soil water content (rSWC) and drying down to ~20% rSWC on five-week-old Col-0 (wild type), *bhlh038-2* and *bhlh038-4* plants. To determine the severity of the stress, daily measurements of relative soil water content (rSWC) were recorded (Fig.15).

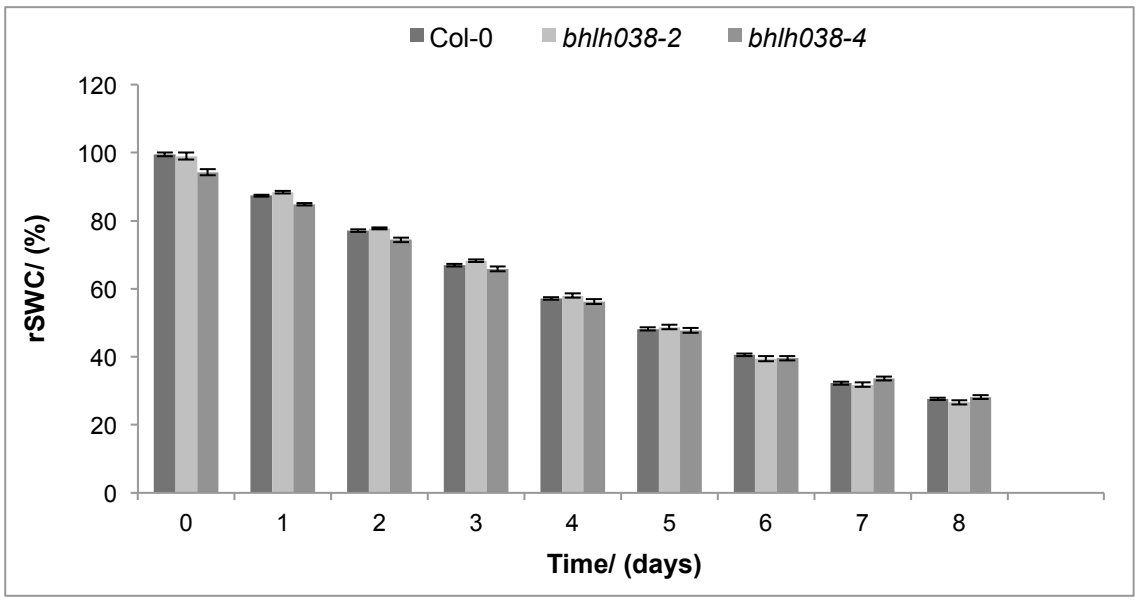

Figure 15: Measurement of relative soil water content (rSWC) in droughted Col-0 (wild type) and the two independent mutant lines five-week-old *Arabidopsis thaliana* plants.

All the values are mean of 15 seedlings (n=15), error bars are standard error ± SE.

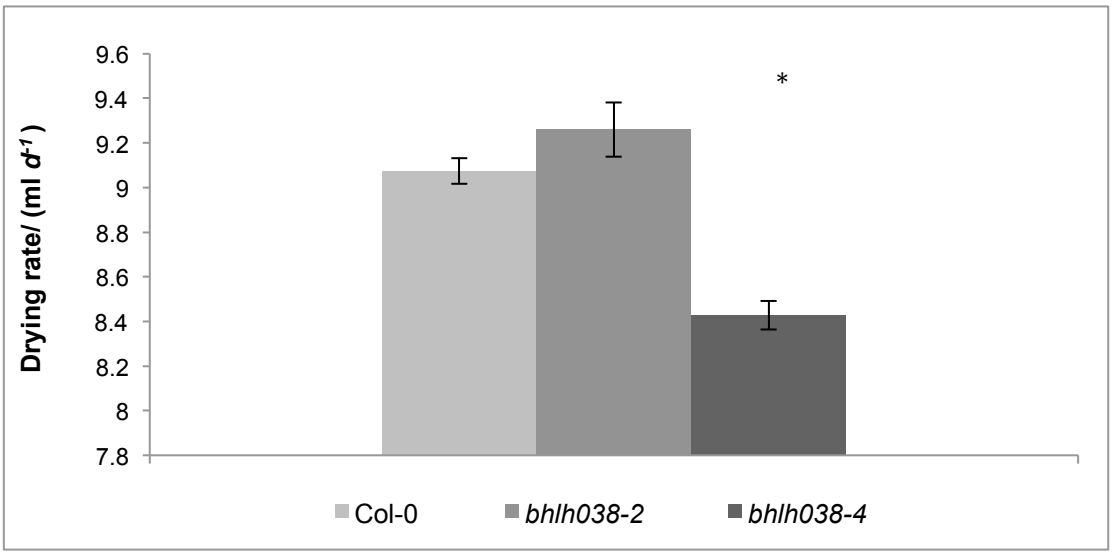

Figure 16: Drying rate for knockouts compared with Wild Type (Col-0) plants.

The values are mean of 15 seedlings; error bars represent standard error ± SE. Statistically significant difference is observed between *bhlh038-4* and Col-0 plants [see *], as well as between the two mutants (P< 0.05, Student's t test).

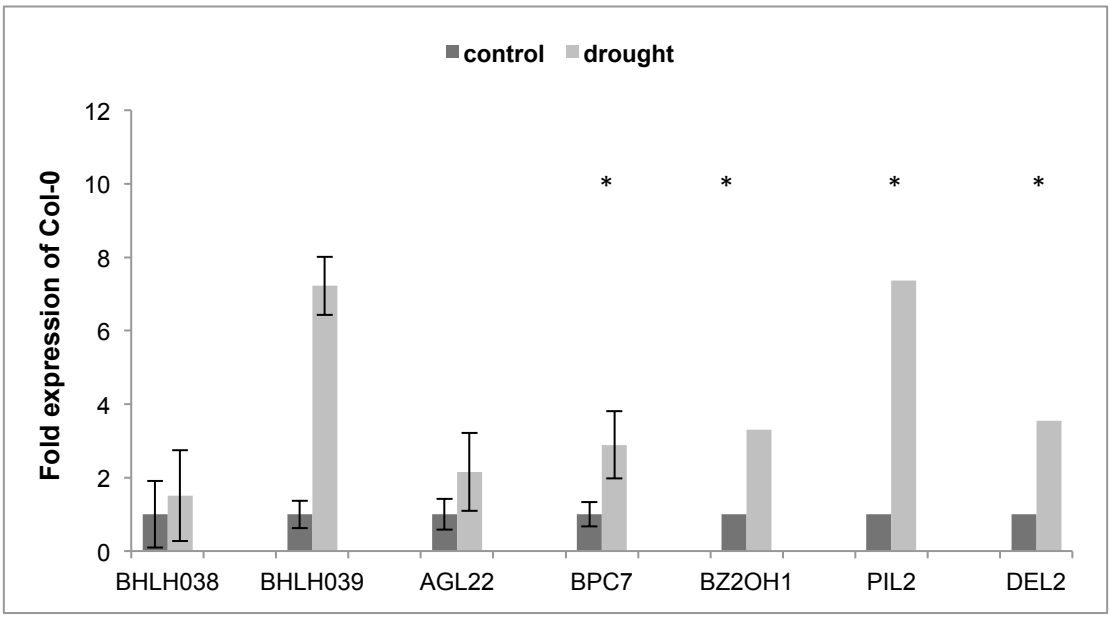

Figure 17: Relative expression of interacting genes in Col-0 Arabidopsis plants grown in control conditions or under drought stress.

Error bars represent standard error (n=6) ± SE. Student's t-test revealed significant difference (see *) >2.78 (df=4)

The two mutants had opposing drying rates compared to Col-0. The drying rate in *bhlh038-4* mutants was significantly slower, while *bhlh038-2* showed a slight increase in drying rate but not significant (Fig. 16).
In order to validate the role of BHLH038 in regulating TF genes involved in the AGL22-centered network interactions (Fig.2, Bechtold et al, 2016) gene expression of network genes were analyzed in both mutants subjected to drought stress. Network genes analyzed

were BPC7 (BASIC PENTACYSTEINE 7), BZ2OH1 (bZIP transcription factor family protein), PIL2 (Phytochrome interacting factor 3-like 2) and DEL2 (DP –E2F –like2). In addition BHLH039 (BASIC HELIX-LOOP-HELIX 39) known as close related bHLH gene to BHLH038 (BASIC HELIX-LOOP-HELIX 38) was also analyzed.

Significant difference in the expression levels of BPC7, BZ2OH1, PIL2 and DEL2 network genes was displayed in Col-0 plants when exposed to progressive soil drought stress compared to control conditions (Fig.17).

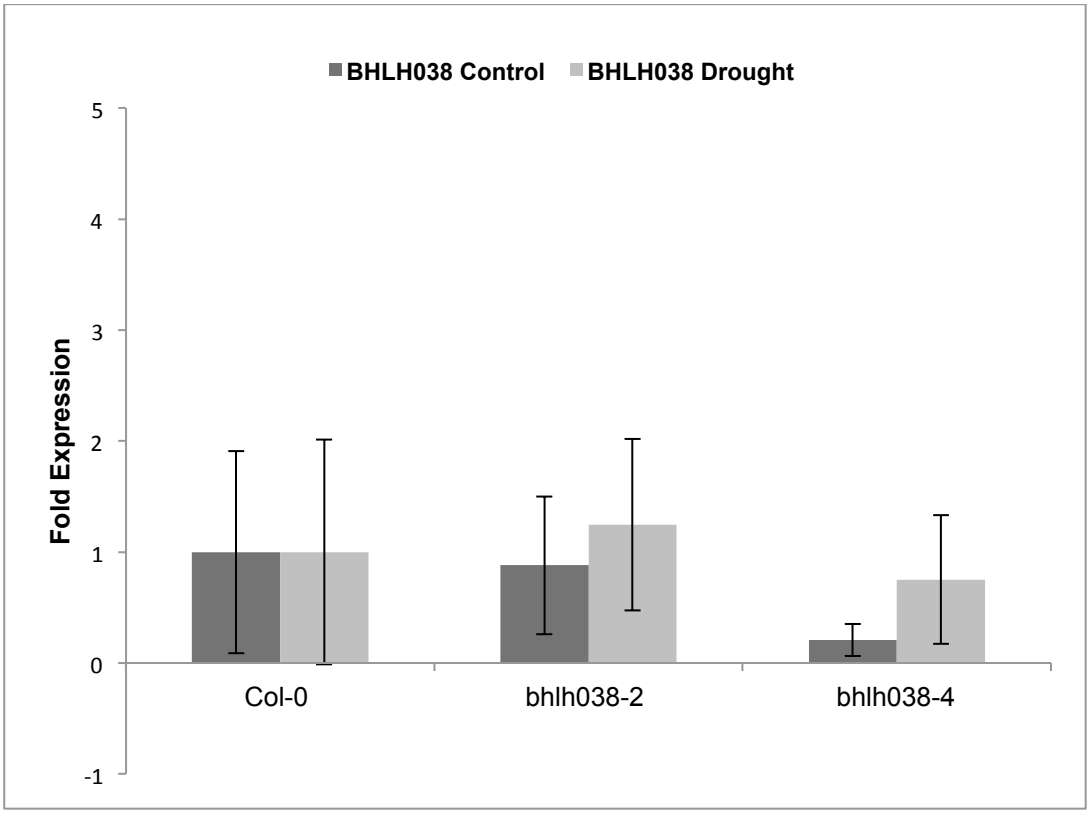

Figure 18: Relative expression levels of BHLH038 in two knockouts compared to Col-0 under drought (~20% rSWC) and control.

Gene expression was analyzed by qPCR. Error bars represent standard error ± SE (n=6). No significant difference observed, two-tails t-test <2.78 (df=4).

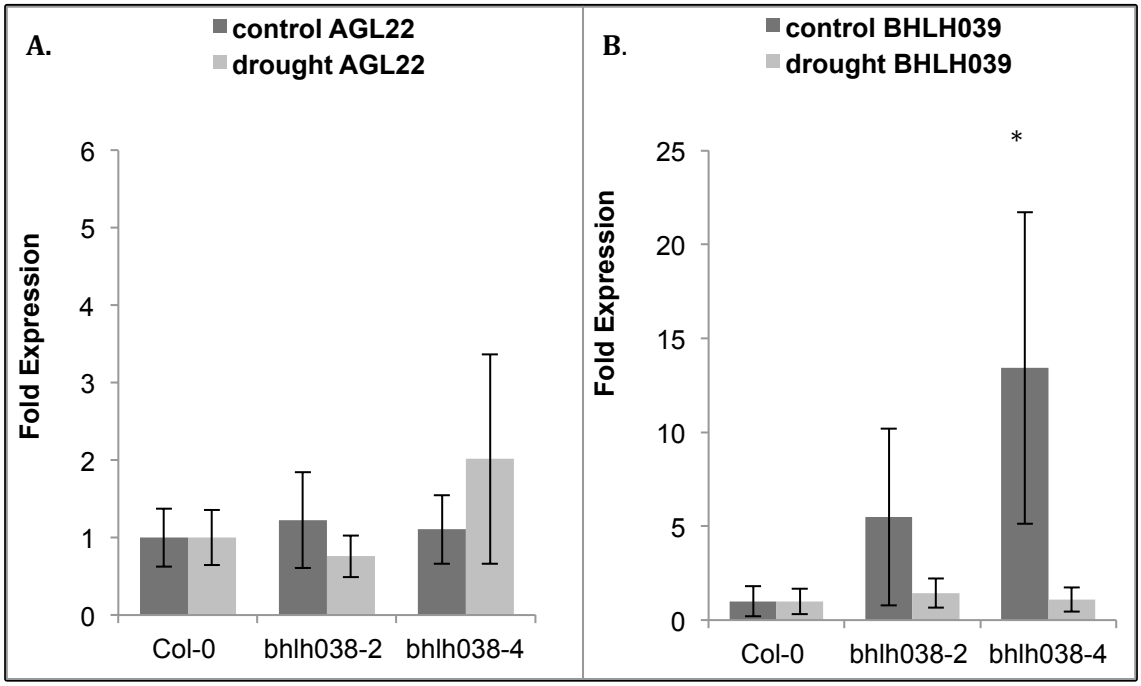

Figure 19: Relative expression levels in *bhlh038-2, bhlh038-4* mutants and Col-0 Arabidopsis plants.

(A) Relative expression levels of AGL22 in *bhlh038-2* and *bhlh038-4* mutants compared to Col-0 in drought (dark bars) and control (light bars) conditions. (B) Relative expression levels of BHLH039 predicted to be regulated by BHLH038 in *bhlh038-2, bhlh038-4* and Col-0 under both drought (light bars) and control (dark bars) conditions. t-test >2.78 (*) shows significant difference in fold changes (df=4). Error bars represent standard error ± SE (n=6).

