IDENTIFICATION AND FUNCTIONAL ANALYSIS OF AN AUTOPHAGY-RELATED GENE, *TdAtg8*, IN WILD EMMER WHEAT UNDER BIOTIC (*Fusarium culmorum)* AND ABIOTIC (Drought) STRESS CONDITIONS

by

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Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
August 2010

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DATE OF APPROVAL : 03/08/2010

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ABSTRACT

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MS Thesis, 2010

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Keywords: Autophagy, Atg8, drought stress, wild emmer wheat, pathogen

Autophagy, literally self eating, is an evolutionary conserved catalytic process for vacuolar degradation of intracellular components, previously examined in yeast, mammals and plants. Abiotic stress factors, including nutrient starvation, oxidative stress, salt stress and osmotic stress have been previously reported to induce autophagy in plants. In this study, for the first time, *Atg8* gene was cloned from wild emmer wheat *(TdAtg8)* and the role of autophagy under biotic and abiotic stress conditions was investigated. Examination of *TdAtg8* expression patterns indicates that *Atg8* expression was immensely upregulated under drought stress, especially in the roots. Monodansylcadaverine (MDC) and Lysotracker Red markers utilized to observe autophagosomes revealed that autophagy is constitutively active in *T.dicoccoides*. Moreover, autophagy was determined to be more active in plants exposed to drought stress when compared to plants grown under normal conditions. *TdAtg8* gene was demonstrated to complement *Atg8* yeast mutants grown under starvation conditions in a drop test assay. For further functional analysis, ATG8 protein from *T. dicoccoides* were expressed in yeast and analyzed with western blotting. *TdAtg8* was also silenced in wild emmer wheat by virus-induced gene silencing and its role was investigated in the presence of a plant pathogen, *Fusarium culmorum.* This response, for the first time, showed that fungi sporulation decreased in *Atg8* silenced plants in comparison to controls. Based on the data obtained, we conclude that the plants become more resistant against the plant pathogen when the autophagy was inhibited.

ÖZET

OTOFAJİ İLE BAĞDAŞTIRILAN *TdAtg8* GENİNİN BİOTİK (*Fusarium culmorum*) VE ABİOTİK (Kuraklık) KOŞULLAR ALTINDA YABANİ BUĞDAYDA TANIMLANMASI VE FONKSİYONEL ANALİZİ

Duygu Kuzuoğlu

Master Tezi, 2010

Doç. Dr. Hikmet Budak (Tez Danışmanı)

Anahtar kelimeler: Otofaji, Atg8, kuraklık stresi, yabani buğday, patojen

Kendi kendini yeme anlamına gelen otofaji, sitoplazma içeriğinin kofullarda ya da lizozomlarda parçalanmasını amaçlayan, evrimsel olarak korunan katalitik bir süreçtir. Daha önce maya, memeli ve bitkilerde tespit edilen otofajinin indüklenmesi ile sitoplazmik bileşenleri içeren çift zarlı, otofagozom adı verilen yapı, kofullara veya lizozonlara yönlendirilir. Tanımlanmış 30 otofaji geninin arasında en yaygın olarak çalışılan *Atg8* geni otofagosomların oluşturulmasında görev almaktadır. Otofajinin; açlık, oksidatif stress, tuz stresi, ozmotik stres gibi çevresel stres koşullarında aktive olduğu belirlenmiştir. Bu çalışmada, bilgimiz dahilinden ilk defa, yabani buğdayın *Atg8* geni tanımlanmış, *TdAtg8* olarak isimlendirilmiş ve otofajinin abiotik ve biotik stres koşullarındaki rolü incelenmiştir. *TdAtg8* geninin ifade profilinin incelenmesi ile, kuraklık koşulları altında *TdAtg8* geninin özellikle köklerde daha çok ifade edildiği belirlenmiştir. Otofagozomların incelenmesi için, bitki çalışmalarında kullanılan, "Monodansylcadaverine" (MDC) ve "Lysotracker" kırmızısı isimli iki ayrı boyadan yararlanılmıştır. Otofagozomların boyanması ile otofajinin temel hücrelerde de aktif olduğu, kuraklık koşullarında ise indüklendiği tespit edilmiştir. *TdAtg8* geninin, mutant maya hücrelerine aktarılması ile *TdAtg8* geninin maya *Atg8* geni ile ortolog olduğu ve fonksiyonel olarak mayada otofajinin aktive olmasını sağladığı gözlenmiştir. TdATG8 proteini mayada ifade edilmiş ve "western blot" yöntemi ile analiz edilmiştir. Yabani buğdayın *TdAtg8* geni, virus indüklenmesi ile gen susturma yöntemi kullanılarak susturulmuş ve otofajinin *Fusarium culmorum* isimli bitki patojeninin varlığında nasıl görev aldığı belirlenmiştir. Yapılan analizde, susturulmuş bitkilerde mantar sporlanmasının çok daha az olduğu gözlenmiştir. Sonuç olarak, *TdAtg8* geninin susturulması halinde, bitkinin patojene karşı daha dayanıklı hale geldiği tespit edilmiştir.

ACKNOWLEDGEMENTS

I would like to express my strong appreciation to my supervisor Assoc. Prof. Hikmet Budak for his guidance and his belief in me during the project. His positive attitude motivated and encouraged me every time. I thank him for sharing his knowledge with me.

I would like to show my gratitude to my thesis commitee members, Assist. Prof. Alpay Taralp, Assist. Prof. Devrim Gözüaçık, Assist. Prof. Halil Kavaklı and Assoc. Prof. Levent Öztürk for their critical suggestions and excellent remarks on my thesis.

This thesis would not have been possible without the endless assistance and precious ideas of my collegues Özge Cebeci Yalçınkaya, Mine Türktaş, Bahar Soğutmaz Özdemir, Turgay Ünver and Stuart Lucas. They were always there to answer my incessant questions.

I would also like to thank Gözde Korkmaz and Gözüaçık Lab members for their help in western blotting analyses.

I am also grateful to my lab members Bala Anı Akpınar, Ayşegül Altıntaş, Mine Bakar, Melda Kantar, Ahmed El Ghareb, Amer Ahmed and all my friends in Sabanci University for making my excessive working hours tolerable. It would be very difficult to finalize this study without them.

I would like to thank to TUBITAK-BIDEB for their financial support during my master study. This project was partially supported by EU-FP6 Cost Action.

I owe my deepest gratitude to my friends Canan Akyüz, Ozan Çağlayan, Hande Erguner, Ezgi Tunç, Çiğdem Vaizoğlu and Emre Yetkin to make my life enjoyable.

Finally, I want to thank my whole family, especially my parents Füsun and Nüvit Kuzuoğlu and my sister İdil Kuzuoğlu for their indefinable help in my life and Can Akın Öztürk to be with me every time I need him.

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ABBREVIATIONS

μM	Micromolar
AD	Activation domain
Amp	Ampicillin
API	Aminopeptidase I
Avr proteins	Avirulence proteins
BSA	bovine serum albumin
BSMV	Barley mosaic stripe virus
cDNA	Complementary DNA
CDS	Coding sequence
Cm	Centimeter
CMA	Chaperone-mediated autophagy
CVT	Cytoplasm-to-vacuole
DEPC	Diethylpyrocarbonate
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraaceticacid
G	Gram
GFP	Green fluorescent protein
H	Hour
HR	Hypersensitive response
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kg	Kilogram
L	Liter
LB	Left border

LB	Luria-Bertani
MDC	Monodansylcadaverine
Mg	Milligram
Min	Minute
Ml	Milliliter
Mmol	Millimol
mRNA	Messanger RNA
MS	Murashige –Skoog basalt salt medium
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAS	Preautophagosomal structure
PBS	phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDS	Phytoene desaturase
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PI3K	Phosphatidylinositol 3-OH kinase
PI3P	Phosphatidylinositol 3-phosphate
PTGS	Post-transcriptional gene silencing
PVX	Potato virus X
Q-RT PCR	Real-time PCR
R proteins	Resistance proteins
RB	Right Border

RISC	RNA interference silencing complex
RNAi	RNA interference
SDS	Sodium Dodecyl Sulfate
siRNA	Small interfering RNA
TdAtg8	*Triticum dicoccoides* Atg8
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TGMV	Tomato golden mosaic virus
TMV	Tobacco mosaic virus
TOR	Target of rapamycin
TRV	Tobacco rattle virus
VIGS	Virus-induced gene silencing
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

1 INTRODUCTION

Plants are organisms which have to cope with several biotic and abiotic stress conditions to survive. For this purpose, they need to develop different mechanisms to protect themselves. Autophagy, self eating, is a mechanism utilized by plants to respond to stress conditions. Previous studies indicated that autophagy is induced under nutrient starvation conditions, such as sucrose, carbon and nitrogen deficiency (Chen et al., 1994; Contento et al., 2004; Doelling et al., 2002; Hanaoka et al., 2002; Xiong et al., 2005), drought stress, salt stress (Liu et al., 2009), oxidative stress (Xiong et al., 2007b) and also in viral infection (Liu et al., 2002a; 2002b; Liu et al., 2005). In this study, we will examine the role of autophagy mechanism in *Triticum dicoccoides,* under different stress conditions, such as drought stress and viral infection. *T. dicoccoides,* wild emmer wheat, was chosen as a model organism, since it is the progenitor of modern wheat (Dvorak & Akhunov, 2005). Wheat is the one of the most valuable crops in the world; it provides more than 60% of human diet together with barley, maize and rice (Harlan, 1992; Zohary & Hopf, 2000). Therefore understanding the protection mechanisms of wheat will help us in plant breeding studies to develop more resistant plants to different stress conditions.

2 OVERVIEW

2.1 *Triticum dicoccoides* as a model organism

Human history analyses have reported obviously the role of wheat in agricultural and economical development of human culture. As a staple crop in more than 40 countries, wheat is one of the main crops of old world agriculture (Harlan, 1992; Williams, 1993; Zohary & Hopf, 2000). Providing more than 60% of human diet together with rice and maize, wheat is domesticated between 12000 and 10000 years ago in Fertile Crescent (Gill et al., 2004; Nesbitt & Samuel, 1996; Tanno & Villcox, 2006). The modern, domesticated forms of wheat are tetraploid durum wheat, *T. durum* (2n=28, AABB) and hexaploid bread wheat, *T. aestivum* (2n=42, AABBDD). Recent studies have indicated that 360,000 years old wild emmer wheat, *Triticum dicoccoides* (AABB) is the progenitor of modern wheat (Dvorak & Akhunov, 2005; Ozkan et al., 2010). , *Triticum dicoccoides* was firstly discovered in 1906 by Aaron Aaronsohn in Northern Israel (Aaronsohn, 1910). Last studies showed that wild emmer wheat still grows in the western Turkey, in the mountain areas in eastern Iraq and western Iran (Ozkan et al., 2010).

As a model organism, wild emmer wheat was found to be resistant to stripe rust (Gerechter-Amitai & Stubbs 1970; Nevo et al., 1986; Fahima et al., 1998), stem rust (Nevo et al., 1991) and powdery mildew (Nevo et al., 1985) and to be tolerant to poor soil and climatic factors (Nevo 1983; 1989; 1995). These characteristics of wild emmer wheat were showed that *Triticum dicoccoides* having rich genetic resource can be used as a model organism in plant breeding studies (Nevo 1983; 1989; 1995; Peng et al., 2000). The evolution tree of polyploid wheat was given in Fig 2.1.