BHLH038 was modelled as a hub gene in a GRN of drought response, where it was predicted to inhibit the expression levels of PIL2 (Phytochrome interacting factor 3-like 2), BPC7 (Basic Pentacysteine 7), BZO2H1 (bZIP transcription factor family protein) and DEL2 (DP –E2F –like2) [Subramaniam, 2016 Fig.20]. Further investigation of relative expression of the above genes in knockouts *bhlh038* lines took place in order to validate BHLH038 as a key regulator of drought stress response (Fig.19, 21 and 22)

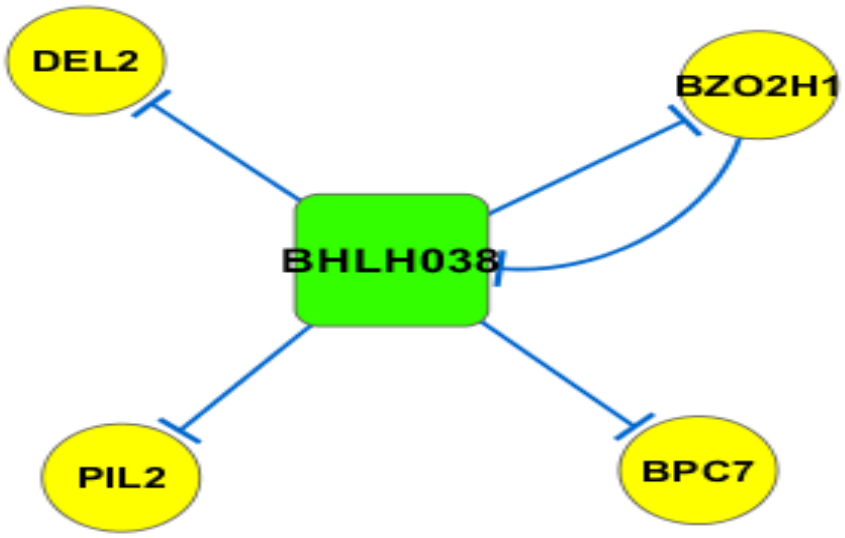

Figure 20: Gene Regulatory network regulated by BHLH038, which was created by VBSSM from Subramaniam (2016). The blue connections indicate that the connections modeled are inhibitory. However no significance was observed (Subramaniam 2016).

The expression levels of TF genes under drought conditions displayed altered gene expression in both mutants *bhlh038-2* and *bhlh038-4* compared to wild type. The use of primers spanning the 3'end region of BHLH038 allowed the investigation of BHLH038 expression where the T-DNA insertion of *bhlh038-2* was found (Fig.18). *bhlh038-4* mutants revealed a down-regulation in BHLH038 expression in both stressed and normal conditions, however the statistical analysis did not show a significant difference in expression compared to Col-0 control and stressed plants. Overall the mutants did not display any significant difference in expression levels relative to Col-0 (Fig.18).

The relative expression of BHLH039 gene in *bhlh038-4* revealed a significant upregulation of ~ 13 fold under control conditions compared to Col-0, while no significant difference was observed in the expression levels of other genotypes (Fig.19B).
Moreover, the mutants did not reveal a significant change in expression levels of AGL22 either in control or drought conditions (Fig.19A).

The gene expression profile of BPC7 and BZ2OH1 in mutant lines *bhlh038-2* and *bhlh038-4* did not reveal any significant difference in either droughted or controlled conditions when compared to Col-0 plants (Fig.21). qPCR analysis of PIL2 and DEL2 gene expression revealed no significant difference between mutant lines *bhlh038-2*, *bhlh038-4* and Col-0 plants under control or stressed conditions (Fig.22).

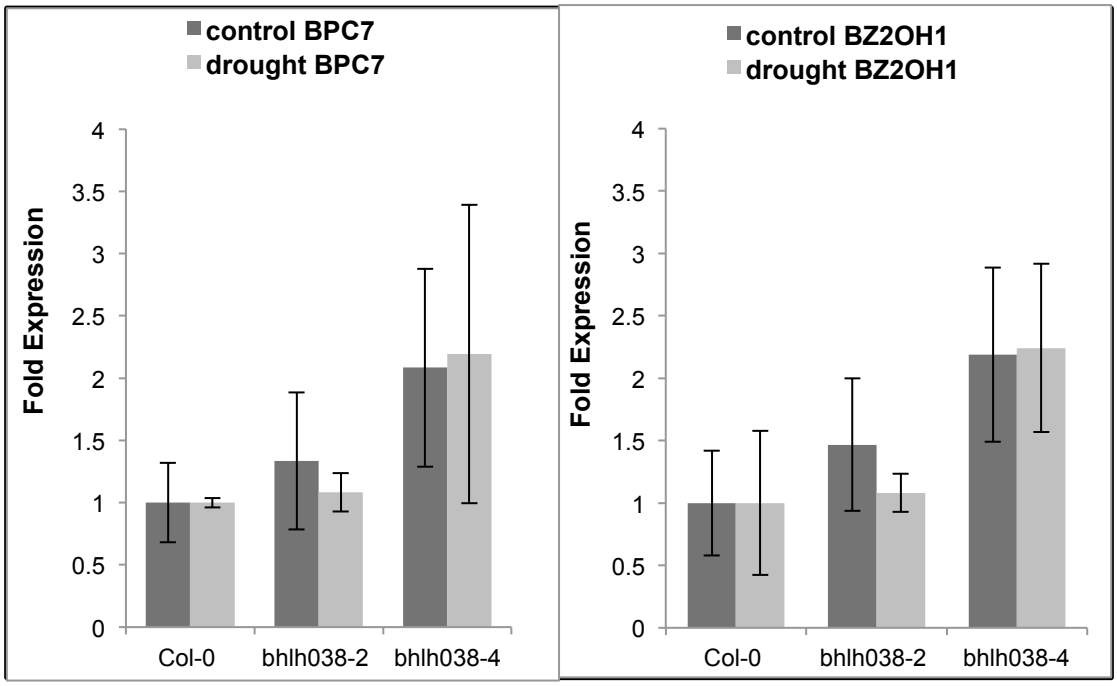

Figure 21: qPCR analysis of BPC7 and BZO2H1 genes identified to be regulated by BHLH038 TF gene, in *bhlh038-2, bhlh038-4* and Col-0 *Arabidopsis* plants subjected to drought.

Error bars represent standard error +/- SE (n=6). (**A**) Expression levels of BPC7 TF gene regulated by BHLH038. No significant difference was observed (df=4). (**B**) Relative Expression of BZO2H1 regulated by BHLH038 in 3 lines of *Arabidopsis thaliana* plants. No significance in gene expression Student t-test <2.78 df=(4).

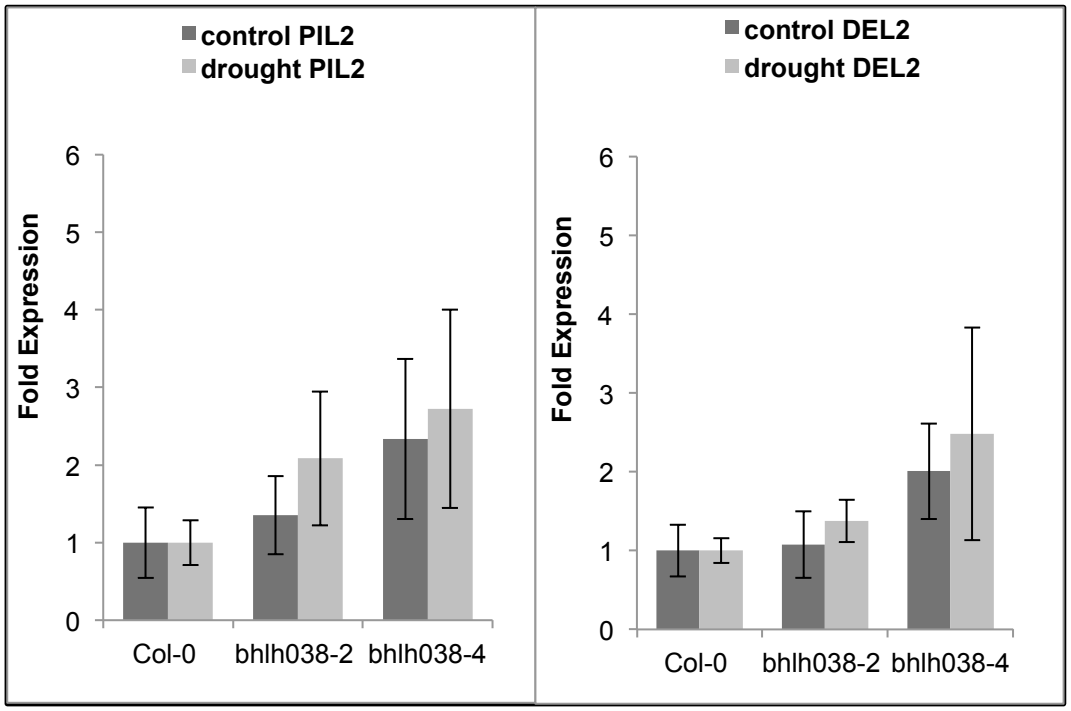

Figure 22: qPCR analysis of PIL2 and DEL2 identified to be regulated by BHLH038 TF gene, in *bhlh038-2, bhlh038-4* and Col-0 *Arabidopsis* plants subjected to drought.

Error bars represent standard error +/- SE (n=6). (**A**) Relative expression of PIL2 gene regulated by BHLH038. (**B**) Relative expression of DEL2 regulated by BHLH038 in 2 lines of *Arabidopsis thaliana* and Col-0 plants. No significant difference in either PIL2 or DEL2, Student's t –test <2.78 (df=4).

3.2.3 Conclusion

Based on the *bhlh038-4* results it could be concluded that BHLH038 acts as a negative regulator of gene expression of genes in the GRN.
If BHLH protein is not present and there is an upregulation in any of the above genes whose expression was analyzed it means that they are either directly or indirectly affected by BHLH038 and that BHLH038 acts as a negative regulator.

This was observed in BHLH039; the expression of this gene in *bhlh038-4* mutants that revealed a phenotype based on the Drying rate results, indicated that the mutation leads to a significant upregulation of BHLH039 compared to Col-0 under control conditions. The altered expression of BHLH039 in *bhlh038-4* mutants under control conditions not only implies that BHLH038 may play a critical role in regulating BHLH039, but also that it may act as a negative regulator. Overall the gene expression profiles differ between the two mutants and it is not clear that either of the insertion is a complete knockout. We need to acknowledge that the expression of the full length of the BHLH038 gene in mutants was not assessed, so the expression profile of BHLH038 still remains unknown and thus the phenotype validation. Further research is needed to confirm the initial results; more replicates as well as better designed primers for BHLH038 should be used for the stress treatment.

3.3 Phenotyping of mutant lines BHLH038 under osmotic stress in soil.

3.3.1 Introduction

3.3.1.1 Chlorophyll fluorescence parameter in Arabidopsis transgenic and wild type plants exposed to osmotic stress in soil

Differences between solid medium plate based analysis and soil experiments affect the development and response of roots to water stress. By the use of soil as the substrate and Mannitol (300mM) to induce precise osmotic stress, the method avoids artifacts resulting from the use of solid -media (½ MS media). In addition, mannitol used in this fashion allows the assessment of the effects and response to osmotic stress independent of other factors that might otherwise vary during progressive soil drying.

In a soil experiment it is hard to assess the root effects of osmotic stress, thus the shoot is the main focus. Imaging of chlorophyll fluorescence is a great tool to assess the responses to such stress in soil grown plants. Imaging of chlorophyll fluorescence is becoming increasingly popular as a screening (Barbagalo et al, 2003). Chlorophyll Fluorescence is a measurement of photosystem II (PSII) efficiency and is a commonly used technique in plant physiology (Murchie and Lawson, 2013). Chlorophyll fluorescence can be used as a measure of abiotic stress, such as drought, salinity or high light stress (Souza et al 2004, Embiale et al, 2016). In this study the two *bhlh038* mutant lines, an overexpressing line and wild type Arabidopsis plants were exposed to water deficit stress when treated with 300mM Mannitol in soil every other day in 5 days overall. The effect of the stress on plants was measured using the Chlorophyll fluorescence parameter Fv/Fm recorded everyday (Fig.23). Fv/Fm for plants grown under control conditions revealed no significance (Fig.3, Appendix). Fv/Fm parameter describes the maximum operating efficiency of PSII in the light adaptive state. At an advanced phase of stress, a down-regulation of PSII activity is expected (Souza et al, 2004).

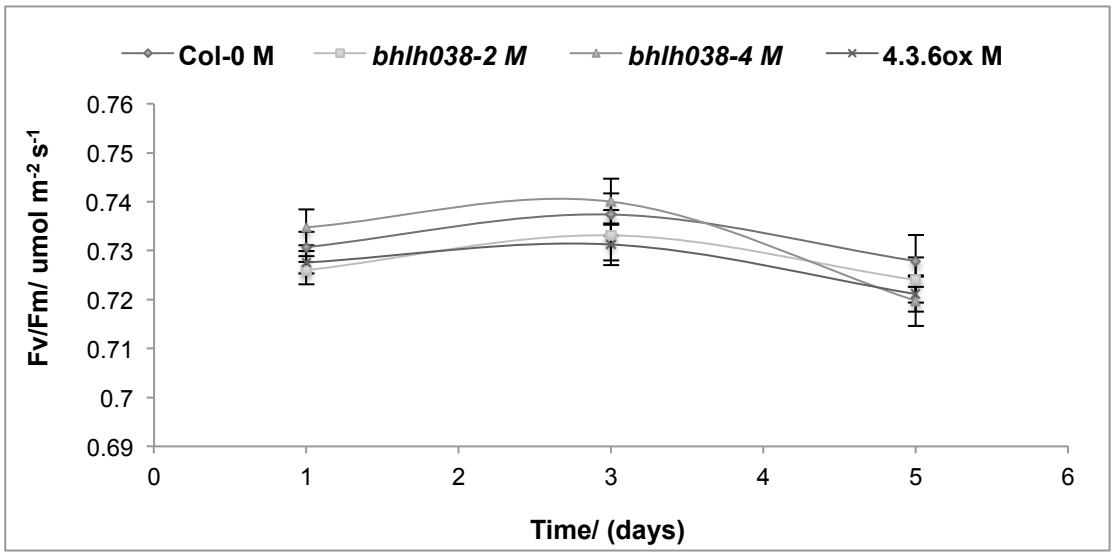

Figure 23: Response of Fv/Fm in rosette leaves of 4 weeks old *Arabidopsis thaliana* plants exposed to water deficit when treated with mannitol in soil (300mM).

Overall, no significant difference is observed in the response Fv/Fm between different genotypes, when exposed to drought stress, by mannitol soil treatment, tukey one-way ANOVA test p >0.05.

3.3.2 Phenotype characterization of BHLH038 under osmotic stress in soil

The expression of BHLH038 in the two independent mutant bHLH038 plants, Col-0 wild type *Arabidopsis* plants and overexpressing 4.3.6ox plants under osmotic stress as well as control conditions was examined using qPCR. The 4 weeks old *Arabidopsis* plants from the above 4 lines, were grown in soil and then they were exposed to osmotic stress via 300mM Mannitol soil treatment for 5 days every other day using the same volume for each plant.

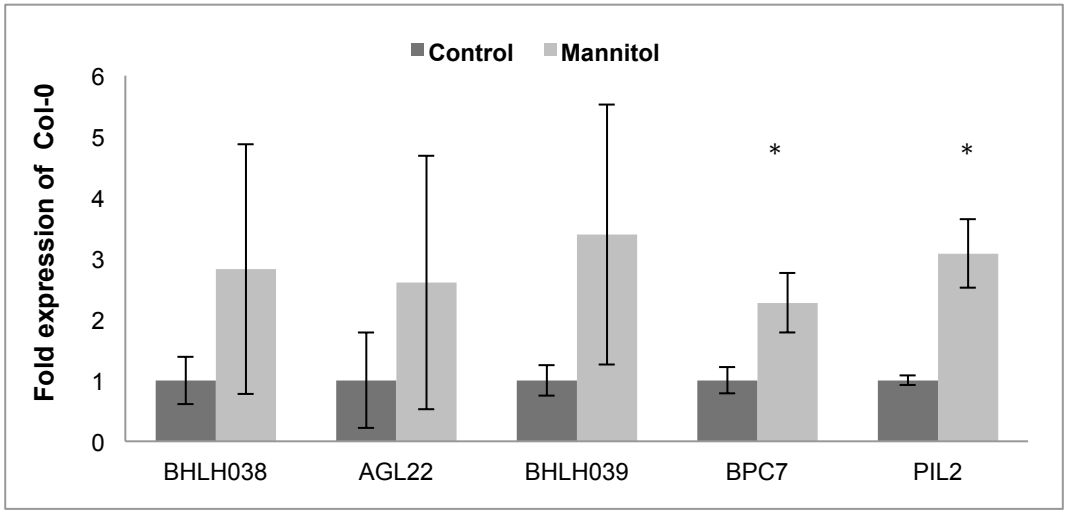

Figure 24: Relative expression levels of 4 genes interacting with BHLH038 in Col-0 Arabidopsis plants exposed to both control conditions and mannitol soil treatment (300mM).

Error bars represent standard error +/- SE (n=6). Significant difference two-tails t-test > 2.78 (df=4) is presented with an asterisk (*).

The effect of the osmotic soil stress alone, on the interaction of BHLH039, AGL22, BPC7 and PIL2 was investigated by assessing the expression profiles of the above genes in Col-0 plants exposed to both normal and stressed conditions (Fig.24). Once again Col-0 stressed plants revealed a significant upregulation of BPC7 and PIL2 (Fig.24).

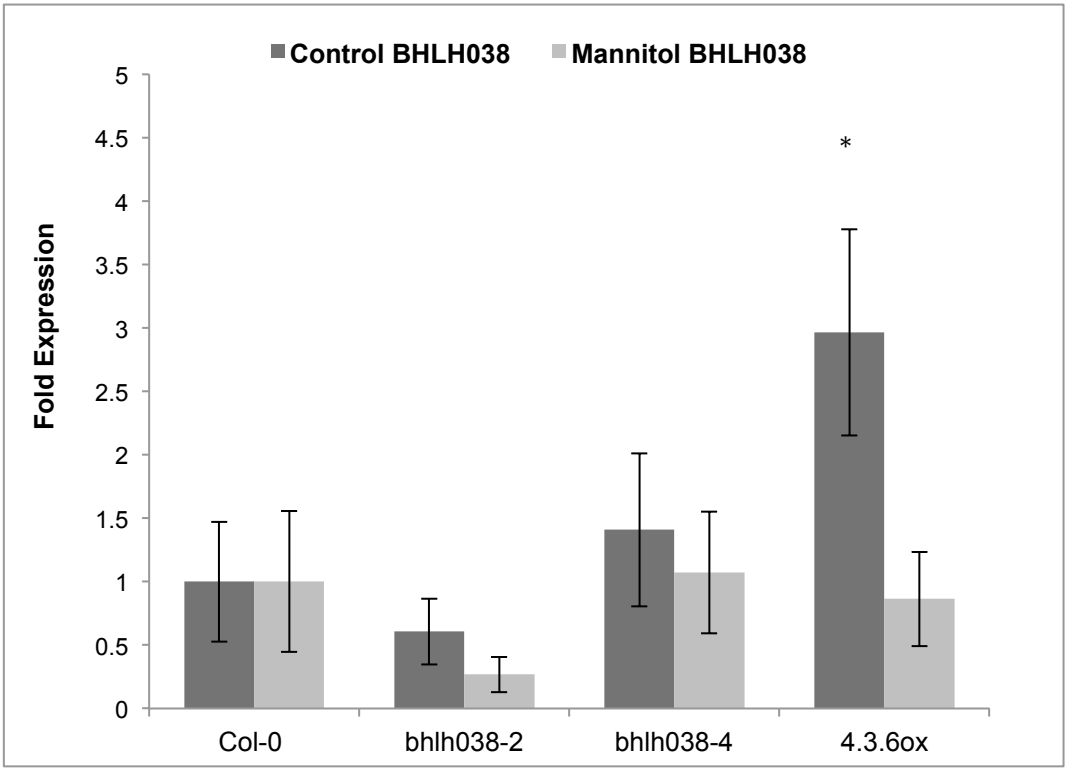

Figure 25: Relative expression levels of BHLH038 in *Arabidopsis thaliana* plants of 4 different lines Col-0, *bhlh038-2, bhlh038-4* and 4.3.6ox under osmotic stress and control conditions.

Primers binding near the STOP codon where T-DNA insertion of *bhlh038-2* mutant line is found. The 4.3.6ox line revealed a significantly different expression level of BHLH038 when exposed to controlled conditions, compared to Col-0 plants, two-tails t-test >2.78 (df= 4). Error bars represent standard error +/- SE (n=6). Gene expression was analyzed using qPCR.

The relative expression of BHLH038 in *bhlh038-2* seems to be slightly suppressed, however the difference in expression levels of BHLH038 between the mutant *bhlh038-2* and the Col-0 *Arabidopsis* plants in control and stressed conditions was not significant (Fig.25). The relative expression levels of BHLH038 in mutant *bhlh038-4* did not vary significantly in comparison to expression levels of Col-0 plants in control or stressed conditions either (Fig.25).
On the other hand the overexpressor line (4.3.6ox) revealed a significantly higher expression level in control conditions of ~3 fold compared to Col-0 plants (Fig.25). The osmotic stress alone confers a significant downregulation in stressed 4.3.6ox plants compared to 4.3.6ox plants grown in control conditions (Fig.25). Expression of ~1.4 fold in *bhlh038-4* mutants in control conditions does not seem to confirm the presence of T-DNA insertion (Fig.25). Likewise, the relative expression of ~0.7 fold

in *bhlh038-2* plants does not seem to confirm the presence of a knockout insertion line.

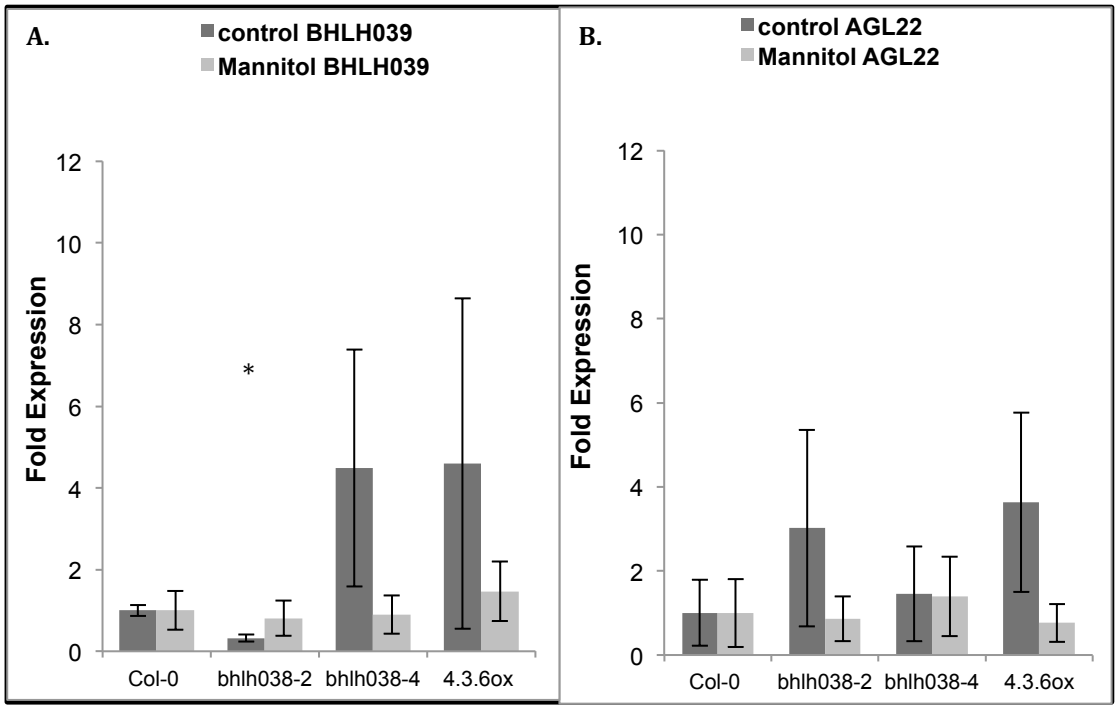

Figure 26: Relative gene expression profiles of BHLH039 and AGL22 genes predicted to be regulated by BHLH038 gene under osmotic stress.

(A) Relative expression of BHLH039. (B) Relative expression of AGL22. Significant difference in BHLH039 expression levels between *bhlh038-2* and Col-0 under control conditions (*) two-tail t-test >2.78 (df=4). The data represent the mean (n=6; error bars are standard error +/- SE). The dark bars represent control conditions and light bars represent mannitol treatment.

BHLH039 was significantly downregulated in *bhlh038-2* mutants compared to Col-0 plants in normal conditions (Fig.26A). The relative expression of BHLH039 in *bhlh038-2* mutants was significantly reduced to ~0.2 fold compared to Col-0 plants in controlled conditions, as opposed to stressed conditions where no significance was observed. The downregulation of BHLH039 in *bhlh038-2* Arabidopsis plants seems to be valid for normal conditions only, since when the stress is applied the expression varies in similar levels as in Col-0 Arabidopsis stressed plants (Fig.26A).

According to the expression profiles of BPC7 and PIL2, significant upregulation was displayed by *bhlh038-4* stressed mutants stressed plants relative to Col-0 *Arabidopsis* plants under osmotic stress (Fig.27 A and B). An increase in the expression levels of BPC7 and PIL2 in *bhlh038-4* mutants would imply an interaction of these two genes with BHLH038 where BHLH038 acts as a negative regulator. However, the same change in expression of BPC7 and PIL2 in overexpressing line indicates that BHLH038 is a positive regulator for these two genes under osmotic stress. A ~2.3 fold expression of BPC7 was displayed in *bhlh038-4* mutants and a ~3.8 fold of PIL2 was revealed in *bhlh038-4* mutants under stressed compared to stressed Col-0 plants (Fig.27). On the other hand, the overexpressor did not reveal any significance in the relative expression levels of BPC7 and PIL2 compared to Col-0 plants (Fig.27). The BPC7 expression in

stressed *bhlh038-4* plants increased by 1 fold compared to *bhlh038-4* mutants grown in normal conditions (Fig.27A). At the same time, in PIL2 expression profile an increase of 3 fold in gene expression was noted between bhlh038-4 plants exposed to stress and control conditions (Fig.27B) This therefore allowed us to investigate the effect of the stress on the interaction of BHLH038 with BPC7 and PIL2.

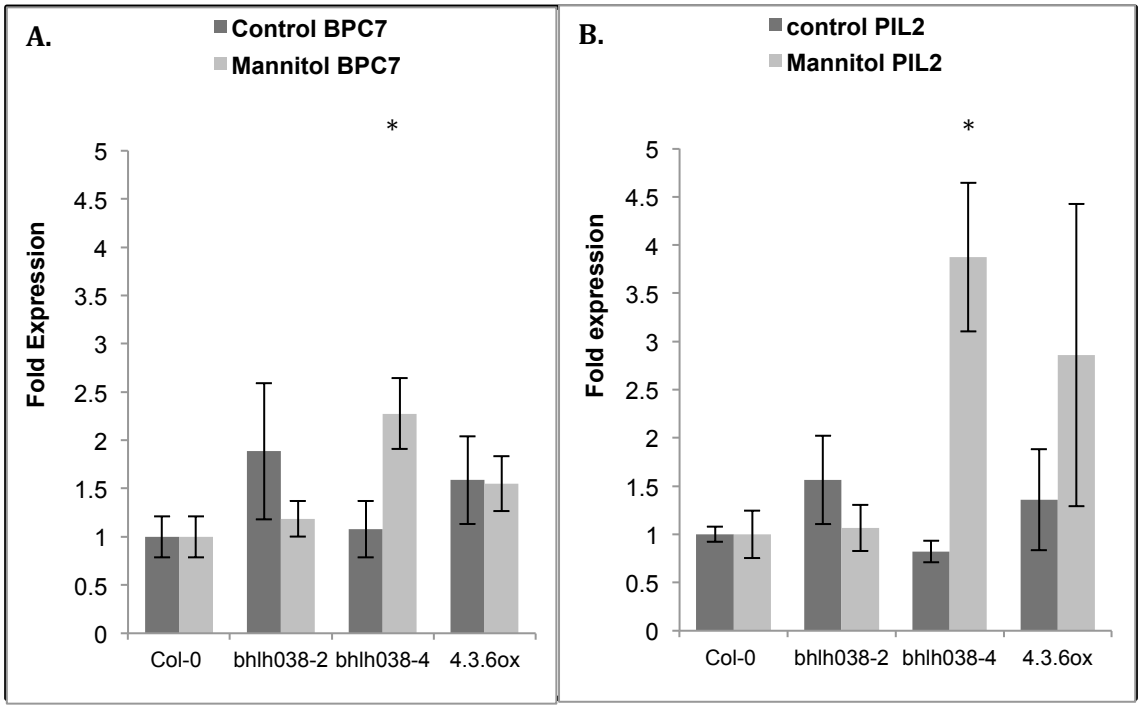

Figure 27: Relative gene expression profiles of BPC7 and PIL2 predicted to be regulated by BHLH038 under osmotic stress

(A) Relative gene expression of BPC7, whereas stressed *bhlh038-4* mutants revealed significance in comparison to Col-0 stressed plants; two-tails t-test > 2.78. (B) Relative gene expression of PIL2. Significant difference in expression level of *bhlh038-4* under stress compared to Col-0 stressed plants, two-tails t-test >2.78 (df=4). The data represent the mean (n=6; +/- SE). The dark bars represent control conditions and light bars represent mannitol treatment.