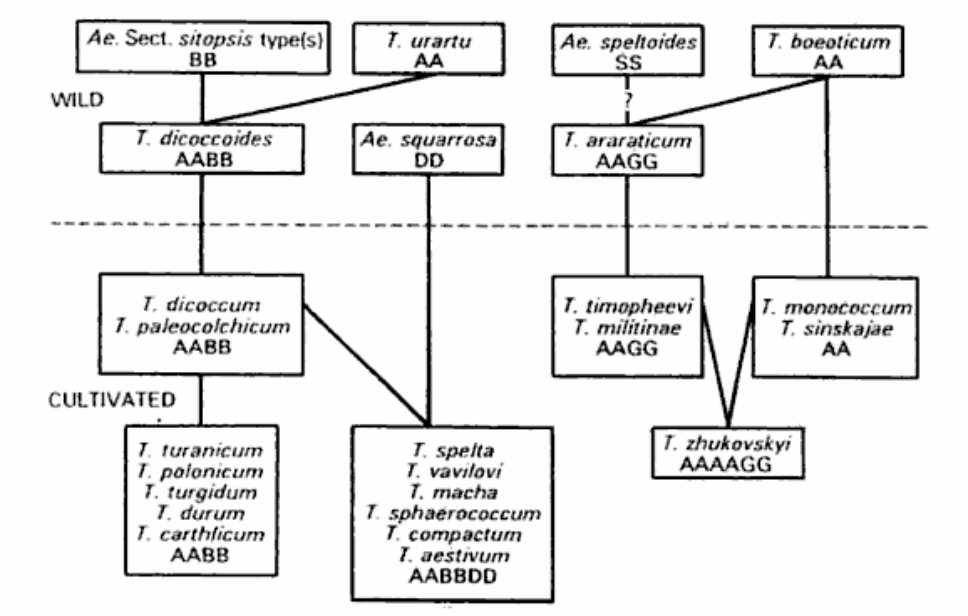

Fig 2.1 Evolution of polyploid wheat (Nevo et al., 2002)

2.2 Autophagy in plants

Autophagy, literally self eating, as a combination of Greek words “phagy” (to eat) and “auto” (oneself), is an evolutionary conserved catalytic process for vacuolar degradation of intracellular components, previously examined in yeast, mammals and plants (Levine & Klionsky, 2004; Mitou et al., 2009). Autophagy provides the balance between the protein synthesis and degradation for the normal cellular development and growth of eukaryotic cells, with the degradation of long-lived cytosolic proteins and organelles (Yorimitsu & Klionsky, 2005). Eukaryotic cells utilize the autophagy pathway to save its resources, to degrade damaged or toxic constituents and to survive under extracellular and intracellular stress conditions (Bassham, 2007; Mitou et al., 2009).

2.2.1 Forms of autophagy

In plants, two forms of autophagy were described based on the transport pathway to vacuole/lysosome: microautophagy and macroautophagy. In microautophagy, the material to be degraded is directly engulfed by the vacuole. This type of autophagy has been observed during seed germination of cotyledon cells of bean seedlings for degradation of starch granules and storage of proteins into the vacuoles (Toyooka et al., 2001; Van der Wilden et al., 1980) and also, during the accumulation of storage proteins in developing wheat seeds (Levanony et al., 1992; Shy et al.; 2001). On the other hand, in macroautophagy, the double-membrane vesicles, called autophagosomes, engulf the material and fuse with the vacuole or the lysosome; following the fusion, the inner material is transferred to the degradative compartment. In plants, the species differ from each other based on the destination of their autophagosomes. *Arabidopsis* have autophagosomes which fuse with the tonoplast and directly transfer to the lumen of the vacuole; however autophagosomes of tobacco cells are transformed firstly into lysosome-like acidic and lytic structures and then fuse with the central vacuole (Inoue et al., 2006; Toyooka et al., 2006). Last studies showed that a macroautophagy derivative pathway, cytoplasm-to-vacuole (CVT) pathway , previously reported only in yeast for the transport of proteins to the vacuole, is also utilized by plant cells (Seay et al., 2006). A different form of autophagy, chaperone-mediated autophagy (CMA), observed only in mammals, deliver the proteins which contain a consensus peptide sequence, KFERQ, to the lytic compartments by chaperone/cochaperone complex (Massey et al., 2006; Mizushima et al., 2008; Bassham, 2007; Klionsky, 2005). General similarities and differences between the types of autophagy were shown in Fig 2.2.

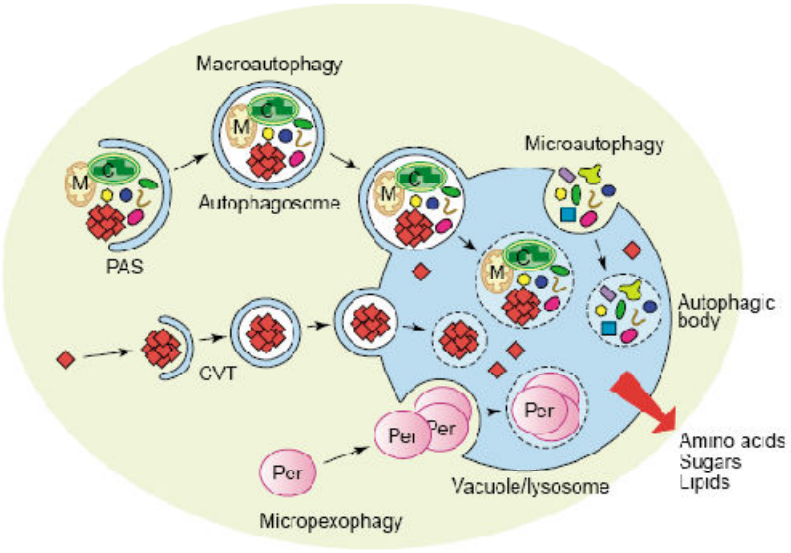

Fig 2.2 Morphological steps of macroautophagy, microautophagy and CVT (Thompson & Vierstra, 2005)

Macroautophagy, the most studied type of autophagy, hereafter simply term as autophagy, has been reported as the major process for cytoplasmic degradation during different stress conditions in several plant species. Autophagy was induced in response to sucrose starvation in rice (Chen et al., 1994), sycamore (Aubert et al., 1996), *Arabidopsis thaliana* (Doelling, 2002; Hanaoka, 2002) and tobacco suspension cultured cells (Moriyasu & Hillmer, 2000); the induction has been also reported in maize in response to carbon starvation (Brouquisse et al., 1998).

2.2.2 Autophagy proteins

Proteins involved in autophagy mechanism have been identified by using a group of autophagy defective yeast mutants (Thumm et al., 1994; Tsukada & Ohsumi, 1993; Harding et al., 1995). By the help of yeast mutants, ~ 30 autophagy related genes, or *Atg* genes, were characterized (Klionsky et al., 2003). Similarities in the autophagy mechanism across species provide to identify orhologs of yeast *Atg* genes. 36 genes with significant homology to yeast *Atg* genes were characterized in *Arabidopsis thaliana* with the help of genome analysis (Seay et al., 2006). Knockout *A. thaliana* mutants of *Atg* genes are used to study the role of autophagy in plants, indicating

sensitivity to nitrogen deficiency and early senescence symptoms (Doelling et al., 2002; Hanaoka et al., 2002; Surpin et al., 2003).

2.2.3 Autophagy machinery

The autophagy machinery which is highly conserved across species, from yeast to higher eukaryotes, can be divided into five phases: induction, nucleation, vesicle expansion and completion, fusion and degradation, and recycling (Mitou et al., 2009).

2.2.3.1 Induction

An appropriate signal is needed to induce autophagy which is inhibited by the major regulatory component, Target of rapamycin (TOR) protein (a serine/threonine kinase) under basal and nutrient-rich conditions (Carerra, 2004). TOR inhibits the autophagy by two different mechanisms. Firstly, it hyperphosphorylates ATG13; because this form of ATG13 has lower affinity for ATG1 which is a kinase; the interaction between ATG13 and ATG1 reduces and autophagy is inhibited (Funakoshi et al., 1997; Kamada et al., 2000). Though, under starvation condition or rapamycin treatment, ATG13 is dephospharyalted and induce autophagy (Abeliovich, 2004; Noda & Ohsumi, 1998). Secondly, TOR plays role in a signal transduction cascade that organizes phosphorylation of different effectors which regulate transcription and translation of some proteins required for autophagy (Klionsky, 2005). Homologous of yeast and mammalian *TOR* and *ATG13* genes were identified in *A. thaliana* (Hanaoka, 2002; Menand et al., 2002).

2.2.3.2 Nucleation

In this step, proteins and lipids come together and build the preautophagosomal structure (PAS) at autophagy organization site. Although that the specific components, lipid donors, for the vesicle formation are not identified yet, a protein complex which functions at the PAS for the initiation of the nucleation was determined in yeast: Vps34, a class of III phosphatidylinositol 3-OH kinase (PI3K) and Atg6/Vps30. Atg6 containing complex together with other regulatory proteins regulate the activity of Vps34 protein which leads the accumulation of phosphatidylinositol 3-phosphate (PI3P) by its PI3K activity. PI3P acts as landing pad on PAS for proteins such as Atg18 and Atg2 to form autophagosomes (Kihara et al., 2001; Klionsky 2005; Petiot et al., 2000; Xie & Klionsky 2007).

2.2.3.3 Vesicle expansion and completion

Two ubiquitin-like conjugation systems, Atg8-phosphatidylethanolamine (Atg8-PE) and Atg12-Atg5 play roles in the biogenesis of autophagic vesicles of yeast, plant and mammals (Ohsumi, 2001). In the first system, ATG12 is conjugated to ATG5 by an irreversible isopeptide bond between C-terminal glycine residue of ATG12 and a central lysine residue of ATG5 (Mizushima et al., 1998). ATG7, a homolog of E1 ubiqutin-activating enzyme Uba1, binds the ATG12 to form a complex via a thioester bond. ATG12 is activated by ATP hydrolysis and activated ATG12 is transferred to the ATG10, a E2 ubiqutin-like conjugating enzyme (Kim et al., 1999; Shintani et al., 1999). ATG12 bond the internal lysine residue of ATG5 by a covalent bond and form a final conjugate which binds ATG12 noncovalently (Mizushima et al., 1999). In the second conjugation system, ATG8 is the key protein, a microtubule-associated protein, which modifies a lipid, PE (Ichimura et al., 2000; Kirisako et al., 2000). ATG4, a cysteine protease, removes the C-terminal arginine residue of ATG8 to expose glycine residue to E1-like enzyme ATG7. Following the activation of ATG8 by ATG7, ATG8 is transferred to ATG3 (E2-like enzyme) and conjugated to PE via an amide bond. In contrast to ATG12-ATG5 complex, ATG8-PE lipidation complex reaction is

irreversible; ATG4 can cleave the conjugate lipid and provide ATG8 recycling (Kirisako, 2000). Although the mechanism is not determined yet, the ATG12-ATG5-ATG16 complex is also required for stability of ATG8-PE complex. Both ATG12-ATG5-ATG16 complex and ATG8-PE are localized on the PAS and play role in the vesicle formation by acting as a coat and as a structural component, respectively. ATG8-PE is observed in intermediate vesicles and completed autophagosomes (Mizushima et al., 2001). The two ubiquitin-like conjugation systems were visualized in Fig 2.3.

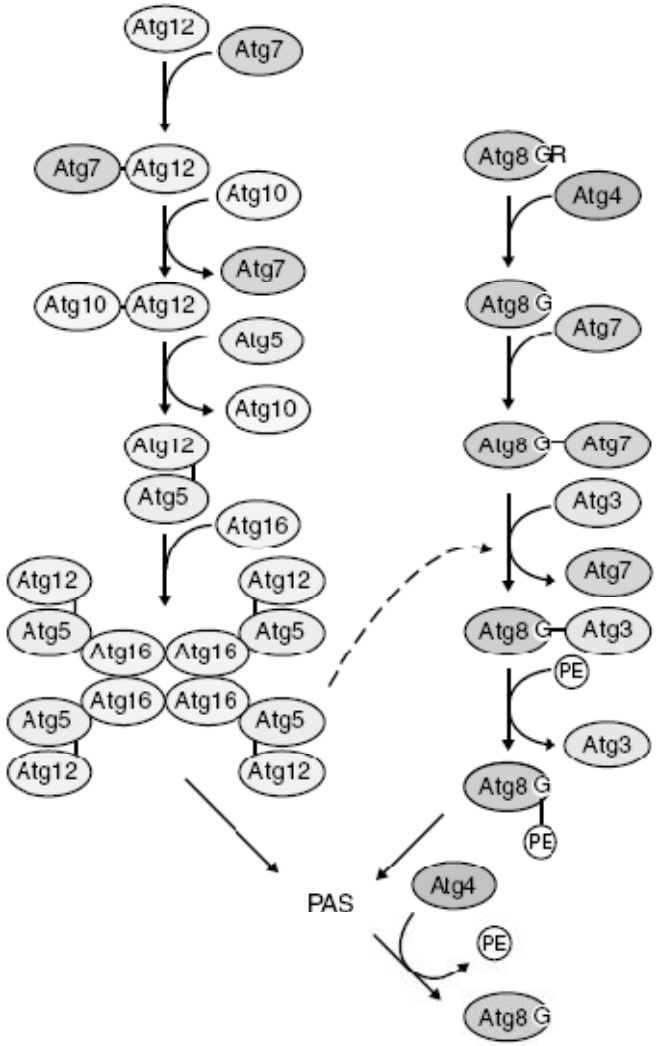

Fig 2.3 Atg8-PE and Atg12-Atg5 ubiquitin-like conjugation systems (Yorimitsu & Klionsky, 2005)

2.2.3.4 Fusion

When double membrane autophagosome is formed, it is transported to the vacuole or the lysosome and fuses its outer membrane with the vacuolar membrane. Ccz1 and Mon1 proteins form the fusion machinery with the SNARE proteins, Vam3, Vti1 and Vam7, the NSF Sec18, the α-SNAP Sec17, the Rab GTPase YPT7 and the class C VPS/HOPS complex (Klionsky, 2005; Wang & Klionsky, 2003; Yorimitsu & Klionsky, 2005). Following the fusion, inner single membrane of autophagosome is released inside the vacuole and form autophagic body.