3.3.4 Conclusion

Another difference between the progressive drought and osmotic soil stress experiment is that the BHLH039 expression seems to be significantly reduced in *bhlh038-2* in osmotic soil stress, while in drought stress, the expression of BHLH039 displayed significant overexpression in *bhlh038-4* mutants compared to Col-0 *Arabidopsis* plants. If we take into account that *bhlh038-4* gave a drought phenotype and that mutation conferred a change in BHLH039 we may assume that it acts as a negative regulator for BHLH039 but BHLH039 is not directly linked to drought signaling since the expression levels when the stress is applied do not vary significantly from Col-0 stressed plants. In soil osmotic stress, *bhlh038-4* stressed

Arabidopsis plants revealed a significant upregulation of BPC7 and PIL2 compared to Col-0 stressed plants as opposed to progressive drought stress. The fact that network genes, BHLH038 and BHLH039 displayed similar expression patterns in Col-0 stressed and controlled conditions in both soil experiments, indicates that any differences between the types of stresses have a minimal effect. However, the fact that *bhlh038-4* mutants revealed overexpression of BPC7 and PIL2 requires further investigation. In conclusion both alleles behave differently when osmotic stress is applied, and yet differently to soil drought stress.

Complementation

3.3.5 Complementation for functional analysis

Since the expression profile of BHLH038 differed between the two mutants in drought and osmotic soil stress experiments, it is unclear whether either of the insertion is a knockout. Thus the genotype of the mutant lines as well as of the transgenic overexpressor remains to be unveiled with further research and analysis. In order to understand whether the phenotype revealed from the mutant lines and the overexpressor, is due to the known T-DNA insertions or due to a possible loss of mutation/ overexpression, or due to a second T-DNA insertion that has taken place, a complementation technique seemed to be ideal. It is known that mutations in the same gene can cause different homozygous phenotypes (Yook, 2005). If a gene functions in many processes, then mutations in this gene may impair each function independently to different degrees (Yook, 2005).

A mutation can affect more than one gene product if the genes are in a complex locus, in this case BHLH039 TF gene is known as closely related gene to BHLH038, both genes are found in chromosome 3 and the loci of both genes are very close; coordinates of BHLH038 are ~21,085kbp while the coordinates of BHLH039 ~ 21,086kbp. Mutations mapped in complex regions such as the region near the STOP codon in *bhlh038-2* mutant, may have an effect on more than one BHLH038 gene product (Fig.1 in Materials and Methods). The T-DNA insertion near the STOP codon in *bhlh038-2* may give rise to a change in the gene expression of BHLH039, which as mentioned above is found in a slightly complex locus to BHLH038. In osmotic soil stress a significant effect in BHLH039 expression was observed only in *bhlh038-2* mutants (Fig.26A). In conclusion, it is important to use the complementation technique so that we can confirm whether the phenotype displayed by each phenotype in the three different sets of experiments varies due to two mutations together that result in a wild-type phenotype. Restoring the gene product BHLH038 using the gene of interest BHLH038 and its native promoter to transform mutant *Arabidopsis thaliana* plants.

The binary vector chosen in this study was pGreen 0229 (~4448bp). This type of binary Ti vector is configured for ease-of-use and to meet the demands of a wide range of transformation procedures for many plant species (Hellens et al, 2000). This plasmid allows any arrangement of selectable marker and reporter gene at the right and left T-DNA borders without compromising the choice of restriction sites for cloning (Hellens et al., 2000). The strain used for the amplification was *E.coli*; the increased efficiencies in routine *in vitro* recombination procedures make this binary vector ideal for this study.

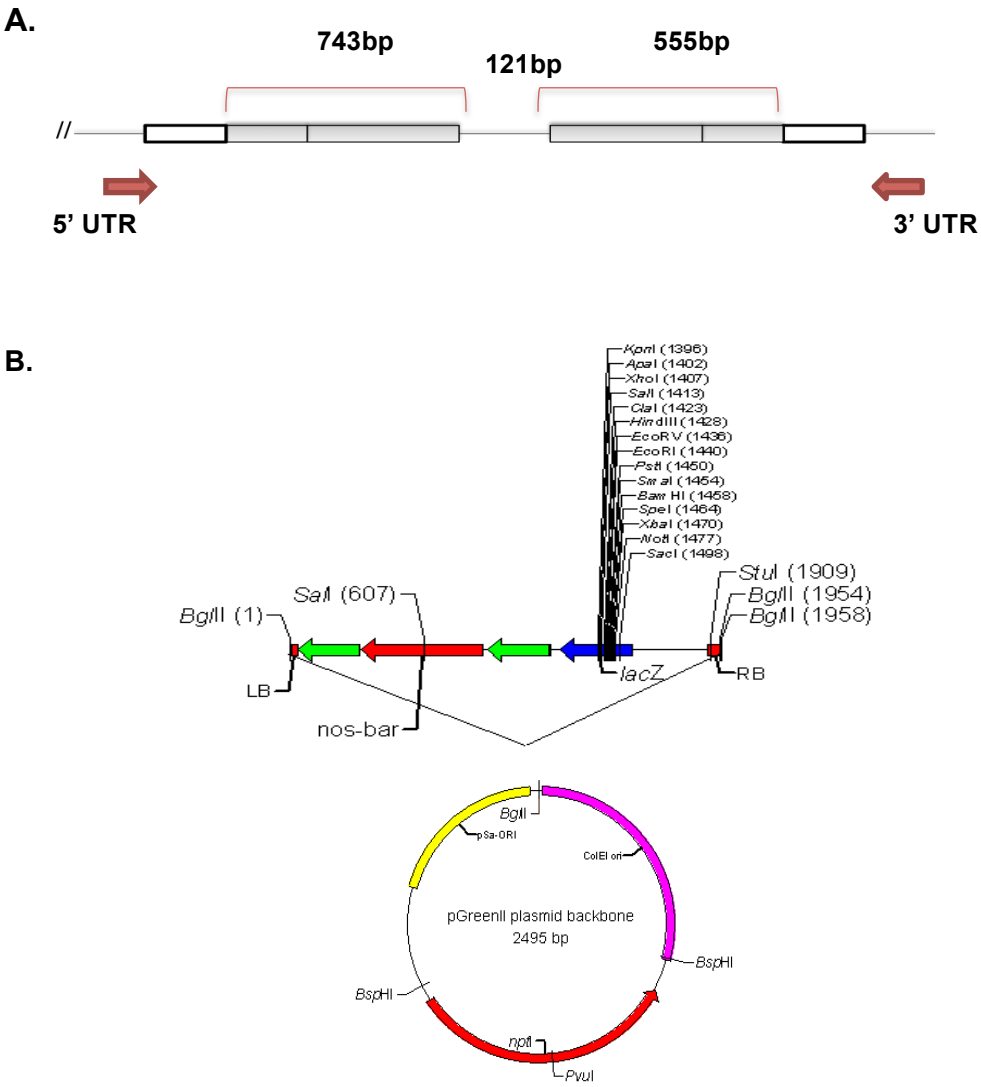

Figure 28: BHLH038 gene and actual construct.

(A) BHLH038 gene; grey boxes are exons, black lines indicate the introns of the gene, while the white boxes are the 3'UTR (STOP) and 5'UTR (containing the native promoter of the gene, 500bp upstream of transcriptional start site), whereas the full genomic length is ~1.2kbp. (B) The actual construct containing the GOI (gene of interest) with its native promoter that is to be found in the pGreen vector.

We placed the plant-selectable marker gene (KanR), opposite to the nopaline synthase gene promoter (Pnos), adjacent to the left border. The restriction enzyme used for this binary vector was SmaI, cutting the vector at ~2080bp, on the lacZa gene and its promoter. This was ideal for the insertion of the gene of interest along with the native promoter fragment that had blunt ends. BHLH038 gene was amplified using specific primers spanning 3'UTR and 5'UTR regions (Table 1, Materials and Methods).

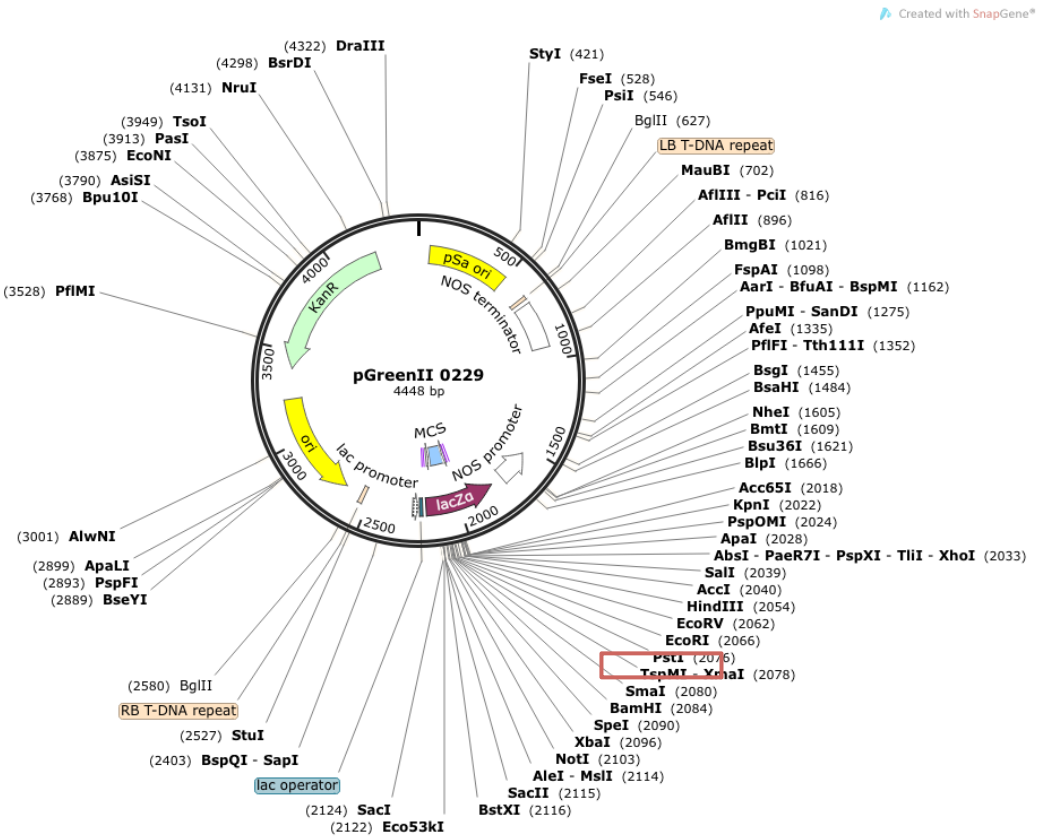

Figure 29: Physical map of pGreen 0229 (~4448bp).

The map was designed using SnapGene and it shows the location of some sites for restriction enzymes that cut the vector once or twice. LB and RB, left and right borders, respectively; Pnos promoter of the nopaline synthase gene; KanR, kanamycin-resistance gene; restriction enzyme cloning site in lacZa gene.

The competent cells were successfully transformed with pGreen 0229 and as a result they successfully acquired antibiotic resistance due to KanR found in pGreen 0229 binary vector. This was revealed by the colony growth on Kanamycin plates. Once a single colony of Kanamycin resistant competent *E.coli* cells was isolated and was left to grow in LB media overnight, the miniprep allowed successful extraction of the *E.coli* DNA containing the pGreen 0229 vector. The plasmid was then successfully digested using SmaI, which resulted in blunt ends at 2080bp of the vector. Furthermore, the gene of interest was amplified and purified along with the native promoter, which was initially extracted from Col-0 *Arabidopsis* plants (Fig.30). Ligation of the amplified PCR product with the linearized vector took place and then colonies containing BHLH038 (the gene of interest along with the native promoter) were grown on Kanamycin plates. The

DNA extracted from these colonies was sent for sequencing in order to confirm that the full sequence of the gene of interest was present. However the sequencing failed and the complementation was not completed.

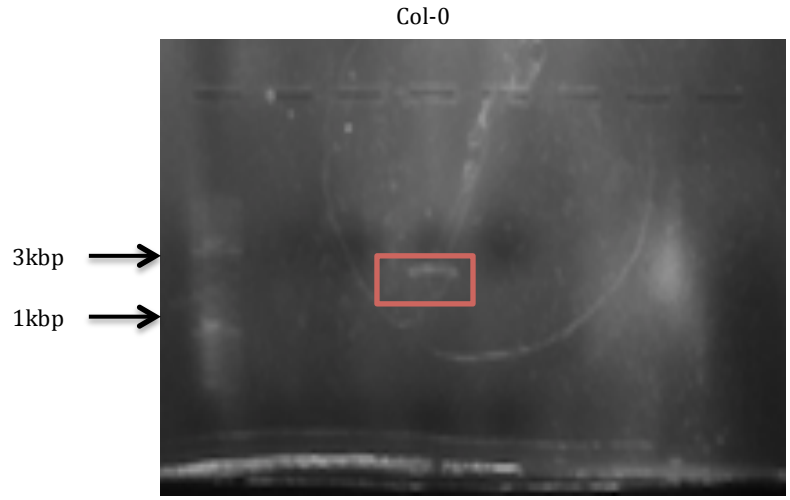

Figure 30: Presence of bHLH038 gene with its native promoter extracted from Col-0 Arabidopsis plants.

PCR product was analyzed on a 1% acrylamide gel and visualized by ethidium bromide staining. Primers used for the amplification spanned the 5' UTR to 3' UTR region (see Materials and Methods, Table 1).