2.2.3.5 Degradation and recycling

The acidic pH of the vacuole lumen and proteinase B (Prb1) control the vesicle lysis step (Nakamura et al., 1997; Takeshige et al., 1992). Additionally, ATG15 and ATG22 proteins are also involved (Epple et al., 2001; Suriapranata et al., 2000; Teter et al., 2001). ATG15, a lipase, play a direct role in vesicle breakdown. ATG22, an intergral membrane protein, is needed only for the degradation of autophagic bodies (Klionsky, 2005). Studies reported that plant vacuoles contain several enzymes, such as proteases, peptidases, nucleases and gluconases, for degradation of autophagic bodies (Marty, 1999).

2.2.4 Roles of autophagy in plants

As in other organisms, autophagy is constitutively active in plant cells. Previous studies have been reported that autophagic vesicles are accumulated in root tips of *A.thaliana* and tobacco cells, using different inhibitors such as E64d, a cysteine protease (Bassham, 2007; Inoue et al., 2006). The mutant *Arabidopsis* seeds and different species were used to identify the roles of autophagy in plants.

2.2.4.1 Autophagy in plant development

Previous researches indicated that autophagy plays an important role in development of several plant species. Recent studies showed that, autophagy-defective plants which have normal embryonic development, germination, shoot and root growth, flower development and seed germination under nutrient-rich condition; perform an increased chlorosis, a dark-induced senescence and a decrease in seed yild under carbon or nitrogen deficient conditions. (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004). Observing shorter primary roots in ATG4 mutant under nitrogen deficiency indicate the role of autophagy in root development (Yoshimoto et al., 2004). Different studies of *Arabidopsis* showed that ATG6/VPS30 regulate pollen germination (Fujiki et al., 2007; Harrison-Lowe & Olsen, 2008; Qin et al., 2007).

The study performed by Yano et al. (2007), suggested that the formation of vacuoles from tobacco BY-2 protobalst involves an autophagy-like pathway, indicating that autophagy plays a role in vacuole biogenesis.

2.2.4.2 Autophagy under abiotic stress

Recent studies indicated that autophagy is induced under nutrient starvation conditions, such as sucrose, carbon and nitrogen deficiency (Chen et al., 1994; Contento et al., 2004; Doelling et al., 2002; Hanaoka et al., 2002; Xiong et al., 2005). The formation of autophagosomes and the degradation of cytoplasmic materials in lytic compartments were observed in cultured plant cells under starvation conditions (Aubert et al., 1996; Moriyasu & Ohsumi, 1996; Rose et al., 2006; Takatsuka et al., 2004).

Autophagy plays also role in oxidative stress response which leads to accumulate highly toxic materails in large amounts under enviromental stress conditions and/or during developmental stages. Study by Xiong et al., 2007a; showed that AtATG18a knockdown transgenic plants perform hypersensitivity to oxidative stress. The accumulation of oxidized proteins was also explained by the reduction in degradation efficiency (Xiong et al., 2007b); indicating that oxidized and damaged cellular materials

during oxidative stress are delivered to the vacuole for autophagic degradation (Xiong et al., 2007b).

2.2.4.3 Autophagy in plant immune system

Plant *Atg* genes were found to be upregulated during defense response to pathogens such as *Pseudomonas syringae* or *Myzus persicea* (Zimmermann et al., 2004). The regulation of *Atg* genes during pathogen infection and programmed cell death (PCD) suggested that autophagy is involved in innate immune responses (Deretic, 2005; Levine, 2005; Liu et al., 2005).

Plants have developed specific mechanism referred as the innate immunity against pathogens. It involves the recognition of pathogen, encoded avirulence (Avr) proteins by plant resistance (R) proteins which causes the hypersensitive response (HR), a form of PCD, at the site of infection (Dangl & Jones, 2001; Jones & Dangl, 2006; Seay et al., 2006). The study by Liu et al. (2005) indicated that autophagy controls HR-PCD. Tobacco ATG6 protein, a homolog of mammalian BECLIN1, was found to be required for the restriction of HR-PCD to TMV-infected sites (Patel et al., 2006; Seay & Dinesh-Kumar, 2005).

2.2.5 Monitoring autophagy

There are several techniques to monitor autophagy in plants. Some of them will be summarized below.

2.2.5.1 ATG8 as a molecular marker

Conjugation of ATG8 to ATG-PE, required for autophagic membrane elongation, was used as a molecular marker to determine active autophagy in several organisms also in plants. The change in molecular weight of ATG8 from 16 kDa (free ATG8) to 18 kDa (ATG8-PE) was easily detected in SDS-PAGE protein gels. (Hanaoka et al., 2002; Kabeya et al., 2000; Yoshimoto et al., 2004; Slavikova et al., 2005).

The fusion protein containing GFP with ATG8 was used to label autophagosomes in many studies. The observation of ATG8-GFP fusion protein by confocal microscopy were demonstrated that ATG8-GFP fusion protein were seen as ring-shaped and punctuate structures corresponding to autophagosomes and intermediates, respectively, in wild type *Arabidopsis* root cells under nutrient deficiency. The absence of these structures in AtATG4a4b-1 double mutant plants was also demonstrated that ATG4 is required for processing and conjugation of ATG8 (Yoshimoto et al., 2004).

2.2.5.2 Fluorescent dyes

Two main acidotrophic fluorescent dyes were generally used to label autophagosomes: Monodansylcadaverine (MDC) and LysoTracker.

MDC is utilized to monitor autophagy in several organisms including plants (Contento et al., 2005; Munafo & Colombo, 2001; Takeuchi et al., 2004; Yu et al., 2006). MDC, a weak base, has the capability to pass through biological membranes and accumulate in acidic organelles, such as autophagosomes (Biederbick et al., 1995). However, recent studies suggested that MDC is not only specific to autophagosomes, but also to endosomes, lysosomes and lamellar bodies (Munafo & Colombo, 2001). Therefore, MDC should be combined with different monitoring techniques.

In several organisms, *Arabidopsis,* barley and tobacco, LysoTracker staining was performed to label autophagosomes (Liu et al., 2005; Moriyasu et al., 2003; Patel & Dinesh-Kumar, 2008). It is a dye, freely permeate to cell membranes typically

accumulates in spherical organelles with low internal pH such as lysosomes and vacuoles.

2.2.5.3 Electron microscopy

Transmission electron microscopy (TEM) is one of the ancient and consistent method used to monitor autophagy (Ashford & Porter, 1962). There are various criteria to detect autophagosomes and autolysosomes in this technique to prevent the misdetection of different organelles such as mitochondria, chloroplast and endoplasmic reticulum (Fengsrud et al., 2000; Klionsky et al., 2008; Eskelinen, 2008). Althoug TEM requires special expertise, it is most reliable method and used in several studies (Ghiglione et al., 2008; Liu et al., 2005; Rose et al., 2006).

2.2.5.4 Test of aminopeptidase I maturation

The role of plant ATG proteins were identified generally by yeast complementation to describe if the target gene is homologous of the yeast ATG and in these studies, test of aminopeptidase I maturation can be used to monitor autophagy. In yeast, as shown in Fig 2.4, the precursor form of aminopeptidase I (prAPI) is found in the cytosol and is targeted to vacuole via CVT for maturation. The presence of mature API (mAPI) demonstrated a functional autophagy pathway in yeast. Therefore, test of API maturation was used in several complementation assay of yeast mutant with plant *Atg* genes (Hanaoka, 2002; Ketelaar et al., 2004).

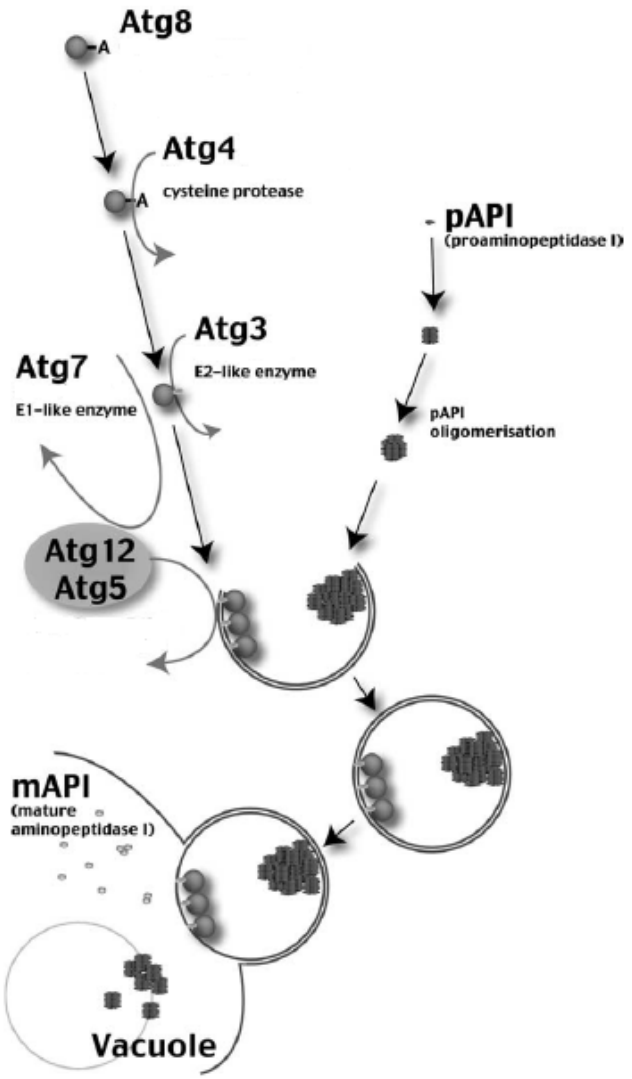

Fig 2.4 Aminopeptidase I maturation in yeast (modified from Ketelaar et al., 2004)

2.3 Virus induced gene silencing

Studies which aim to determine the function of a target gene were accelerated by the discovery of T-DNA knockout and T-DNA activation libraries in *Arabidopsis thaliana* (Weigel et al., 2000). Several lines with T-DNA interruption to inactivate a specific gene were generated in knockout libraries. On the other hand, in T-DNA activation libraries, T-DNAs which contain enhancer elements near their borders were used to activate the expression of gene located near the T-DNA insertion site. Following the selection of plants based on phenotype, the target gene was identified. Although

these methods are very useful for the gene function studies in dicots, they are not applicable for monocots such as *Triticum aestivum* (wheat) and *Hordeum vulgare* (barley). Transformation efficiency was found very low in these plants because of their huge genome. Additionally, the polyploidy of wheat causes also the gain-of-function of mutated gene by its homologues and prevents the selection according to the phenotype (Cakir et al., 2010). Therefore, virus-induced gene silencing (VIGS) was developed as an alternative method for gene function analysis in cereal crops, specially wheat and barley.

VIGS is a reverse genetic technique to form knockdown phenotypes which are used to identify the function of the silenced gene (Baulcombe, 1999; Kumagai et al., 1995; Ratcliff et al., 1997). VIGS was developed based on the plant defense system. Following the viral infection, viral RNAs accumulate in the cell and form double stranded RNAs (dsRNA) which are degraded by plant host-defense system. Researchers utilized the same mechanism to degrade/silence the target gene by using engineered viral vectors that contain the gene-of-interest. Several advantages of VIGS make it a forceful tool for plants: i) The silencing procedure is very rapid. ii) Full length coding sequence is not required. iii) It is applicable in polyploid plants since homologous genes that show ~85% similarity, are silenced by vectors containing information about their conserved regions. iv) The effect of silencing is easily observed thanks to the viral infection. v) It is applicable in species which are difficult to transform for RNAi studies (Cakir et al., 2010; Unver & Budak, 2009).

2.3.1 Post-transcriptional gene silencing machinery

RNA-induced gene silencing was described differently in several organisms: Post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNA interference (RNAi) in animals (Cogoni, C. & Macino, 1999; Fire et al., 1998). PTGS machinery is also acting in VIGS. As shown in Fig 2.5, double-stranded RNA (dsRNA) is degraded into ~21 nucleotide long dsRNA fragments named as small interfering RNAs (siRNA) by Dicer, a RNase-like enzyme. siRNAs are incorportated into RNAi silencing complex (RISC), a nuclease-containing complex and degrade the mRNA fragment having sequence complementary with the siRNA.

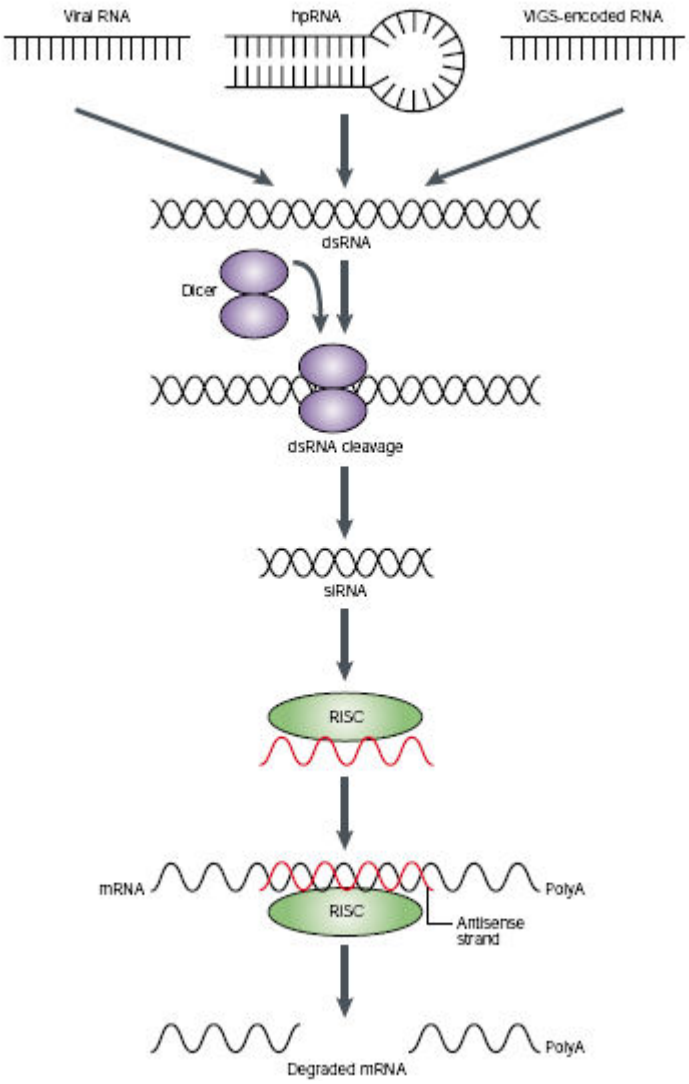

Fig 2.5 PTGS machinery (Waterhouse & Helliwell, 2002).

The replication of virus genomic RNA by the virus-encoded RNA-dependent RNA polymerase to produce sense and antisense, make VIGS an efficient method in functional genomics (Baulcombe 1999; Covey et al., 1997; Ruiz et al., 1998).

2.3.2 Viral vectors used in VIGS

Several viral vectors were developed for the infection of different species. In the first VIGS study, tobacco mosaic virus (TMV) containing a fragment of an endogenous gene, phytoene desaturase (*PDS*) which encodes an enzyme that protects chlorophyll from photo-bleaching, was used. Tobacco plants were infected by modified TMV and *PDS* silencing was phenotypically observed in leaf tissues (Kumagai et al., 1995). Potato virus X (PVX) and tobacco rattle virus (TRV) were also modified and successfully performed in VIGS studies (Liu et al., 2002(a); Liu et al., 2002(b); Ruiz et al., 1998; Ratcliff et al., 2001). TRV-based VIGS was used to silence different genes in several organisms since it is located between Right Border (RB) and Left Border (LB) sites of T-DNA and inserted into *Agrobacterium tumefaciens* (Liu et al., 2002(b); Ratcliff et al., 2001). In addition to these vectors, geminiviruse-derived DNA vectors, such as tomato golden mosaic virus (TGMV), were also developed for functional studies in *Nicotiana benthamiana* (Peele et al., 2001).

With the discovery of modified barley mosaic stripe virus (BSMV), VIGS become applicable in monocot plants. The efficient *PDS* silencing was demonstrated in barley (Holzberg et al., 2002) and wheat (Scofield et al., 2005). BSMV is a tripartitate single-stranded RNA virus containing α, β and γ RNAs. cDNAs of three RNAs were synthesized and cloned into DNA plasmid to allow the insertion of target gene into γ plasmid. α, β and γ plasmids were *in vitro* transcribed and mixed before the inoculation. β plasmid were modified to prevent the production of coat proteins. The procedure of BSMV mediated VIGS was described in Fig 2.6.

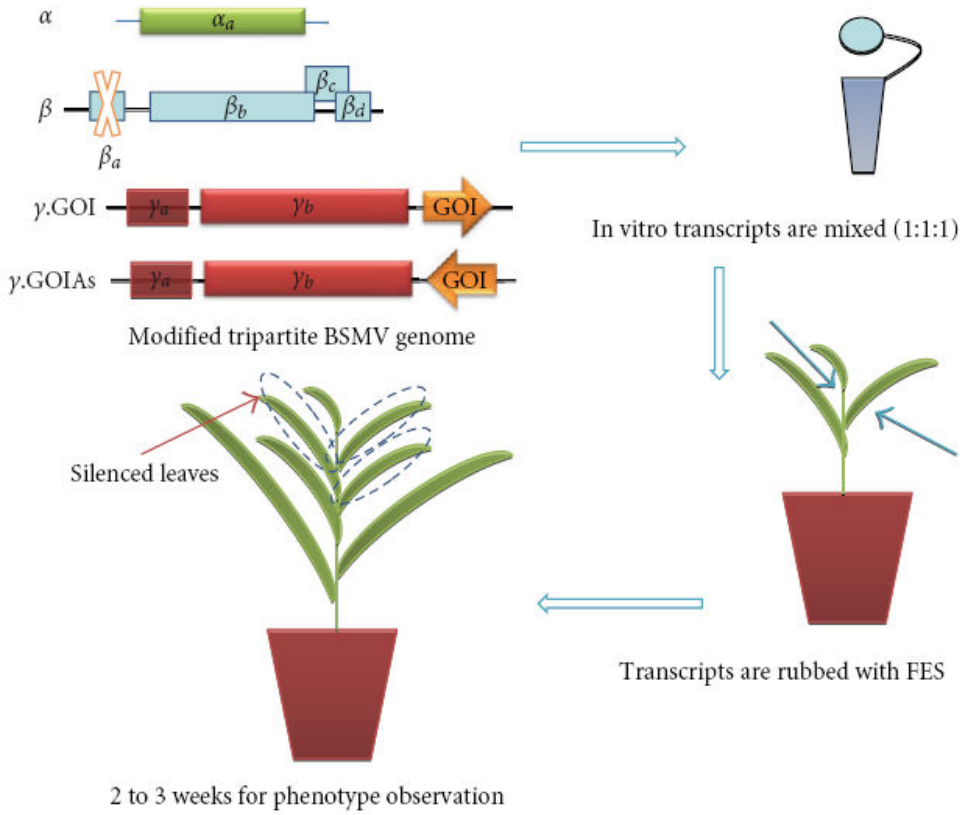

Fig 2.6 BSMV-mediated virus-induced gene silencing (Unver & Budak, 2009)

Recent studies indicated that the optimum length of the target gene in the γ plasmid is 120-500 bp (Holzberg et al., 2002). Although it was demonstrated that inserted sequence less than 120 bp reduce the silencing efficiency (Bruun-Rasmussen et al., 2007; Scofield et al., 2005), the upper size limit is not well-defined. However, it was thought that longer than 500 bp inserted fragment may be lost with higher frequency (Bruun-Rasmussen et al., 2007; Cakir & Scofield, 2008) since the virus replication is not stable in plants (Pogue et al., 2002).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

In this experiments, *Triticum dicoccoides*, genotype TTD-03, TR38827 and TR62248 were used (Ergen & Budak, 2009). Nullisomic-tetrasomic wheat lines were a kind gift of Prof. Bikram Gill from Kansas Stated University (www.k-state.edu/media/mediaguide/bios/gillbio.html).

3.1.2 Yeast strain and plasmid

In this study, yeast *Atg8* mutant strain (BY4741, atg8Δ::*kanMX*, *MATa*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and pRS316 plasmid with yeast *Atg8* gene provided by Dr. Nakatogawa were used.

3.1.3 Fungi material

Fusarium culmorum fungi samples were prepared at Selcuk University, Faculty of Agriculture, Department of Plant Protection, Konya, Turkey.

3.1.4 Antibodies

Polyclonal anti-API antibody used in this study was a kind gift of Dr. Klionsky (http://www.biochem.med.umich.edu/?q=klionsky). Monoclonal anti-AtATG8 antibody was kindly provided by Dr. Vierstra (http://vierstra.genetics.wisc.edu/people.rick.vierstra.php)

3.1.5 Vectors

Barley mosaic stripe virus (BSMV) vectors used in this study were obtained from Large Scale Biology Corporation (CA, USA). Sequences of pα, pβΔβa, pγ and pγPDS were given in Appendix A.

3.1.6 Chemicals and Commersial Kits

All chemicals were obtained from Merck (Germany, www.merck.com), SIGMA-ALDRICH (USA, www.sigmaaldrich.com), Fluka (Switzerland) and Riedel de Häel (Germany). All chemicals and commercial kits used in this study were listed in Appendix F and G, respectively.

3.1.7 Growth Media, Buffers and Solutions

The growth media, buffers and solutions used in this study were prepared according to the procols in Sambrook et al., 2001, unless otherwise stated.

3.1.8 Primers

All primers were commercially synthesized in Integrated DNA Technologies (USA).

3.1.9 Equipments

All equipments used in this study were listed in Appendix E

3.2 Methods

3.2.1 Plant growth conditions and polyethylene glycol application

Seeds of *T. dicoccoides* were sterilized by washing with 70% ethanol for 5 min, rinsing with sterile dH_2O for three times, washing with NaOCl for 20 min and finally rinsing with sterile dH_2O for five times (Filiz et al., 2009). Surface sterilized seeds were planted on solid medium (Murashige-Skoog Vitamin and Salt Mixture (Duchefa), 2% (w/v) sucrose (Duchefa), 0.8% plant agar (Duchefa)) in Magenta boxes. All plants were grown under 16 h light for 4 weeks including germination period. For the drought treatment, 50 ml of 40% PEG 6000 was added on 50 ml solid medium agar, incubated at dark for 16 h and then discarded. Plants were transferred to new mediums and when the first symptoms became visible on plants, leaf and root samples were collected. Samples were frozen in liquid nitrogen and stored at -80°C until further use.