DISCUSSION

4.1 Importance of abiotic stresses, the plant responses and evaluation

It is well known that abiotic stresses such as drought and salt are the major environmental factors limiting crop productivity worldwide (Liu et al., 213). Plants have evolved multifaceted strategies that involve biochemical, morphological as well as physiological adaptations in order to avoid or reduce the unfavorable effects of various abiotic stresses (Liu et al., 2013). Gene regulation under abiotic stresses is mediated by multiple transcriptional cascades (Liu et al., 2013). These cascades involve the induction of a transcription factor gene, which either activates or represses downstream target genes that are crucial for abiotic resistance (Liu et al., 2013). A recent drought transcriptome analysis of *Arabidopsis thaliana* identified drought responsive gene that have a potential role in early and late drought responses (Bechtold et al., 2016). AtbHLH038 TF gene was one of the genes involved in a TF GRN (Gene Regulatory Network) regulating many genes that play a crucial role in early and late drought responses (Bechtold et al., 2016).

Gene knockouts that arise from mutations as well as overexpression permit the gene sequence to be linked to a phenotype from which the function of the gene can be clearly deduced (Srinivasan et al., 2005). In this study in order to evaluate the role of AtbHLH038 in drought and salt stress response, two independent mutants were used. However, based on the BHLH038 expression profiles it is obvious that the two mutants demonstrate differential expression of BHLH038 expression and the other genes whose expression was assessed (Fig.19 & 26, Fig. 21, 22 & 27) giving rise to the question whether there is a knockout T-DNA insertion present in either one of the two *bhlh038* lines or even both. The presence of a knockout T-DNA insertion in one of the two lines may justify the phenotypic differences between the two independent mutants. In addition another aspect that is associated with phenotypic variation between mutant lines is the random rise of multiple insertions, the complex arrangement of T-DNA, gene or chromosomal duplication mutations, insertion of vector backbone sequences and rearrangements or a combination of all the above. Thus these are great disadvantages, which are related to T-DNA lines (Srinivasan et al., 2005).
Previous studies have concluded that inconsistencies in the T-DNA integration can complicate efforts to clone and identify adjacent plant DNA since larger tags of vector backbone sequences have been detected, vector DNA sequences located far beyond the defined T-DNA region restricted by border sequences have been reported in various studies in the past (Srinivasan et al., 2005).
In many cases, various knockout mutants have no readily identifiable phenotype; another reason for the lack of identifiable phenotype linked to the knockout mutations is the functional redundancy among the members of a gene family (Krysan et al., 1999). In this study, *bhlh038-4* plants gave a phenotype in soil drought experiment and *bhlh038-2* plants gave a phenotype according to the plate assays, whilst both lines were identical to WT under control/ optimal growth conditions. Following these results assessing gene expression of these two lines is considered to be beneficial, since this research may contribute to the elimination of gaps in the role of BHLH038 TF gene in drought stress in the literature. Following that, gene expression profiles established revealed that BHLH039 was substantially upregulated in *bhlh038-4* mutants under control conditions (Fig. 19B), suggesting that this TF was indirectly regulated through BHLH038, since altered gene expression of BHLH038 does not give rise to a differential expression of BHLH039 (Fig. 19B), relative expression of BHLH038 in *bhlh038-4* was not significantly altered as opposed to BHLH039 therefore an altered expression of BHLH039 in the mutants that gave the drought phenotype leads to the conclusion stated above. BHLH038 and BHLH039 are involved in the maintenance of iron ion homeostasis and uptake (Wang et al., 2007), and they are known to be very similar in sequence. Thus, there

could be some functional redundancy between the two genes, which may result in the lack of phenotype in the *bhlh038-2* mutant line, whose T-DNA insertion is found in the 3' end of BHLH038, very close to BHLH039 gene (Fig. 1, Materials and Methods). Another important factor in the effectiveness of a T-DNA insertion is its location within the gene of interest. Previous studies have revealed that insertions upstream of the stop codons depending on the number of bp upstream or downstream of the stop codon of the target gene, lead to a knock out which has no effect on the plant, unless gene is crucial for plant's survival (Wang, 2008). Hurtado, Farrona and Reyes (2006) revealed that it is possible for an insertion placed a few bp before the stop codon in the target gene to have a negligible effect on the transcription of the gene. Insertion toward the 3' end of a gene can simply produce a weaker phenotype (Wang, 2008). The *bhlh038-2* mutants possibly demonstrate this phenomenon. During soil progressive drought and soil osmotic stress treatments, no phenotype was revealed, the expression was not significantly altered and the location of the T-DNA found extremely close to 3' end of target gene (Fig. 1, Materials and Methods). This further confirms that the lack of phenotype is not dependent on the type of stress but it is directly linked to the insertion location.

4.2 Phenotypic evaluation on early and late developmental/ growth stages

A series of growth stages has been used in this study in order to collect phenotypic data. Growth stages serve both as developmental landmarks for the collection of detailed morphological data (Boyes et al 2001). This method has helped to establish a data set representative of wild type Col-0 *Arabidopsis thaliana* plants under standardized and stressed environmental conditions and revealed and characterized the phenotype of *bhlh038* mutants through the characterization of single-gene mutations (Fig. 1, Materials and Methods). The characterization of early phenotypes was undertaken on plate-based platform since morphological observation in soil-based platform may not be possible during early stages of development (Boyes et al 2001). Plate-based platform analysis was ideal for the collection of quantitative data regarding the development of shoots and roots under normal, salt and osmotic stresses. Once again the two mutants revealed different phenotypes under salt and osmotic stress. Mutants *bhlh038-2* behave as knockouts since the early development of the seedlings as well as the root growth is significantly altered under both stresses as opposed to *bhlh038-4* which display wild-type characteristics (Fig. 4, 5, 10 & 11). Whether, the stress response revealed by *bhlh038-2* mutants is directly linked to altered expression levels of BHLH038 or due to indirect link between BHLH038 and the regulation of other TF genes remains unknown.

Moreover it may seem that shoot growth is more sensitive than root growth when exposed to salt-induced osmotic stress and this is due to the fact that a reduction in the leaf area development relative to the root growth would lead to the decrease of water use by the plant, and so soil moisture level would be maintained by the plant but also salt concentration would be prevented in the medium (Carillo et al 2011). Seedlings undergo the first phase of salt stress, which is osmotic phase where the rate of shoot growth reduces significantly (Carillo et al, 2011). This phenomenon is observed in the phenotypic analysis of mutant *bhlh038-2* regarding the early stage development under salt and osmotic stress (Fig. 4 & 5). However it is difficult to make a causal link between the assumed genotype of *bhlh038-2* and the phenotype presented and therefore draw conclusions on the role of BHLH038 in salt and osmotic stress response.

Another aspect that plays a significant role is the concentration of exogenous chemicals applied to confer either an osmotic or salt stress. The concentrations used may confer an osmotic potential stress, which may differ between the two treatments (salt and mannitol). It is clear that a concentration of 100mM NaCl confers a greater stress to the roots of *bhlh038-2* than a concentration of 75mM NaCl (Fig. 10 & 13). In addition there are two ways in which high salt concentration may affect plants; high salt concentrations in the soil disturb the efficiency of roots to extract water, and the toxicity due to high salt concentrations within the plant increases, as a result many physiological and biochemical processes such as assimilation and nutrient uptake are inhibited (Carillo et al., 2011). Both ways in which high salinity affects plants mentioned above lead to reduction of plant growth, development and survival (Carillo et al., 2011). However this is observed mostly in soil salinity experiments, where there is a greater variety of parameters that may well characterize any modifications in metabolic processes, rosette area, oxidative stress among others (Carillo et al., 2011).

The evaluation of a drought phenotype in late developmental/ growth stages was examined by inducing a water deficit in 5 weeks old Arabidopsis thaliana plants. Previous studies have reported that approximately after 35 days of growth the Arabidopsis plants have entered a flowering period where 10% of flowers to be produced have opened (Boyes et al., 2001). Therefore taking into account that some of the most effective and efficient ways to measure drought resistance or adaptation to osmotic and drought stresses in plants is by assessing metabolic based apart from physiological-based parameters (Pillitteri and Torrii, 2012), other basic responses to tolerate or resist drought and osmotic stress, are reduction of transpiration and leaf rolling (Ahmad et al., 2016), photosynthetic capacity (Tuberosa, 2012; Sharma et al., 2014) and leaf temperature. As a result the plants must have reached a developmental stage where all the above parameters can be properly assessed, and this stage is when they are 5 weeks old. None of the above parameters was measured in this study but it is important to acknowledge that when plants are 4-5 weeks old they have reached the late vegetative state before entering reproductive and flowering phase, where root system has fully developed and can support growth (Park et al., 2017).

In addition, during the reproductive phase which is entered on week 6 after germination, the focus in on the reproductive organs and as a result the genes studied are linked to alterations in metabolic and physiological pathways of flowering, the focus of this study is on the adult vegetative stage of Arabidopsis plants (Baürle and Dean, 2006). The slow progressive drought experiment performed in this study allowed us to investigate the physiological and transcriptional responses in a single fully expanded *Arabidopsis* leaf (Bechtold et al., 2016). Moreover, the constant decrease of rSWC, which was measured, confirmed the stress applied to the plants and likewise the drying rate allowed us to monitor the progression and the degree of drought stress in relation the physiological and transcriptome changes. We need to acknowledge the fact that the drought stress demonstrated in this study was progressive and not sudden but it seemed to confer a certain degree of stress to the plants, allowing the plants to acclimate to the drought and /or display a drought response. Evidence from previous studies has shown that exposure of plants to various rSWC levels, represent statistically significant levels of water stress and in this study it is clear that a drop of rSWC from 100% to ~20% confers a significant water stress (Earl, 2003).

The ability of plants to endure low tissue water content through the evolution of adaptive traits is known as drought tolerance (Basu et al., 2016). The maintenance of osmotic turgor is the main goal of these adaptive traits established by the plant, which could be achieved by the cellular elasticity, the osmotic adjustment as well as

the increasing protoplasmic resistance (Basu et al., 2016). Evidence has previously shown that the slow dehydration of plants leads to the increase in dehydration tolerance by permitting drought- induced acclimation (Levitt, 1985). This phenomenon is well presented by the reduced drying rate (ml/day) in *bhlh038-4* mutants (Fig.16). Apart from the drying rate evaluation the photosynthetic efficiency is another great measure that could reveal a drought phenotype. The chlorophyll fluorescence parameter Fv/Fm reflects the maximum quantum efficiency of the PSII photochemistry and has been widely used for early stress detection in plants (Sharma et al., 2014). Various studies revealed that good indicators of a drought phenotype involve altered stomatal conductance, transpiration rate and photosynthetic capacity/ efficiency (Tuberosa, 2012; Sharma et al., 2014), so we used the parameter Fv/Fm as an efficient measure of drought phenotype evaluation.

A recent study revealed that stomatal conductance and net photosynthetic rate are reduced as plants were subjected to different extent drought stresses in which involved some signals such as ABA; photosynthetic apparatus on the other hand may be damaged under sever/ extreme drought/ osmotic stress, thus leading to declines in PSII photochemical efficiency and enhancing peroxidation (Xu, Zhou and Shimizu, 2010). Taking into account the above findings, the degree of drought and osmotic stress in the two soil experiments conducted in this study is questioned. Another study (Zivcak et al., 2014), reported that maximum quantum efficiency of PSII photochemistry (Fv/Fm) parameter remains unaffected by dehydration until a sever water stress, which leads to the conclusion that 5 days of mannitol treatment in soil were enough to confer a significant osmotic stress that would be demonstrated by Fv/Fm photosynthetic parameter. The stress was significant on day 5 of the experiment (Table 3.9, Appendix).

According to Bechtold et al., (2016), altered drying rates suggest that there may be an altered drought phenotype, but further investigation is needed to determine if this is due to developmental or metabolic changes. In the study we conducted the photosynthetic parameter Fv/Fm, which represented the photosynthetic efficiency could be linked to the metabolic changes that did not reveal a modification in mutant lines and so no drought response was observed, however the mutations might contribute in developmental changes, which give a drought response. In conclusion, when using physiological measures to investigate a drought response, experiments that assess both the developmental and metabolic changes are critical. The assessment of parameters such as stomatal aperture (Liu et al., 2013), H_20_2 measurement (Terzi et al., 2014) among others have been proven to well-characterize drought phenotypes over the years.

As mentioned above one of the main differences between young and old Arabidopsis plants is that during early developmental stages the root systems has not fully formed (Park et al., 2007) and during the development of the plant many hormonal signaling as well as transcription factor genes play an important role to the regulation of seedling germination and growth. In this study the plate based platform analysis could be implying that the relative contribution of bHLH038 in osmotic and salt stress response is significant during early developmental stages. Previous studies have shown that particular genes are expressed mostly during specific stages of early flower development (Wellmer et al., 2006). Relatively high expression levels of BHLH038 in roots of WT Arabidopsis plants, with or without the apex revealed (Fig. 4, Appendix) may indicate the role of BHLH038 in hormonal signaling cross talk, affecting levels of auxin which is known to be a key regulator of growth and development (Band et al., 2014), as well as the importance in triggering a response of the root apex to stresses in soil, such as Fe deficiency (Guangjie, Kronjucker and Weiming, 2016).

ABA and GA content during seed development and imbibition seem to play an important role for the proliferation and expansion of cells and thus the root and shoot development in Arabidopsis plants (Wani et al., 2016). A recent study showed that ABA level increases at early and middle seed developmental stages, while at late developmental stage it declines (Liu et al., 2014). Moreover studies have shown that drought stress mildly affects cell proliferation during early leaf developmental stages, in which cells are only proliferating and not yet expanding (Skirycz et al., 2011). Hormonal signaling is involved in stress responses but also during the life cycle of a plant.

According to the results generated in this study, phenotypic differences exist between early developmental stages of mutant Arabidopsis seedlings displays a drought/ osmotic stress phenotype whilst this phenotype is not present in older plants. An assumption that could be made according to the results of this study, is that BHLH038 may contribute in the cross talk, hormonal signalling and thus regulation of other TFs genes during early developmental stages, that give rise to a drought response. Hence the phenotype displayed by *bhlh038-2* Arabidopsis seedlings in early vegetative stages. However, genotyping of the two alleles used in this study would have made this conclusion reliable.

4.3 Importance of genotyping mutants and failure of T-DNA insertions

The correlation between a mutation and a certain phenotype has always been the goal of reverse genetics, however whether there is a causal link between the two factors (mutation and phenotype) in a particular genes is of great interest too. One of the most widely used techniques to prove definitely that the insertional mutation causes a phenotype is Complementation. In this study it is obvious that conclusions on the correlation of mutations in BHLH038 TF gene linked to a drought phenotype cannot be drawn easily, however we need to take into account the rate of T-DNA insertion failures, over the years recorded in various studies.

Garg et al., (2016), showed that two drought-related genotypes might exhibit differences in the phenology at early reproductive (ER) and late reproductive (LR) stages. This may be the case for the two independent mutant lines of BHLH038 in this study. The T-DNA insertion may have an effect in the early developmental and growth stages of *Arabidopsis thaliana* seedlings, where the T-DNA insertion in *bhlh038-2* mutants being drought- sensitive, exhibited thinner and shorter roots for better proliferation of root biomass. Although the root- to –shoot ratio tends to increase under drought conditions, the biomass of fine roots in particular is often reduced as a consequence of reduced transpiration and respiration rates (Brunner et al., 2015). On the other hand, in late plant growth/ development a different T-DNA insertion seemed to give a drought-tolerant phenotype, revealing slower drying rate. Thus, the plant growth stage seems to contribute to the drought adaptation (Garg et al., 2016). In addition according to Liu et al., (2013) it is possible for a mutant line to have induced gene transcript levels in both shoots and roots by osmotic stress (200mM Mannitol) and salt stress but not by drought treatment, this could apply on *bhlh038-2* mutant which gives a phenotype under osmotic and salt stress in plates but not drought in soil. Another study showed that allelic T-DNA lines might differ for many traits at a specific Arabidopsis gene but not at another (Valentine et al., 2012). Valentine et al., (2012) found out that even when a single insertion locus is identified via screening for homozygosity, there may still be additional inserts at unknown loci, and it is possible that a second locus may cause a phenotype of interest or may even alter the phenotypic effect of the initial knockout mutation. As mentioned above part of the variation between the two lines in our study could be attributed to

differences in the location of the insertion within the gene of interest (Fig.1, Materials and Methods).

Evidence has shown that insertions within exons are more likely to have a phenotype than insertions in other regions (Valentine et al., 2012). This may be the case in *bhlh038-4* mutants, which are found in an exon, as opposed to *bhlh038-2* found near the 3' UTR region (Fig.1, Materials and Methods). However, an additional insert may have affected an unknown locus. Many studies have reported high frequency transfers of sequences beyond the T-DNA border, which can complicate efforts to clones and identify adjacent plant DNA (Srinivasan et al., 2005), T-DNA may be present in the genomes of transformed host plants as single units or in multiple tandem arrays, whilst T-DNA regions beyond the border repeat were also found to be stably integrated into plant genomes at high frequencies (Srinivasan, Nath Radhamony and Mohan Prasad, 2005). Even DNA sequences of the vector also known as binary vector backbone sequences, from far beyond the defined T-DNA region delimited by the border sequences have been detected and flanking sequence amplification from T-DNA tagged mutants has displayed the ability to amplify considerably larger tags of vector backbone sequences than expected (Srinivasan et al., 2005). This was displayed almost by 75% of transgenic tobacco plants generated using Agrobacterium mediated T-DNA transfer containing vector sequences integrated into the plant genome (Kononov et al., 1997). As a result it is possible that the T-DNA of a large percentage of T-DNA tagged Arabidopsis plants in the study we conducted did not co segregate with the mutant phenotype. In the past a large percentage of T-DNA tagged *Arabidopsis* plants revealed that the T-DNA did not co segregate with the mutant phenotype. In these cases it is possible that the mutation could have been caused by the insertion of backbone sequences independent of the T-DNA in these lines (Srinivasan et al., 2005). Another reason why T-DNA insertions have shown a failure rate is due to rearrangements that interfere with reverse-genetic analyses, phenotypes that arise from an unexpected high frequency of duplication/ transpositions that therefore provide misleading information on the molecular basis of mutant phenotypes. Genetic mapping of T-DNA insertion sites as well as genetic and molecular characterization of mutant alleles is necessary for the phenotype portrayed (Srinivasan et al., 2005).

The use of mutagenesis to find and study plant genes is commonly being used in functional genomics. Both forward and reverse genetic strategies are based mainly on T-DNA insertion mutations that provide crucial information for the study of gene function, those T-DNA insertion mutations are considered as a source of sequence tags from large collections of mutant lines. A disadvantage of mutagenesis technique is the partial analysis of genes that are crucial for many stages of development, functionally redundant genes or highly pleiotropic (Srinivasan et al., 2005).
The genotyping of mutants with the process of PCR screening for individual knockout mutations has been proven to be an efficient approach to reverse genetics. For this reason the design and testing of gene-specific primers in order to identify suitable primers specific for the particular T-DNA insertion sequence is crucial. In this study, the primers used in order to assess the expression profile of BHLH038 were not ideally designed to screen the full length of the gene of interest, thus the primers used in PCR and RT-PCR reveal and confirm the correlation of the mutation present in the gene with the phenotype observed.

4.4 Why study AtbHLH038 for these specific abiotic factors, other closely related factors linked to osmotic/ salt and drought stress make the link

Although very little information is available on the AtbHLH038 TF gene, especially regarding its role in the abiotic stress response of Arabidopsis thaliana, many closely related genes have been reported to be involved in abiotic stress signalling. A recent study (Guo and Wang 2017) showed that bHLH TFs with similar structures might have similar functions. Most bHLH TFs genes from the same subfamily had similar expression patterns, suggesting that bHLH TFs in different subfamilies that have identical expression patterns may participate in the same network to cooperatively regulate plant processes. (Guo and Wang, 2017).
The basic helix-loop-helix (bHLH) TFs are considered to be the largest group in plants and are induced under different abiotic stresses. The bHLH genes regulate plant responses to various abiotic stresses such as cold (Chinnusamy et al., 2003; Wang et al., 2003), salinity (Mao et al., 2017), drought (Seo et al., 2011) and iron deficiency (Zhang et al., 2015) among others.
The bHLH TF family is known to be associated with root growth and plant developmental processes, Abe et al. (2003) reported the up-regulation of the bHLH gene, AtMYC2 during the early stages of drought stress and showed a steady increase under salinity stress. In addition, Liu et al. (2015) reported the AtbHLH112 gene to play the role of a transcriptional activator that regulating the expression of many genes via binding to their GCG- or E-boxes leading to abiotic stress tolerance. Liu et al. (2013) also revealed that bHLH122 acts as a positive regulator under drought and osmotic stress. Furthermore, the bHLH genes play an important role in regulating multiple signal transduction pathways and impacting biosynthesis (Zhao et al., 2018). Taken together this highlights the important role bHLH TFs play during stress defense and strengthens the argument that BHLH038 may also be a key player in osmotic stress defenses.

In addition various studies have demonstrated the variety of function the bHLH transcription factors have in plant growth and development. Loss of function mutation in RGE1 (Retarded Growth of Embryo 1) gene, a bHLH TF gene, displayed retarded growth of the embryo and small and shriveled seeds (Kondou et al., 2008). Groszmann et al. (2010) reported another bHLH gene, SPATULA (SPT) to be involved in carpel development, seedling germination and lateral organ growth in *Arabidopsis thaliana*. bHLH TF genes are characterized as key regulators in hormonal signaling during abiotic stress, a closely related gene AtbHLH129 in Arabidopsis, demonstrated a key regulatory role in ABA signaling, mainly expressed and affecting the root elongation of Arabidopsis plants (Tian et al., 2015). Thus the above result is further evidence of the bHLH TF genes are important integrating stress with development and the results suggest a similar role of AtbHLH038 in stress signaling during abiotic stress such as drought and salt stress. Further evidence that AtbHLH038 may act as a key role in drought/salt stress signaling in Arabidopsis, is the study that revealed that AtMYC2, a well known MYC TF gene which belongs to the bHLH TF family of Arabidopsis, which was therefore found to interact with R2R3 MYB TF (AtMYB2) to activate the expression of ABA response gene RESPONSIVE TO DEHYDRATION 22 (RD22) (Abe et al., 2003). Taking the above findings into consideration, we need to acknowledge the fact that there is great potential fin AtbHLH038 TF gene for future work.

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APPENDIX

1.1 Developmental stages statistical analysis

Table 1.1: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.1 in control

time_taken

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.391	2	.696	1.836	.162
Within Groups	80.715	213	.379		
Total	82.106	215			

Table 1.2: Post-Hoc test Multiple Comparisons

Dependent Variable: time_taken

Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bhlh038-2	-.17361	.10260	.211	-.4158	.0685
	bhlh038-4	-.00694	.10260	.997	-.2491	.2352
bhlh038-2	Col-0	.17361	.10260	.211	-.0685	.4158
	bhlh038-4	.16667	.10260	.238	-.0755	.4088
bhlh038-4	Col-0	.00694	.10260	.997	-.2352	.2491
	bhlh038-2	-.16667	.10260	.238	-.4088	.0755

Table 1.3: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.5.

time_taken

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.029	2	1.015	1.741	.178
Within Groups	115.966	199	.583		
Total	117.995	201			

Table 1.4: Post Hoc Multiple Comparisons

Dependent Variable: time taken

Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bhlh038-2	-.23320	.13327	.189	-.5479	.0815
	bhlh038-4	-.03461	.12858	.961	-.3382	.2690
bhlh038-2	Col-0	.23320	.13327	.189	-.0815	.5479
	bhlh038-4	.19859	.13371	.300	-.1171	.5143
bhlh038-4	Col-0	.03461	.12858	.961	-.2690	.3382
	bhlh038-2	-.19859	.13371	.300	-.5143	.1171

Table 1.5: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.7 in control.

time_taken

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.685	2	.843	1.195	.305
Within Groups	140.295	199	.705		
Total	141.980	201			

Table 1.6: Multiple Comparisons

Dependent Variable: time_taken

Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bhlh038-2	-.18275	.14658	.427	-.5289	.1634
	bhlh038-4	.02907	.14142	.977	-.3049	.3630
bhlh038-2	Col-0	.18275	.14658	.427	-.1634	.5289
	bhlh038-4	.21183	.14707	.322	-.1355	.5591
bhlh038-4	Col-0	-.02907	.14142	.977	-.3630	.3049
	bhlh038-2	-.21183	.14707	.322	-.5591	.1355

Table 1.7: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 1.0 in control.

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	1.566	2	.783	.560	.572
Within Groups	274.206	196	1.399		
Total	275.771	198			

Table 1.8: Multiple Comparisons

Dependent Variable: time_taken
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bhlh038-2	-.16731	.20934	.704	-.6617	.3271
	bhlh038-4	.04648	.19922	.970	-.4240	.5170
bhlh038-2	Col-0	.16731	.20934	.704	-.3271	.6617
	bhlh038-4	.21379	.21002	.566	-.2822	.7098
bhlh038-4	Col-0	-.04648	.19922	.970	-.5170	.4240
	bhlh038-2	-.21379	.21002	.566	-.7098	.2822

Table 1.9: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.1 in ½ MS with 75mM NaCl

time_taken

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	***10.461***	***2***	***5.230***	***3.720***	***.026***
Within Groups	***216.539***	***154***	***1.406***		
Total	***227.000***	***156***			

Table 1.10: Multiple Comparisons*:*

Dependent Variable: time_taken
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	*bhlh038-2*	*-.67410**	.24787	.020	-1.2607	-.0875
	bhlh038-4	*-.21667*	.21649	.577	-.7290	.2957
bhlh038-2	*Col-0*	*.67410**	.24787	.020	.0875	1.2607
	bhlh038-4	*.45743*	.24787	.158	-.1292	1.0440
bhlh038-4	*Col-0*	*.21667*	.21649	.577	-.2957	.7290
	bhlh038-2	*-.45743*	.24787	.158	-1.0440	.1292

**. The mean difference is significant at the 0.05 level.*

Table 1.11: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.5 in ½ MS with 75mM NaCl

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	***57.782***	***2***	***28.891***	***19.741***	***.000***
Within Groups	***220.985***	***151***	***1.463***		
Total	***278.766***	***153***			

Table 1.12: Multiple Comparisons

Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-1.39080*	.25811	.000	-2.0017	-.7799
	bhlh038-4	.12825	.22180	.832	-.3968	.6533
bhlh038-2	Col-0	1.39080*	.25811	.000	.7799	2.0017
	bhlh038-4	1.51905*	.25730	.000	.9100	2.1281
bhlh038-4	Col-0	-.12825	.22180	.832	-.6533	.3968
	bhlh038-2	-1.51905*	.25730	.000	-2.1281	-.9100

*. The mean difference is significant at the 0.05 level.

Table 1.13: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.7 in ½ MS with 75mM NaCl

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	58.013	2	29.007	19.093	.000
Within Groups	230.922	152	1.519		
Total	***288.935***	***154***			

Table 1.14: Multiple Comparisons

Dependent Variable: time_taken
Tukey HSD

(I) genotype	*(J) genotype*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*	*95% Confidence Interval*	
					Lower Bound	*Upper Bound*
Col-0	bhlh038-2	-1.27262*	.26216	.000	-1.8931	-.6521
	bhlh038-4	.30833	.22504	.359	-.2243	.8410
bhlh038-2	Col-0	1.27262*	.26216	.000	.6521	1.8931
	bhlh038-4	1.58095*	.26216	.000	.9605	2.2015
bhlh038-4	Col-0	-.30833	.22504	.359	-.8410	.2243
	bhlh038-2	-1.58095*	.26216	.000	-2.2015	-.9605

***.** The mean difference is significant at the 0.05 level**.**

Table 1.15: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 1.0 in ½ MS with 75mM NaCl

time_taken

	Sum of Squares	*df*	*Mean Square*	*F*	*Sig.*
Between Groups	8.363	2	4.181	4.915	.009
Within Groups	117.396	138	.851		
Total	125.759	140			

Table 1.16: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

(I) genotype	*(J) genotype*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*	*95% Confidence Interval*	
					Lower Bound	*Upper Bound*
Col-0	bhlh038-2	-.58485*	.22988	.032	-1.1295	-.0402
	bhlh038-4	.13164	.16911	.717	-.2690	.5323
bhlh038-2	Col-0	.58485*	.22988	.032	.0402	1.1295
	bhlh038-4	.71649*	.23041	.006	.1706	1.2624
bhlh038-4	Col-0	-.13164	.16911	.717	-.5323	.2690
	bhlh038-2	-.71649*	.23041	.006	-1.2624	-.1706

*. The mean difference is significant at the 0.05 level.