3.2.2 Total RNA isolation

Total RNA isolation was performed by TRIzol® reagent (Invitrogen) according to the manufacturer's instruction with minor modifications. Briefly, 200 mg of tissue was homogenized in 1.5 ml TRIzol® reagent (Invitrogen) and 1 ml of liquid was transferred to an eppendorf tube, which was kept on ice while processing the other samples. 0.4 ml chloroform was added, tubes was shake by hand and incubated at room temperature for 7 min. After the centrifugation at 12,000 x g for 15 min at 4°C, the supernatant was transferred to a new eppendorf tube and 0.5 ml isopropanol was added. After shaking by hand again, tubes were incubated at room temperature for 10 min. Samples were centrifuged at 12,000 x g for 10 min at 4°C; pellets were washed with 1 ml 75% DEPC treated ethanol. Samples were mixes and spun at 7,500 x g for 5 min at 4°C. Supernatants were discarded and pellets were dried for 10 min. RNA pellets were dissolved in 30µl RNase free water at 55°C for 1 h.

Total RNA isolations were performed from each tissue type (leaf and root tissues) and treatment (control and 20% PEG application). RNA concentrations were measured by Nanodrop spectrophotometry and the quality of RNA samples were controlled by agarose gel electrophoresis.

3.2.3 cDNA synthesis

First strand cDNA synthesis was performed by RevertAid™ H minus M-MulV reverse transcriptase (Fermentas). 1 µg total RNA from root and leave samples with and without PEG treatment was reverse transcribed according to manufacturer's instruction using oligodT primers (Fermentas).

3.2.4 Semi-quantitative analysis

Primers were designed according to full coding sequence (CDS) of *Triticum aestivum Atg8* (Appendix B). To compare expression levels of *Atg8* in control and stress tissues, 20 µl PCR reaction was performed by using 5'AAG CTT CAT GGC CAA GAC TTG CTT A 3' forward and 5' CTC GAG TTA GGC AGA GCC GAA AGT 3' reverse primers. Each reaction contained 1 µl (1:5 diluted) first strand cDNA, 1X PCR buffer without $MgCl_2$, 2 mM $MgCl_2$, 0.25 mM dNTP mix, 0.25 µM of each primer and 1 unit of Taq DNA polymerase (Fermentas). PCR program was started with the initial denaturation at 94°C for 4 min, continued with 30 cycles of amplification (94°C for 45 s., 60°C for 1 min, 72°C for 45 s.) and terminated by final extension at 72°C for 7 min. PCR products were analyzed on 1% agarose gel.

3.2.5 Quantitative analysis by Q-RT

Quantitative analysis of *TdAtg8* expression level in different tissue samples under different conditions was performed by real-time PCR to detect the effect of drought stress in *T. dicoccoides*.

20 µl of PCR reaction was performed by using 1 µl of synthesized cDNA (1:5 diluted), 0.35 µM of each primer (described in section 3.2.4) and 1X FastStart Universal SYBR green PCR master mix (Roche) with Icycler Multicolor Realtime PCR Detection Systems (BioRad Laboratories). Standardization of the analysis was provided by using 4 different dilutions (1:5, 1:10, 1:20 and 1:40) for one sample. 18srRNA (F: 5' GTGACGGGTGACGGAGAATT 3' and R: 5' GACACTAATGCGCCCGGTAT 3') primers were used for normalization.

The templates were amplified at 95°C for 10 minutes, followed by 40 cycles of amplification (95°C for 45s, 60°C for 1 minute). The quantification was performed according to previous studies (Simon, 2003).

3.2.6 Amplification of full CDS of *TdAtg8* gene

Forward and reverse primers used to amplify coding sequence of *TdAtg8* gene contained *HindIII* and *XhoI* restriction enzyme sites, respectively, shown as bold characters (F: 5'**AAG CTT** CAT GGC CAA GAC TTG CTT A 3', R: 5' **CTC GAG** TTA GGC AGA GCC GAA AGT 3'). Those primers have been designed to be in frame with GAL1 promoter of pYES2 (Invitrogen) yeast expression vector. The map of the vector is given in Appendix C. 50 µl of PCR reaction contained 1µl (1:5 diluted) first strand cDNA, 1X PCR buffer without $MgSO_4$, 1 mM $MgSO_4$, 0.25 mM dNTP mix, 0.2 µM of each primers and 1.8 unit Platinum® *Pfx*DNA polymerase (Invitrogen). The templates were amplified at 94°C for 5 minutes, followed by 30 cycles of amplification (94°C for 45s, 57°C for 1.5 min, 68°C for 45s) and the reaction was terminated with 68°C for 7 minutes. PCR product was analyzed on 1% agarose gel and extracted from the gel. The amplicon was purified by Qiaquick® gel extraction kit (Qiagen) according to manufacturer's instruction. Purified fragment was polyadenylated using 1X PCR buffer, 2 mM $MgCl_2$, 0.25 mM dATP and 1 unit of Taq DNA polymerase (Fermentas) at 72°C for 15 min.

3.2.7 TA cloning

pGEM®-T Easy Vector System I (Promega) was used to clone polyadenylated *TdAtg8* fragment. The map of pGEM® T-Easy vector is provided in Appendix D. DH5α strain of *Escherichia coli (E.coli)* cells were transformed with the recombinant plasmids. Positive clones were selected and plasmids were purified to obtain large amount of *TdAtg8*.

3.2.7.1 Ligation

Polyadenylated *TdAtg8* fragment was ligated to pGEM®-T easy vector (Promega) according to 3:1 insert/vector ratio, as described in the kit manual. The reaction was incubated at room temperature for one hour. Positive and negative controls of ligation reaction were also performed.

3.2.7.2 Chemically competent cell preparation

Single colony of *E.coli* cell (DH5α strain) was inoculated into 50 ml Luria-Bertani (LB) broth and grown overnight (~16 h) in 200 ml flask at 37°C with moderate shaking (250 rpm). 4 ml of the culture was transferred into 400 ml LB medium in a sterile 2 L flask and grown at similar conditions, until OD_{590} was reached to 0.375. Culture was aliquoted into eight pre-chilled sterile polypropylene tubes (50 ml) and tubes were incubated on ice for 5-10 min. The tubes were spun at 2,700 x g for 7 min at 4°C. Supernatants were discarded and pellets were resuspended in 10 ml ice-cold $CaCl_2$ solution, containing 60 mM $CaCl_2$, 15% glycerol and 10 mM PIPES (pH 7.0). Tubes were centrifuged at 1,800 x g for 5 min at 4°C. Supernatants were discarded; pellets were resuspended in 10 ml ice-cold $CaCl_2$ solution and kept on ice for 30 min. The tubes were spun at 1,800 x g for 5 min at 4°C. Supernatants were discarded; pellets were resuspended in 2 ml ice-cold $CaCl_2$ solution. 200 µl aliquots of cells were dispensed into pre-chilled, sterile eppendorf tubes and freeze in liquid nitrogen immediately. Competent cells were stored at -80°C for further use. The transformation efficiency of competent cells was checked by using 1 ng and 1 pg of pUC plasmid (Invitrogen). Competent cells with minimum 10^7 transformation efficiency were used in this study.

3.2.7.3 Transformation

2 µl of ligation reaction was mixed with 100 µl chemically competent *E.coli* cells according to pGEM®-T easy manufacturer's instruction (Promega). Cells were kept on ice for 20 min. and incubated at 42°C for 1 minute. After cooling on ice for 2 min, 800 µl LB medium was added and cells were grown at 37°C for 1 hour. Since the vector has Ampicillin resistance and *LacZ* genes, 200 µl of culture was spread on LB agar/Amp/IPTG/X-Gal plate containing 20 µl of 100 µg/ml ampicillin, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal and incubated at 37°C for 16 hours.

3.2.7.4 Colony selection

Positive clones were chosen according to blue/white selection since the vector contains *LacZ* gene.

3.2.7.5 Colony PCR

Selected white colonies were used as PCR templates and PCR reaction was performed as described in section 3.2.4 with Taq polymerase enzyme (Fermentas) to confirm that the transformation was correctly done.

3.2.7.6 Preparation of glycerol stock

Glycerol stocks of transformants were prepared in 15% sterile glycerol and stored at -80°C.

3.2.7.7 Plasmid isolation

Colonies, confirmed by colony PCR, were inoculated in 5 ml of LB broth containing 100 µg/ml of ampicillin in sterile culture tubes. Cells were grown in a shaker incubator (270 rpm) at 37°C for 16 hours. Plasmids were purified by High Pure plasmid isolation kit (Roche) according to the manufacturer's protocol. The concentration and the quality of isolated plasmids were checked by Nanodrop spectrophotometry.

3.2.7.8 Restriction enzyme digestion

Purified plasmids containing *TdAtg8* fragment was digested with *NotI* restriction enzyme to check the transformation. 50 µl of digestion reaction was set up with 1 µg of plasmid, 1X buffer and 1 unit of *NotI* (NEB) and incubated at 37°C for 3 hours. The presence of *TdAtg8* was controlled by agarose gel electrophoresis.

3.2.7.9 DNA sequence analysis

Sequence analysis of isolated plasmid containing *TdAtg8* was commercially provided by Refgen (Ankara, Turkey, www.refgen.com) using M13F and M13R primers.

3.2.8 Analysis of intron-exon organization in *Atg8*

Different *Triticum* samples, *T. dicoccoides, T.monococcum* and *T.durum,* were used for the analysis of *Atg8* full length open reading frame (ORF). The aim was to identify introns and exons of *Atg8* and construct the phylogenetic tree of these species according to their *Atg8* sequence.

3.2.8.1 DNA isolation

DNA isolation of *T. dicoccoides, T.monococcum* and *T.durum* leaf samples was performed by using Wizard® Genomic DNA purification kit (Promega) according to the manufacturer's instruction with minor modifications. Briefly, ~100 g leaf tissues, freezed with liquid nitrogen, were homogenized by Tissue Lyser (Qiagen) and were wet with 600 µl of nuclei lysis solution by vortex. After the incubation at 65°C for 15 min, 3 µl of RNase solution was added to the lysates and the samples were mixed by inverting the tubes 2-5 times. Following the incubation at 37°C for 15 min, samples were cooled at room temperature. 200µl of protein precipitation solution was added and the tubes were vortexed vigorously at high speed for 20 seconds. Proteins were precipitated after the centrifugation at 14,000 x g for 15 min and the superantants were removed. Adding 600 µl of room temperature isopropanol makes the DNAs visible. DNAs were precipitated with centrifugation step at 14,000 x g for 1 min. at room temperature and the pellets were washed with 70% ethanol. After the final centrifugation at 14,000 x g for 1 min., DNA pellets were dried and solved in 100µl of DNA rehydration solution incubating at 65°C for 1 hour. The concentration of the isolated DNA samples was measured by Nanodrop spectrometry and the quality was checked by agarose gel electrophoresis.

3.2.8.2 Amplification of full length ORF of *Atg8* gene

The PCR reaction was repeated with the same primer pairs to amplify full length ORF of *Atg8* gene from genomic DNA, as described in section 3.2.6. 200 ng of DNA was used as a template. Amplified *Atg8* fragments were extracted from the gel and introduced into pGEM-T easy vector to perform TA cloning, as described in section 3.2.7.

3.2.8.3 DNA sequence analysis

Plasmids were sent to Refgen to be sequenced by using M13F and M13R primers. Firstly, sequences were exposed to "VecScreen" algorithm (www.ncbi.nlm.nih.gov) to prevent vector contamination. DNAstar software (www.dnastar.com) was used to align genomic DNA sequences of different *Atg8* genes with CDS of *T. aestivum Atg8,* resulting intron fragments present into ORF of *Atg8.* Finally, CDSs of *T. dicoccoides, T.monococcum, T.durum* and *T.aestivum Atg8* were identified and aligned to construct a phylogenetic tree.

3.2.9 Chromosomal localization of Atg8

Nullisomic-tetrasomic wheat lines were used to determine the chromosomal localization of *Atg8* gene and to identify the copy number of *Atg8* in polyploid wheat.

3.2.9.1 DNA isolation of nullisomic-tetrasomic wheat lines

DNAs were isolated from leaf tissues of 38 different nullisomic-tetrasomic wheat lines as described in section 3.2.8.1.