Table 1.17: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.1 in ½ MS with 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	18.327	2	9.163	5.553	.004
Within Groups	476.881	289	1.650		
Total	495.208	291			

Table 1.18: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.55198*	.18783	.010	-.9945	-.1095
	bhlh038-4	-.00098	.17857	1.000	-.4217	.4197
bhlh038-2	Col-0	.55198*	.18783	.010	.1095	.9945
	bhlh038-4	.55100*	.18824	.010	.1075	.9945
bhlh038-4	Col-0	.00098	.17857	1.000	-.4197	.4217
	bhlh038-2	-.55100*	.18824	.010	-.9945	-.1075

****. The mean difference is significant at the 0.05 level.***

Table 1.19: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.5 in ½ MS with 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	37.523	2	18.762	19.012	.000
Within Groups	282.240	286	.987		
Total	319.763	288			

Table 1.20: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Col-0	bhlh038-2	-.71918*	.14669	.000	-1.0648	-.3736
	bhlh038-4	.12881	.13878	.623	-.1982	.4558
bhlh038-2	Col-0	.71918*	.14669	.000	.3736	1.0648
	bhlh038-4	.84799*	.14573	.000	.5046	1.1913
bhlh038-4	Col-0	-.12881	.13878	.623	-.4558	.1982
	bhlh038-2	***-.84799****	***.14573***	***.000***	***-1.1913***	***-.5046***

. The mean difference is significant at the 0.05 level.

Table 1.21: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 0.7 in ½ MS with 200mM Mannitol

time_taken

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	56.376	2	28.188	19.704	.000
Within Groups	407.704	285	1.431		
Total	464.080	287			

Table 1.22: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Col-0	bhlh038-2	-.91847*	.17720	.000	-1.3359	-.5010
	bhlh038-4	.10534	.16709	.803	-.2883	.4990
bhlh038-2	Col-0	.91847*	.17720	.000	.5010	1.3359
	bhlh038-4	1.02381*	.17604	.000	.6090	1.4386
bhlh038-4	Col-0	-.10534	.16709	.803	-.4990	.2883
	bhlh038-2	-1.02381*	.17604	.000	-1.4386	-.6090

*. The mean difference is significant at the 0.05 level.

Table 1.23: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/bhlh038-4*) to reach Developmental Stage 1.0 in ½ MS with 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	103.854	2	51.927	18.161	.000
Within Groups	786.290	275	2.859		
Total	890.145	277			

Table 1.24: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-1.29409*	.25874	.000	-1.9038	-.6844
	bhlh038-4	.15894	.23679	.780	-.3990	.7169
bhlh038-2	Col-0	1.29409*	.25874	.000	.6844	1.9038
	bhlh038-4	1.45303*	.25768	.000	.8458	2.0602
bhlh038-4	Col-0	-.15894	.23679	.780	-.7169	.3990
	bhlh038-2	-1.45303*	.25768	.000	-2.0602	-.8458

*. The mean difference is significant at the 0.05 level.

Table 1.25: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/*4.3.6ox) to reach Developmental Stage 0.1 in control

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	1.116	2	.558	2.387	.096
Within Groups	29.446	126	.234		
Total	30.562	128			

Table 1.26: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.18328	.10488	.192	-.4320	.0655
	4.3.6ox	.02722	.10366	.963	-.2186	.2731
bhlh038-2	Col-0	.18328	.10488	.192	-.0655	.4320
	4.3.6ox	.21050	.10429	.112	-.0368	.4578
4.3.6ox	Col-0	-.02722	.10366	.963	-.2731	.2186
	bhlh038-2	***-.21050***	***.10429***	***.112***	***-.4578***	***.0368***

Table 1.27: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.5 in control

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	1.109	2	.555	1.494	.228
Within Groups	46.778	126	.371		
Total	47.888	128			

Table 1.28: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.21899	.13219	.226	-.5325	.0945
	4.3.6ox	-.05233	.13066	.915	-.3622	.2576
bhlh038-2	Col-0	.21899	.13219	.226	-.0945	.5325
	4.3.6ox	.16667	.13144	.416	-.1451	.4784
4.3.6ox	Col-0	.05233	.13066	.915	-.2576	.3622
	bhlh038-2	***-.16667***	***.13144***	***.416***	***-.4784***	***.1451***

Table 1.29: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.7 in control

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	.350	2	.175	.337	.715
Within Groups	65.456	126	.519		
Total	65.806	128			

Table 1.30: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.11240	.15637	.753	-.4833	.2585
	4.3.6ox	-.10862	.15456	.762	-.4752	.2580
bhlh038-2	Col-0	.11240	.15637	.753	-.2585	.4833
	4.3.6ox	.00379	.15548	1.000	-.3650	.3726
4.3.6ox	Col-0	.10862	.15456	.762	-.2580	.4752
	bhlh038-2	***-.00379***	***.15548***	***1.000***	***-.3726***	***.3650***

Table 1.31: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 1.0 in control

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	.221	2	.111	.102	.903
Within Groups	131.239	121	1.085		
Total	131.460	123			

***Table 1.32*: Multiple Comparisons**
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.09398	.23317	.914	-.6473	.4593
	4.3.6ox	-.08442	.22467	.925	-.6175	.4487
bhlh038-2	Col-0	.09398	.23317	.914	-.4593	.6473
	4.3.6ox	.00957	.23064	.999	-.5377	.5568
4.3.6ox	Col-0	.08442	.22467	.925	-.4487	.6175
	bhlh038-2	-.00957	.23064	.999	-.5568	.5377

Table 1.33: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.1 in 100mM NaCl

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	55.400	2	27.700	16.218	.000
Within Groups	181.041	106	1.708		
Total	236.440	108			

***Table 1.34:* Multiple Comparisons**
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-1.07143*	.31240	.002	-1.8140	-.3288
	4.3.6ox	.65165	.30429	.086	-.0717	1.3750
bhlh038-2	Col-0	1.07143*	.31240	.002	.3288	1.8140
	4.3.6ox	1.72308*	.30429	.000	.9998	2.4464
4.3.6ox	Col-0	-.65165	.30429	.086	-1.3750	.0717
	bhlh038-2	-1.72308*	.30429	.000	-2.4464	-.9998

*. The mean difference is significant at the 0.05 level.

Table 1.35: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.5 in 100mM NaCl

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	121.903	2	60.951	28.798	.000
Within Groups	196.837	93	2.117		
Total	318.740	95			

Table 1.36: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-1.84048*	.36197	.000	-2.7026	-.9783
	4.3.6ox	.93963*	.35881	.028	.0850	1.7943
bhlh038-2	Col-0	1.84048*	.36197	.000	.9783	2.7026
	4.3.6ox	2.78011*	.37259	.000	1.8927	3.6676
4.3.6ox	Col-0	-.93963*	.35881	.028	-1.7943	-.0850
	bhlh038-2	-2.78011*	.37259	.000	-3.6676	-1.8927

*. The mean difference is significant at the 0.05 level.

Table 1.37: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.7 in 100mM NaCl

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	73.290	2	36.645	21.278	.000
Within Groups	160.168	93	1.722		
Total	233.458	95			

Table 1.38: **Multiple Comparisons**
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-1.43214*	.33274	.000	-2.2247	-.6396
	4.3.6ox	.74589	.31843	.055	-.0125	1.5043
bhlh038-2	Col-0	1.43214*	.33274	.000	.6396	2.2247
	4.3.6ox	2.17803*	.33719	.000	1.3749	2.9812
4.3.6ox	Col-0	-.74589	.31843	.055	-1.5043	.0125
	bhlh038-2	***-2.17803****	***.33719***	***.000***	***-2.9812***	***-1.3749***

****. The mean difference is significant at the 0.05 level.***

Table 1.39: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 1.0 in 100mM NaCl

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	***43.321***	***2***	***21.660***	***19.174***	***.000***
Within Groups	***100.538***	***89***	***1.130***		
Total	***143.859***	***91***			

Table 1.40: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.1 in 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	***61.712***	***2***	***30.856***	***53.120***	***.000***
Within Groups	***71.447***	***123***	***.581***		
Total	***133.159***	***125***			

Table 1.41: **Multiple Comparisons**
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-1.45122*	.16833	.000	-1.8506	-1.0519
	4.3.6ox	.07899	.16544	.882	-.3135	.4715
bhlh038-2	Col-0	1.45122*	.16833	.000	1.0519	1.8506
	4.3.6ox	1.53021*	.16544	.000	1.1377	1.9227
4.3.6ox	Col-0	-.07899	.16544	.882	-.4715	.3135
	bhlh038-2	-1.53021*	.16544	.000	-1.9227	-1.1377

*. The mean difference is significant at the 0.05 level.

Table 1.42: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.5 in 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	22.699	2	11.350	10.619	.000
Within Groups	125.048	117	1.069		
Total	147.748	119			

Table 1.43: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 0.7 in 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	12.683	2	6.342	7.747	.001
Within Groups	99.049	121	.819		
Total	111.732	123			

Table 1.44: Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.65238*	.19989	.004	-1.1267	-.1781
	4.3.6ox	.05952	.19743	.951	-.4090	.5280
bhlh038-2	Col-0	.65238*	.19989	.004	.1781	1.1267
	4.3.6ox	.71190*	.19989	.002	.2376	1.1862
4.3.6ox	Col-0	-.05952	.19743	.951	-.5280	.4090
	bhlh038-2	-.71190*	.19989	.002	-1.1862	-.2376

*. The mean difference is significant at the 0.05 level.

Table 1.45: One-Way ANOVA test for time taken by seedlings (Col-0/ *bhlh038-2/4.3.6ox*) to reach Developmental Stage 1.0 in 200mM Mannitol

time_taken

	Sum of Squares	***df***	***Mean Square***	***F***	***Sig.***
Between Groups	14.831	2	7.416	5.787	.004
Within Groups	146.092	114	1.282		
Total	160.923	116			

Table 1.46 Multiple Comparisons
Dependent Variable: time_taken
Tukey HSD

					95% Confidence Interval	
(I) genotype	***(J) genotype***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***Lower Bound***	***Upper Bound***
Col-0	bhlh038-2	-.57778	.26007	.072	-1.1954	.0398
	4.3.6ox	.29146	.25158	.480	-.3060	.8889
bhlh038-2	Col-0	.57778	.26007	.072	-.0398	1.1954
	4.3.6ox	.86924*	.25856	.003	.2552	1.4832
4.3.6ox	Col-0	-.29146	.25158	.480	-.8889	.3060
	bhlh038-2	-.86924*	.25856	.003	-1.4832	-.2552

*. The mean difference is significant at the 0.05 level.

2.1 Root growth and statistical analysis

Table 2.1: Pairwise Comparisons of average root length between different genotypes of *Arabidopsis* seedlings in ½ MS media (control)
Measure: root_length

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
1	2	2.376	2.354	.953	-3.460	8.213
	3	1.614	2.217	1.000	-3.883	7.111
2	1	-2.376	2.354	.953	-8.213	3.460
	3	-.763	1.379	1.000	-4.181	2.656
3	1	-1.614	2.217	1.000	-7.111	3.883
	2	.763	1.379	1.000	-2.656	4.181

Based on estimated marginal means
*Genotype 1= Col-0, 2= *bhlh038-2* and 3= *bhlh038-4*
a. Adjustment for multiple comparisons: Bonferroni.

Table 2.2: Pairwise Comparisons in average root length between different genotypes of Arabidopsis seedlings exposed to ½ MS media supplemented with 75mM NaCl.
Measure: root_length_

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a] Lower Bound	Upper Bound
1	2	.196	1.370	1.000	-3.199	3.591
	3	-2.390	1.704	.501	-6.613	1.833
2	1	-.196	1.370	1.000	-3.591	3.199
	3	-2.586	2.038	.632	-7.639	2.467
3	1	2.390	1.704	.501	-1.833	6.613
	2	2.586	2.038	.632	-2.467	7.639

Based on estimated marginal means
*Genotype 1= Col-0, 2= *bhlh038-2* and 3= *bhlh038-4*
a. Adjustment for multiple comparisons: Bonferroni.

Table 2.3: Pairwise Comparisons in average root length between different genotypes of Arabidopsis seedlings exposed to ½ MS media supplemented with 200mM Mannitol
Measure: _root_length

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
1	2	.736*	.269	.026	.070	1.403
	3	.493	.276	.240	-.191	1.176
2	1	-.736*	.269	.026	-1.403	-.070
	3	-.244	.247	.984	-.855	.368
3	1	-.493	.276	.240	-1.176	.191
	2	.244	.247	.984	-.368	.855

Based on estimated marginal means
*. The mean difference is significant at the .05 level.
*Genotype 1= Col-0, 2= *bhlh038-2* and 3= *bhlh038-4*
b. Adjustment for multiple comparisons: Bonferroni.

Table 2.4: Pairwise Comparisons in average root length between different genotypes of Arabidopsis seedlings grown in ½ MS media (control).
Measure: Root length

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
1	2	3.770	1.749	.115	-.634	8.173
	3	-5.309	2.500	.123	-11.606	.988
2	1	-3.770	1.749	.115	-8.173	.634
	3	-9.079*	1.876	.000	-13.803	-4.354
3	1	5.309	2.500	.123	-.988	11.606
	2	9.079*	1.876	.000	4.354	13.803

Based on estimated marginal means
*. The mean difference is significant at the .05 level.
*Genotype 1= Col-0, 2= *bhlh038-2* and 3= 4.3.6ox
b. Adjustment for multiple comparisons: Bonferroni.

Table 2.5: Pairwise Comparisons in average root length between different genotypes of Arabidopsis seedlings exposed to ½ MS media supplemented with 100mM NaCl.
Measure: Root length

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
1	2	4.464*	.875	.000	2.260	6.669
	3	-6.398*	1.052	.000	-9.046	-3.750
2	1	-4.464*	.875	.000	-6.669	-2.260
	3	-10.863*	.817	.000	-12.921	-8.804
3	1	6.398*	1.052	.000	3.750	9.046
	2	10.863*	.817	.000	8.804	12.921

Based on estimated marginal means
*. The mean difference is significant at the .05 level.
*Genotype 1= Col-0, 2= *bhlh038-2* and 3= 4.3.6ox
b. Adjustment for multiple comparisons: Bonferroni.

Table 2.6: Pairwise Comparisons in average root length between different genotypes of Arabidopsis seedlings exposed to ½ MS media supplemented with 200mM Mannitol.
Measure: _root_length_

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
1	2	1.209*	.382	.010	.247	2.170
	3	-.011	.489	1.000	-1.243	1.222
2	1	-1.209*	.382	.010	-2.170	-.247
	3	-1.220*	.419	.019	-2.276	-.164
3	1	.011	.489	1.000	-1.222	1.243
	2	1.220*	.419	.019	.164	2.276

Based on estimated marginal means
*. The mean difference is significant at the .05 level.
*Genotype 1= Col-0, 2= *bhlh038-2* and 3= 4.3.6ox
b. Adjustment for multiple comparisons: Bonferroni.