Table 3.1 Nullisomic-tetrasomic wheat lines

Sample ID	Line Name	Sample ID	Line Name
1	N1A- T1B	20	N3B-T3D
2	N1A- T1D	23	N5B-T5A
3	N2A- T2B	24	N5B-T5D
4	N2A- T2D	26	N6B-T6D
5	N3A-T3B	27	N7B-T7A
6	N3A-T3D	28	N7B-T7D
7	N4A-T4B	29	N1D-T1A
8	N4A-T4D	30	N1D-T1B
9	N5A-T5B	31	N2D-T2A
10	N5A-T5D	32	N2D-T2B
11	N6A-T6B	33	N3D-T3A
12	N6A-T6D	34	N3D-T3B
13	N7A-T7B	36	N4D-T4B
14	N7A-T7D	37	N5D-T5A
15	N1B-T1D	38	N5D-T5B
16	N1B-T1A	39	N6D-T6A
17	N2B-T2A	40	N6D-T6B
18	N2B-T2D	41	N7D-T7A
19	N3B-T3A	42	N7D-T7B

3.2.9.2 Amplification of *Atg8* gene

Atg8 gene was amplified using each line as template as described in section 3.2.6. PCR products were firsly observed on 0.8% agarose gel and then on 12% polyacrylamide gel to determine clearly the deleted fragment.

3.2.10 Complementation assay of yeast atg8Δ mutant with *TdAtg8* gene

TdAtg8 was cloned into pYES2 yeast expression vector and the function of *TdAtg8* was observed under nitrogen deficiency.

3.2.10.1 Digestion of *TdAtg8* and pYES2

Plasmids containing full CDS of *TdAtg8*gene and pYES2 yeast expression vector (Invitrogen) were double digested with *XhoI* (Fermentas) and *HindIII* (Fermentas) restriction enzymes using 1X R Buffer at 37°C for 3 hours.

3.2.10.2 Ligation

XhoI/HindIII digested *TdAtg8* fragment was ligated with *XhoI/HindIII* digested pYES2 vector (Invitrogen). Two different insert:vector ratio (1:1 and 3:1) was performed according to T4 DNA ligase (Fermentas) protocol. The ligation reaction was incubated at 16°C for 16 hours.

3.2.10.3 Transformation

Yeast *Atg8* mutant strain (BY4741, atg8Δ::*kanMX*, *MATa*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) was used in this study (Kawamata et al., 2005). pYES2-*TdAtg8* recombinant vector was transformed into yeast *Atg8* mutant strain according to LiAc/SS-DNA/PEG TRAFO protocol (Gietz and Woods, 2002). pRS316 vector containing yeast *Atg8* gene was also transformed into yeast *Atg8* mutant strain, as a positive control. Since pYES and pRS316 vectors contained *URA3* gene, uracil (URA) was used as the auxotrophic marker. 50 µl and 200 µl of transformation mixtures were spreaded on SC-URA selective medium plates containing 0.67% yeast nitrogen base, drop-out (DO) supplement without URA, 2% glucose and 2% agar. Positive colonies that have pYES2-*TdAtg8* recombinant vector was selected from plates, after 3-4 days. Selection was confirmed by colony PCR, as described in section 3.2.7.5. Selected colonies were inoculated in SC-URA medium containing 2% galactose and 1% raffinose at 29°C for 16 hour. Glycerol stocks of transformants were prepared in 15% glycerol and stored at -80°C until use.

3.2.10.4 Drop test assay

Yeast transformants were grown in SC-URA liquid medium and 5 µl of them were spotted in serial dilutions (1:1, 1:5, 1:10, 1:100, 1:1000) on standard rich growth medium YPD containing yeast extract, peptone, dextrose, for control of equal loading and viability. They were also spotted on synthetic minimal medium without nitrogen containing 0.67% yeast nitrogen base without ammonium sulfate and amino acids, 2% galactose and 1% raffinose, for detecting the effect of TdAtg8 on growth of yeast, under N deficiency condition.

3.2.11 Protein expression and western blot analysis with polyclonal anti aminopeptidease I (API) antibody

Recombinant yeasts were grown in SC-URA medium for 48 hours and cultures were diluted to have an OD_{600} of 0.4 in 50 ml induction medium without nitrogen (0.67% yeast nitrogen base without ammonium sulfate and amino acids, 2% galactose and 1% raffinose) to provide N deficiency conditions. The induction of *TdAtg8* was provided by galactose according to manufacturer's instructions (pYES2 manual, Invitrogen), except the expansion of the time for galactose induction to 24 and 48 hours. After protein expression, preparation of cell lysates was done in accordance with producer's instructions (pYES2 manual, Invitrogen). The concentration of cell lysates was measured by Bradford analysis using bovine serum albumin (BSA) as a standard (0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml).

100 µg of proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane using wet transfer method. The membrane was blocked by phosphate-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 hour and, probed with a specific rabbit anti-API antibody (1:1000) overnight at 4°C and after washing, exposed to horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:100000) for 1 hour. The visualization of bound antibodies was performed using ECL western blotting substrate and enhancer according to manufacturer's instructions (PIERCE, USA).

3.2.12 *TdAtg8* cloning into pACT2 yeast expression vector

Forward and reverse primers used to amplify *TdAtg8* gene contained *SpeI* and *SmaI* restriction enzyme sites, respectively, shown as bold characters (F: 5'**ACT AGT** GGA TGG CCA AGA CTT GCT TCA AGA 3', R: 5' **CCC GGG** AAG GCA GAG CCG AA 3'). Those primers have been designed to be in frame with ADH1 promoter of pACT2 yeast expression vector (Clontech) containing hemaglutinin (HA) epitope tag for western analysis. The map of this vector is provided in Appendix E.

50 µl of PCR reaction contained 1µl (1:5 diluted) first strand cDNA, 1X PCR buffer without $MgSO_4$, 1.5 mM $MgSO_4$, 0.25 mM dNTP mix, 0.25 µM of each primers and 2.5 unit *Pfu* DNA polymerase (Fermentas). The templates were amplified at 94°C for 5 minutes, followed by 30 cycles of amplification (94°C for 45s, 59°C for 1 min, 72°C for 45s) and the reaction was terminated with 72°C for 7 minutes. PCR product was analyzed on 1% agarose gel and extracted from the gel. The amplicon was purified by Qiaquick® gel extraction kit (Qiagen) according to manufacturer's instruction. Purified fragment was polyadenylated using 1X PCR buffer, 2 mM $MgCl_2$, 0.25 mM dATP and 1 unit of Taq DNA polymerase (Fermentas) at 72°C for 15 min.

Amplified fragment was cloned into pGEM-T easy vector as described in section 3.2.7. pGEM-T easy vector containing full CDS of *TdAtg8*gene and pACT2 yeast expression vector (Clontech) were double digested with *SmaI* (Fermentas) and *SacI* (Fermentas) restriction enzymes using 1X R Buffer at 37°C for 3 hours.

As described in section 3.2.8., *SmaI/SacI* digested *TdAtg8* fragment was ligated with *SmaI/SacI* digested pACT2 vector (Clontech). pACT2-*TdAtg8* recombinant vector was transformed into yeast *Atg8* mutant strain as described previously. The transformation was controlled by colony PCR. Colonies containing pACT2-*TdAtg8* recombinant vector was grown in YPD medium at 29°C for 5 hour. Yeast protein purification was performed according to pYES2 manual (Invitrogen). The concentration of cell lysates was measured by Bradford analysis using bovine serum albumin (BSA) as a standard (0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml).

50 µg of proteins were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane using wet transfer method. The membrane was blocked by phosphate-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1

hour and, probed with a specific rabbit anti-HA antibody (1:1000) overnight at 4°C and after washing, exposed to horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:100000) for 1 hour. The visualization of bound antibodies was performed using ECL western blotting substrate and enhancer based on manufacturer's instructions (PIERCE, USA)

3.2.13 Protein expression and western blot analysis with anti-AtAtg8 antibody

Proteins were extracted from both control and 20% PEG treated leaf and root tissues according to the protocol described in Bieri et al., 2004. 200 µg of extracted proteins were used for western blot analysis with the antibody designed according to *Arabidopsis thaliana* ATG8, as described in section 3.2.12.

3.2.14 Monitoring autophagy

3.2.14.1 Monodansylcadaverine (MDC) Staining

Root tips (about 1-2 cm in lenght) were collected from control and 20% PEG treated *Triticum dicoccoides* samples and incubated in 0.05 mM MDC (Sigma) in phosphate buffered saline (PBS) for 20 min. Root tips were washed two times with PBS (Contento et al., 2005). MDC stained root tips were observed under fluorescence microscopy (Olympus, BX-60).

3.2.14.2 LysoTracker red staining

Root tips (about 1-2 cm in length) was collected from control and 20% PEG treated *Triticum dicoccoides* samples and incubated in nutrient medium (2.1 g/L MS, 3% sucrose, pH 5.8) containing 100 mM E64d (Sigma) cysteine protease inhibitor at

room temperature for 16 hour with rotation. After the removal of E64d, root tips were stained with LystoTracker Red in 10 mM HEPES-Na (pH 7.5) for 1 h. with rotation and was fixed in a solution containing 50 mM Na phosphate, 5 mM EGTA, 3.7% formaldehyde and 0.02% NaN_3, as described by Moriyasu et al., 2003. Lysotracker red stained root tips were observed under confocal microscope in Istanbul Technical University (Leica Microsystems Heidelberg GmbH).

3.2.15 PDS silencing of T.dicoccoides

TdAtg8 gene was silenced by virus-induced gene silencing (VIGS) method and its role was analyzed in the presence of pathogen. Prior to functional analysis of *TdAtg8,* the applicability of VIGS in *Triticum dicoccoides* was controlled by silencing *Phytoene desaturase (Pds)* gene.

3.2.15.1 Preparation of BSMV vectors

Barley mosaic stripe virus (BSMV) is composed of three parties: pα, pβΔβa and pγ. Green fluorescent protein (GFP) was used to control the virus spreading in *T.dicoccoides*. *PDS* silencing was performed by pγPDS vector containing *Hordeum vulgare PDS* fragment, 185 bp in length, constructed by Holzberg et al., 2002. The fragment was given in Appendix A.

Chemically competent *E.coli* cells*,* strain *DH5α,* were transformed with pα, pβΔβa, pγ, pγGFP and pγPDS vectors as described in section 3.2.7.3. Selection was performed according to Ampicillin resistance gene in the vectors. Cells were spread on LB/Amp plates. Positive colonies were grown in liquid LB/Amp medium for 16 hours and plasmids were purified as described in section 3.2.7.7.

3.2.15.2 Linearization of BSMV vectors

BSMV vectors were linearized by different restriction enzymes. pα, pβΔβa and pγ (pγ, pγGFP and pγPDS) vectors were digested with *MluI* (Fermentas), *BcuI* (Fermentas) and *BsshII* (NEB) restriction enzymes respectively, according to manufacturers' manuals using maximum amount of plasmids for 50 µl reactions. Digested vectors were loaded on 0.8% agarose gel and extracted from gel by Agarose extraction kit (Qiagen).

3.2.15.3 *In vitro* transcription

Linearized vectors were transcribed by mMessage mMachine® T7 High Yield Capped RNA Transcription kit (Ambion, 1344). 80 ng of each plasmid was mixed with 1X reaction buffer, 1X NTP and 0.15 µl T7 RNA polymerase mix and incubated at 37°C for 2 hours. DNase treatment was performed by adding 0.4 U Turbo DNaseI (Ambion) in the last 15 minutes of the incubation.

Transcription reaction was controlled by 2% agarose gel and repeated for 800 ng or 1200 ng of plasmids according the number of plants that will be inoculated. Transcripts were purified by RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

3.2.15.4 Inoculation

Triticum dicoccoides, genotype TR38827, was used for BSMV mediated silencing. The third leaves (5-7 cm in length, approximately 10-12 days after germination) were inoculated with BSMV for silencing. 1 µg of each pα, pβΔβa and pγ transcripts were mixed in 1:1:1 ratio. Concentrations of transcripts were determined by NanoDrop spectroscopy. Transcription mix was combined with 30 µl FES which is

composed of 1X autoclaved GP (10X GP contains 3, 75 g glycine and 6,8 g $K_2HPO_4.3H_2O$ in 100 ml dH_2O), 1 g sodium pyrophosphate, 1 g bentonite, 1 g celite for 100 ml dH_2O according to previous study (Pogue et al., 1998) and directly applied to the leaf with two light strokes. A systemic spread was determined by the appearance of mosaic symptoms on leaves after 21 days post inoculation (dpi).

Table 3.2 BSMV vectors using in *PDS silencing*

	pα	**pβΔβa**	**pγ**	**pγPDS**	**pγGFP**	**FES**
BSMV:00	1 µg	1 µg	1 µg	-	-	30 µl
BSMV:PDS	1 µg	1 µg	-	1 µg	-	30 µl
BSMV:GFP	1 µg	1 µg	-	-	1 µg	30 µl
FES	-	-	-	-	-	30 µl

3.2.15.5 GFP analysis

Leaves were collected after 5 dpi from BSMV:GFP inoculated samples and observed under fluorescence microscopy.

3.2.15.6 Quantification of *PDS* expression level

Leaves were collected from inoculated samples and total RNA isolation was performed as described in section 3.2.2. After the cDNA synthesis, as described in section 3.2.3, real-time PCR was performed with forward 5'CCCTGACGAGTTATCCATGCAG3' and reverse 5'GGACCTCACCACCCAA AGACT3' PDS primers at 60°C annealing temperature to measure the expression level of *PDS* in silenced and non silenced plants as described in 3.2.5. 18S rRNA primers were used for normalization.

3.2.16 Atg8 silencing of T.dicoccoides

BSMV mediated gene silencing was used to silence *Atg8* of *Triticum dicoccoides.*

3.2.16.1 *TdAtg8* cloning into BSMV

Forward and reverse primers used to amplify *TdAtg8* gene contained *PacI* (NEB) and *NotI* (NEB) restriction enzyme sites, respectively, shown as bold characters (F: 5' **TTAATTAA**CTGAGCCCAGAAAAGGCCATCTT 3' and R: 5' **GCGGCCGC**TG TTCTCGCCACTGTAAGTCATGTA 3'). Primers were designed to amplify 133 bp long region of *Atg8* gene near to its 3' region to be more specific. 50 µl of PCR reaction contained 1µl (1:5 diluted) first strand cDNA, 1X PCR buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix, 0.25 µM of each primers and 0.2 unit *Taq* DNA polymerase (Fermentas). The templates were amplified at 94°C for 5 minutes, followed by 30 cycles of amplification (94°C for 45s, 58.2°C for 1 min, 72°C for 45s) and the reaction was terminated with 72°C for 7 minutes. PCR product was analyzed on 1% agarose gel and extracted from the gel. The amplicon was purified by Qiaquick® gel extraction kit (Qiagen) according to manufacturer's instruction. *TdAtg8* fragment was inserted into pGEM-T® easy vector and was cloned into *E.coli* as described previously. TA cloning was checked by colony PCR and plasmids were purified. The insertion of fragment was also tested by sequencing (Refgen, Ankara, Turkey).

TdAtg8 fragment and pγ vector were first digested with *PacI* (NEB) using 1X NEB1 buffer at 37°C for 16 hours. Digested fragment was extracted from 0.8% agarose gel and purified by Qiaquick® gel extraction kit (Qiagen) according to manufacturer's instruction. Second digestion was performed by using 1X NEB3 buffer with *NotI* restriction enzyme for 16 hours. Double digested *TdAtg8* fragment and pγ vector were extracted from 0.8% agarose gel and purified by Qiaquick® gel extraction kit (Qiagen). *PacI/NotI* digested *TdAtg8* fragment was ligated with *PacI/NotI* digested pγ vector by using T4 DNA ligase (Fermentas) as described in section 3.2.8. pγ-*TdAtg8* recombinant vector (pγAtg8) was cloned into *E.coli* and plasmids were purified as described in section 3.2.7.

3.2.16.2 Linearization of pγAtg8

pγAtg8 vector was digested with *BsshII* (NEB) restriction enzyme according to manufacturer's instructions. After overnight incubation at 50°C, linearized vector was extracted from 0.8% agarose gel and purified by Qiaquick® gel extraction kit (Qiagen). Linearization of pα and pβΔβa vectors was described previously in section 3.2.14.2.

3.2.16.3 *In vitro* transcription of pγAtg8

Linearized vectors, pα, pβΔβa and pγTdAtg8, were transcribed by mMessage mMachine® T7 High Yield Capped RNA Transcription kit (Ambion, 1344) as described in section 3.2.14.3

3.2.16.4 Inoculation

Triticum dicoccoides, genotype TR 62248, were inoculated with 1 µg of each transcript, pα, pβΔβa and pγTdAtg8 combined with 30 µl FES as described in section 3.2.14.4. The inoculation was applied to three different plants for each transcript.

Table 3.3 BSMV vectors using in *Atg8* silencing

	pα	**pβΔβa**	**pγ**	**pγAtg8**	**FES**
BSMV:00	1 µg	1 µg	1 µg	-	30 µl
BSMV:TdAtg8	1 µg	1 µg	-	1 µg	30 µl
FES	-	-	-	-	30 µl

3.2.16.5 Fusarium culmorum infection

A plant pathogen, *Fusarium culmorum*, was grown on potato dextrose agar (PDA) medium containing 4 g potato starch, 20 g dextrose and 15 g agar in 1 L dH_2O for 12-15 days at room temperature. The spores were collected with 3-5 µl dH_2O and counted by hemocytometer under light microscope. The concentration was calculated as $5x10^6$ spores/ml. 20 ml of spores were sprayed on BSMV inoculated plants after 14 days of virus inoculation.

3.2.16.6 Trypan blue staining

Disease symptoms were visible after 19 days of BSMV inoculation and after 5 days of fungi infection. Infected leaves were collected. 3-4 cm of each leaf tips were cut and stained with Trypan blue. The remainders of the leaves were stored at -80°C for RNA isolation. Leaf tips were boiled for 2 minutes in alcoholic lactophenol trypan blue (20 mL ethanol, 10 mL phenol, 10 mL water, 10 mL lactic acid (83%), and 10 mg trypan blue). Stained leaves were cleared in chloral hydrate (2.5 g dissolved in 1 mL of water) overnight at room temperature (Koch and Slusarenko, 1990). Leaf samples were observed under light microscope.

3.2.16.7 Quantification of *Atg8* expression level

Total RNA isolation was performed from the remainder parts of Trypan blue stained leaves as described in 3.2.2. cDNA was synthesized and real-time PCR was used to quantify *Atg8* expression level. Full lenght ORF *Atg8* primers were used for amplification.

4 RESULTS

4.1 *TdAtg8* expression pattern analysis

4.1.1 Semi-quantitative analysis

Semi-quantitative analysis was performed to detect *TdAtg8* expression level in both control and 20% PEG treated leaf and root tissues. As shown in Fig 4.1, *TdAtg8* expression was detected in leaves and roots in both control and 20% PEG treated plants; signifying that *TdAtg8* is constitutively expressed in whole plants. Moreover, semi-quantitative analysis indicated that the expression of *TdAtg8* is higher in PEG treated plant in comparison to the control in *T. dicoccoides*.

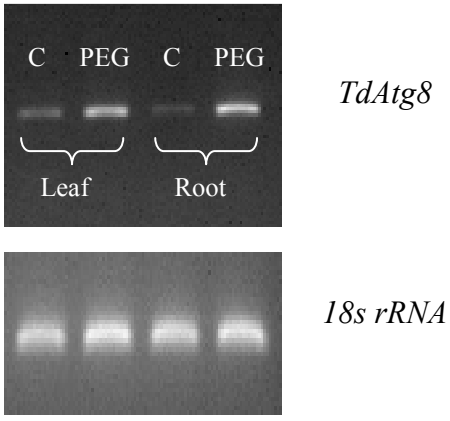

Fig 4.1 RT-PCR analysis of *TdAtg8* gene in leaf and root tissues of control and 20% PEG treated plants. 18S rRNA was used as a control

4.1.2 Quantitative analysis

The effect of drought stress on the expression pattern was also detected by real-time PCR. *TdAtg8* expression level was measured in order to analyze the effect of 20% PEG treatment in different tissues. 18S rRNA was used as an internal reference for normalization of data. The quantification was performed according to previous studies (Simon, 2003). Q-RT PCR results were in well-agreement with RT-PCR results. *TdAtg8* expression was detected in root and leaf tissues and under both control and drought stress conditions. These results indicated that *TdAtg8* is constitutively expressed in whole plants. In addition to this result, the higher expression level in 20% PEG treated samples was also demonstrated. As shown in Fig 4.2, with PEG treatment, the expression level of *Atg8* increases 24 fold and 40 fold in leaves and in roots, respectively.

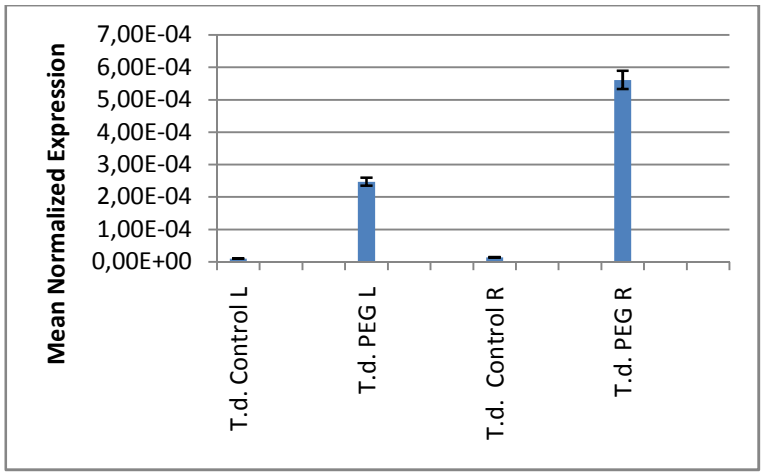

Fig 4.2 Quantitative analysis of *TdAtg8* expression level in both control and stress samples

4.2 Analysis of intron-exon organization in *Atg8*

The full length ORF of *Atg8* gene was amplified and sequenced from different species: *T. dicoccoides, T.monococcum* and *T.durum.* The sequences were aligned with CDS of *TdAtg8* (Fig 4.3) and the intron regions were determined using DNAStar

Lasergene 8. The alignment demonstrates that ORF of *Atg8* contains 5 exons and 4 introns.

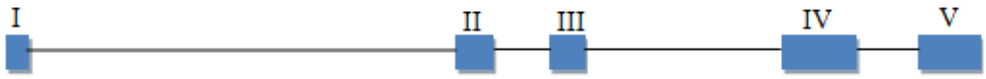

Fig 4.3 Intron-exon organization of *TdAtg8*. Boxes and solid lines represent exons and introns respectively

The protein sequences were aligned by ClustalW. Phylogenetic trees were constructed based on their coding sequences.