3.1 Photosynthetic Parameters

Table 3.1: t-test (Tukey) **Fv/Fm** of mannitol treated 4-week-old Arabidopsis plants of different genotypes (Col-0/ *bhlh038-2/ bhlh038-4*/ 4.3.6ox) on day 1 of treatment.

Fv_Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	1.607	.210
Within Groups	.002	28	.000		
Total	.002	31			

Table 3.2: Multiple Comparisons
Dependent Variable: Fv_Fm
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bh-2	.004750	.004300	.690	-.00699	.01649
	bh-4	-.004000	.004300	.789	-.01574	.00774
	4.3.6ox	.003125	.004300	.886	-.00862	.01487
bh-2	Col-0	-.004750	.004300	.690	-.01649	.00699
	bh-4	-.008750	.004300	.200	-.02049	.00299
	4.3.6ox	-.001625	.004300	.981	-.01337	.01012
bh-4	Col-0	.004000	.004300	.789	-.00774	.01574
	bh-2	.008750	.004300	.200	-.00299	.02049
	4.3.6ox	.007125	.004300	.365	-.00462	.01887
4.3.6ox	Col-0	-.003125	.004300	.886	-.01487	.00862
	bh-2	.001625	.004300	.981	-.01012	.01337
	bh-4	-.007125	.004300	.365	-.01887	.00462

Table 3.3: t-test (Tukey) **Fv/Fm** of mannitol treated 4-week-old Arabidopsis plants of different genotypes (Col-0/ *bhlh038-2/ bhlh038-4*/ 4.3.6ox) on day 3 of treatment.
Fv_Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.746	.534
Within Groups	.005	28	.000		
Total	.005	31			

Table 3.4: Multiple Comparisons
Dependent Variable: Fv_Fm
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bh-2	.004250	.006514	.914	-.01353	.02203
	bh-4	-.002625	.006514	.977	-.02041	.01516
	4.3.6ox	.006125	.006514	.784	-.01166	.02391
bh-2	Col-0	-.004250	.006514	.914	-.02203	.01353
	bh-4	-.006875	.006514	.719	-.02466	.01091
	4.3.6ox	.001875	.006514	.992	-.01591	.01966
bh-4	Col-0	.002625	.006514	.977	-.01516	.02041
	bh-2	.006875	.006514	.719	-.01091	.02466
	4.3.6ox	.008750	.006514	.544	-.00903	.02653
4.3.6ox	Col-0	-.006125	.006514	.784	-.02391	.01166
	bh-2	-.001875	.006514	.992	-.01966	.01591
	bh-4	-.008750	.006514	.544	-.02653	.00903

Table 3.5: t-test (Tukey) **Fv/Fm** of mannitol treated 4-week-old Arabidopsis plants of different genotypes (Col-0/ *bhlh038-2/ bhlh038-4*/ 4.3.6ox) on day 1 of treatment.

Fv_Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	3	.000	.368	.777
Within Groups	.005	27	.000		
Total	.005	30			

Table 3.6: Multiple Comparisons
Dependent Variable: Fv_Fm
Tukey HSD

(I) genotype	(J) genotype	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Col-0	bh-2	.002125	.006881	.990	-.01671	.02096
	bh-4	.006375	.006648	.773	-.01182	.02457
	4.3.6ox	.005000	.006648	.875	-.01319	.02319
bh-2	Col-0	-.002125	.006881	.990	-.02096	.01671
	bh-4	.004250	.006881	.926	-.01458	.02308
	4.3.6ox	.002875	.006881	.975	-.01596	.02171
bh-4	Col-0	-.006375	.006648	.773	-.02457	.01182
	bh-2	-.004250	.006881	.926	-.02308	.01458
	4.3.6ox	-.001375	.006648	.997	-.01957	.01682
4.3.6ox	Col-0	-.005000	.006648	.875	-.02319	.01319
	bh-2	-.002875	.006881	.975	-.02171	.01596
	bh-4	.001375	.006648	.997	-.01682	.01957

Table 3.7: t-test (Tukey) **Fv/Fm** of control and mannitol treated 4-week-old Arabidopsis Col-0 plants on day 1 of treatment.

Fv_Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.720	.410
Within Groups	.002	14	.000		
Total	.002	15			

Table 3.8: t-test (Tukey) **Fv/Fm** of control and mannitol treated 4-week-old Arabidopsis Col-0 plants on day 3 of treatment.

Fv_Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.084	.777
Within Groups	.002	14	.000		
Total	.002	15			

Table 3.9: t-test (Tukey) **Fv/Fm** of control and mannitol treated 4-week-old Arabidopsis Col-0 plants on day 5 of treatment.

Fv_Fm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	1	.001	5.278	.038
Within Groups	.002	14	.000		
Total	.003	15			

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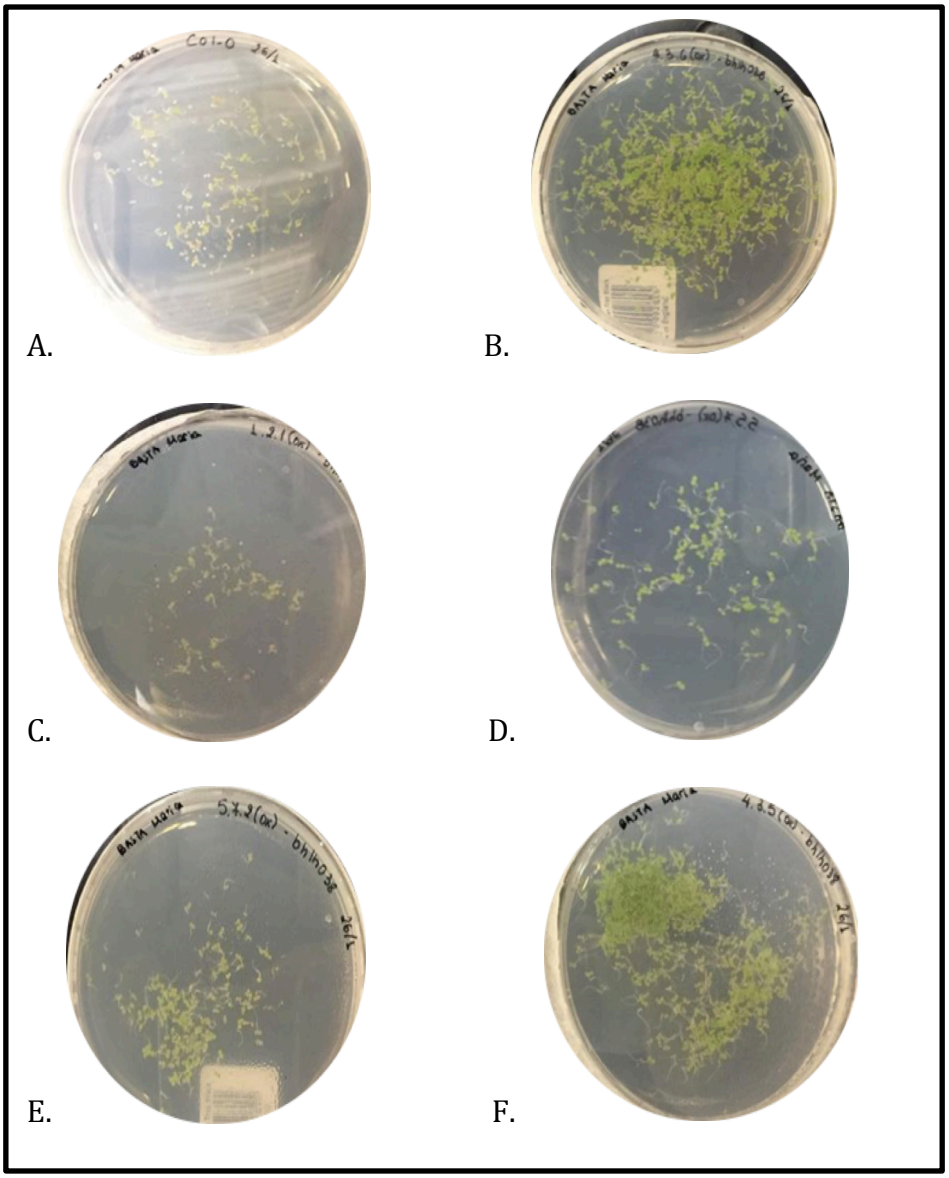

Fig.1: ½ MS media plates supplemented with BASTA for selection of Arabidopsis transformants. (A) Col-0 seedlings (B) 4.3.6ox (overexpressing line of BHLH038) (C) 1.2.1ox (D) 5.5.7ox (E) 5.7.2ox (F) 4.3.5ox

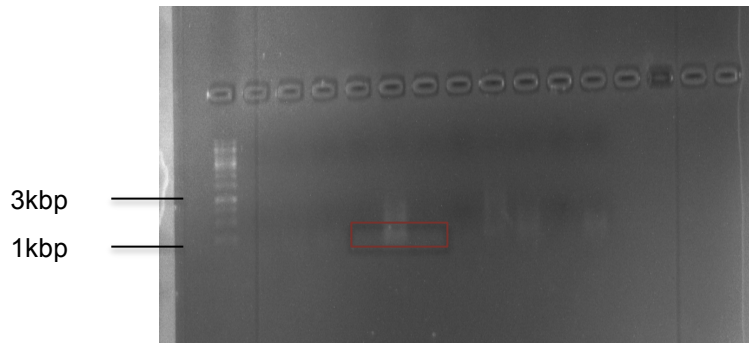

Fig.2: Screening for T-DNA insertion lines for BHLH039. No T-DNA insertions are present. DNA band present is ~1700bp, which is the full genomic length of BHLH039 TF gene.

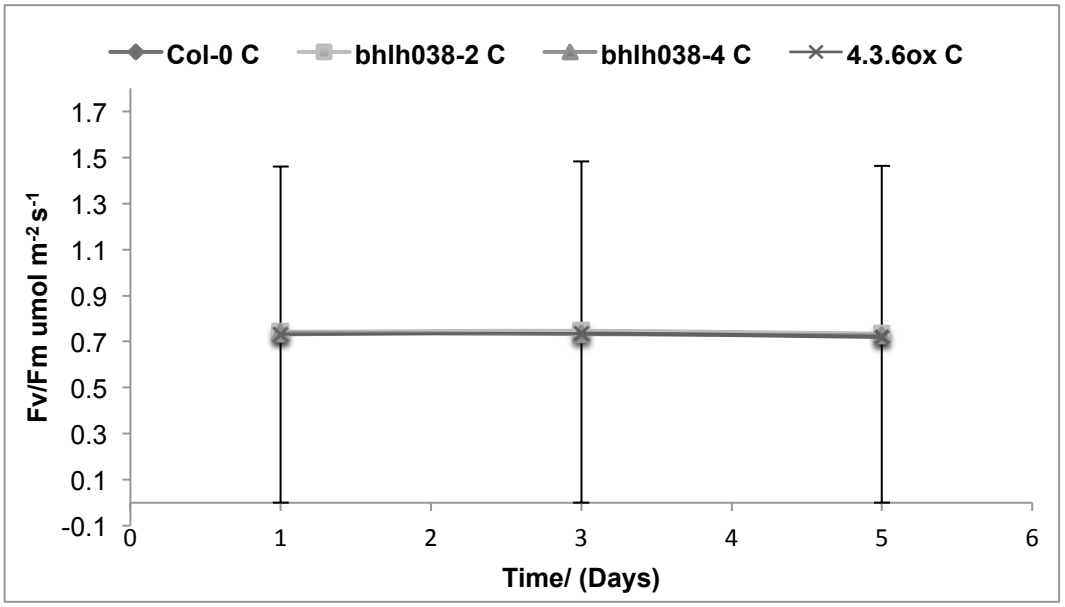

Fig.3: Fv/Fm in rosette leaves of 4 weeks old Arabidopsis thaliana plants under control conditions. No significance observed.

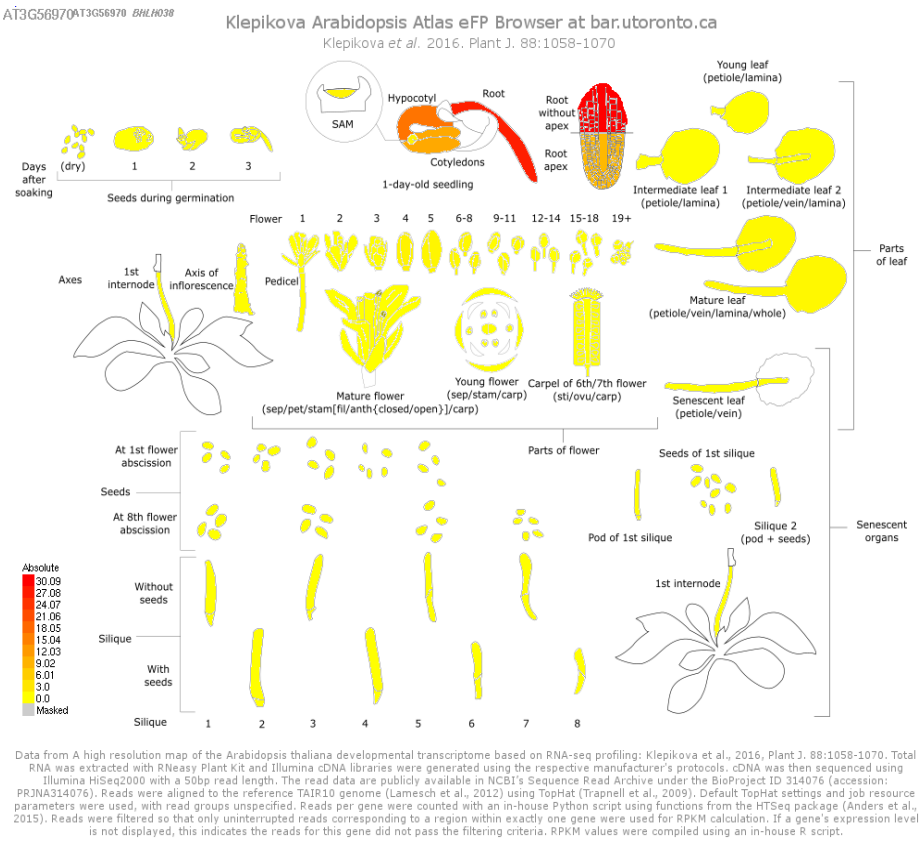

Fig.4: Graphic representation of bHLH038 absolute gene expression in Col-0 (WT) *Arabidopsis thaliana* plants, in different tissues of plant. Generated by eFP (Winter, D., 2007).