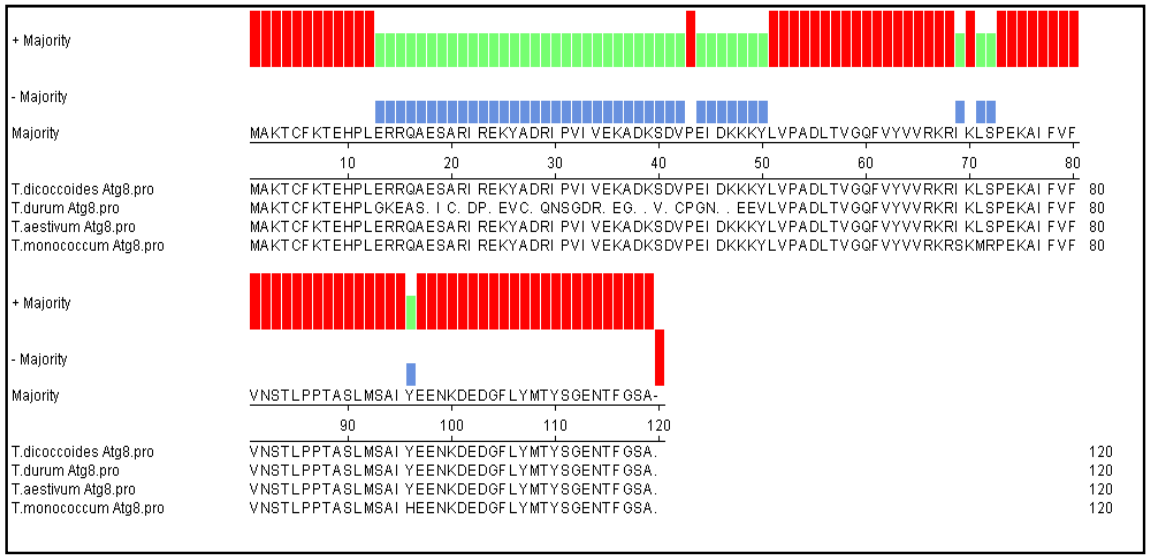

Fig 4.4 Multiple sequence alignment of ATG8 protein sequences

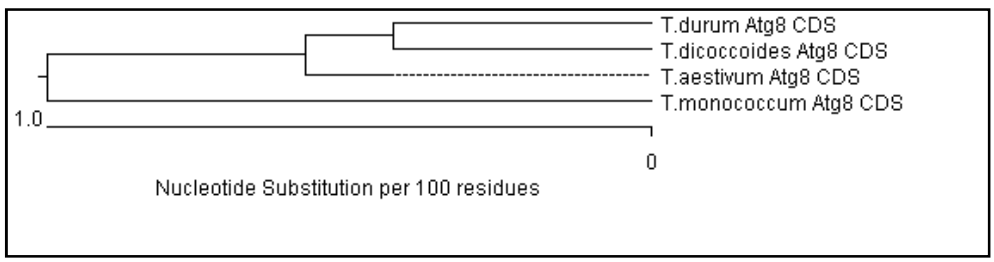

Fig 4.5 Phylogenetic tree constructed based on CDS

4.3 Chromosomal localization of *Atg8* gene

Nullisomic-tetrasomic wheat lines were used to determine chromosomal localization of *Atg8* gene. *Atg8* was amplified and PCR products were run on 0.8% agarose gel at 100V for 1 h. Three different fragments were obtained indicating that three different copies of *Atg8* are present in the *T. aestivum* genome (AABBDD). Although, observing only one band in samples N2A-T2D and N2D-T2A (shown with arrows) was explained as the gene may localize in chromosome 2, since the deletion of 2A and 2D may result in amplification of only two copies of Atg8 (Fig 4.6).

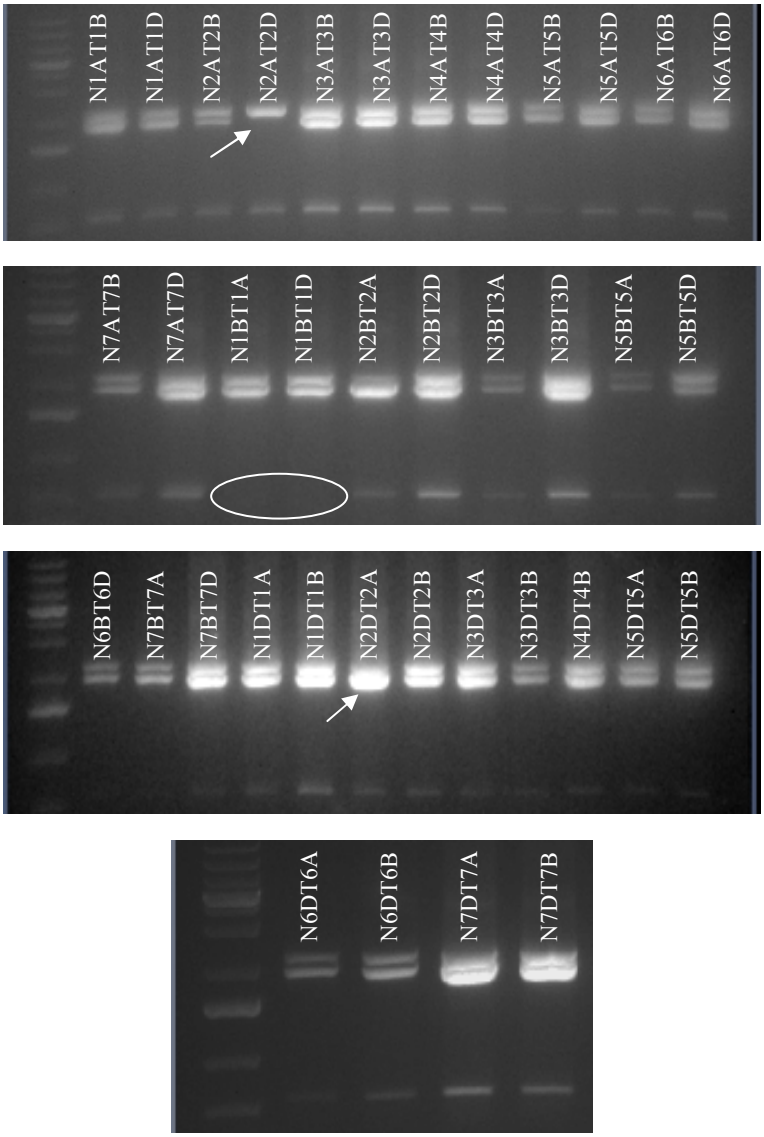

Fig 4.6 Agarose gel electrophoresis results of Atg8 amplification from nullisomic-tetrasomic wheat lines

The third fragment was observed in every *T. aestivum* samples, except N1B-T1A and N1B-T1D (marked with the circle in Fig 4.6), indicating that there will be another copy which is located on chromosome 1B.

The samples that contain a deletion in the chromosome 2 were also analyzed on polyacrylamide gel. *Atg8* fragments obtained from different species, *T. monococcum* (AA), *T.dicoccoides* (AABB), *T.durum* (AABB), *Aegilops tauschii* (DD), were also loaded to determine the genomic source of each copy. Three copies of *Atg8* were labeled as a, b and c, as shown in Fig 4.7 The copy (a) was obtained from genome D since it is the only fragment present in *A. tauschii* (DD) and the copy (b) was amplified from genome A since it is the only fragment present in *T. monococcum* (AA). The lack of copies (a) and (b) in samples N2A-T2D and N2B-T2A, respectively, indicate that *Atg8* is located on chromosomes 2A, 2B and 2D. The presence of copy (a) in samples N2D-T2A and N2D-T2B shows that the genome B contain the same copy of *Atg8* as the genome D and it was verified by the samples obtained from *T. durum* (AABB) and *T. dicoccoides* (AABB) having the copies (a) and (b). The third copy (c) was observed every *T. aestivum* samples, except N1B-T1A and N1B-T1D, indicating that the gene was also located on chromosome 1B.

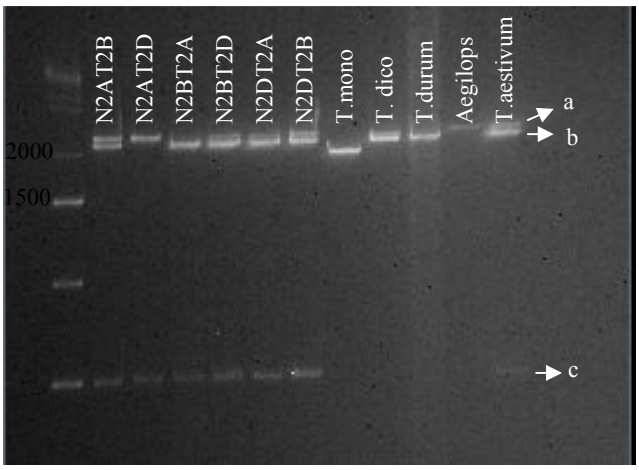

Fig 4.7 Polyacrylamide gel electrophoresis result of Atg8 amplification from nullisomic-tetrasomic wheat lines. *Atg8* fragments obtained from different species, *T. monococcum* (AA), *T.dicoccoides* (AABB), *T.durum* (AABB), *Aegilops tauschii* (DD), were also loaded as control.

4.4 Monitoring autophagy in Triticum dicoccoides

4.4.1 Monodansylcadaverine (MDC) staining

MDC is a fluorescent dye used to monitor autophagosomes in plant studies. Control and 20% PEG treated *T. dicoccoides* root tips were stained with MDC and observed under fluorescence microscope (Olympus BX60, Japan). As shown in Fig 4.8, MDC fluorescence signal was found to be higher in roots of plants treated with 20% PEG and the number of MDC stained vesicles increased in these plants.

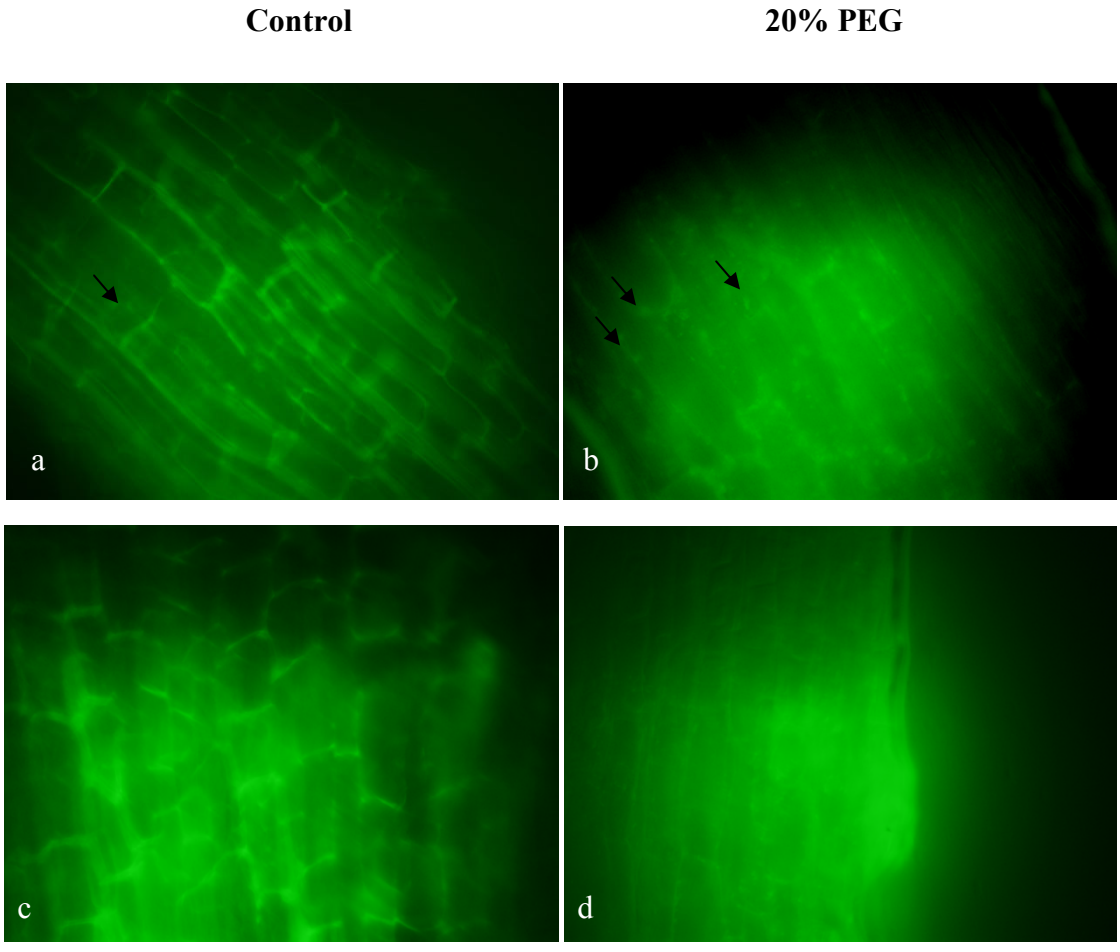

Fig 4.8 MDC staining of *T. dicoccoides* roots. (a) MDC stained control root sample, (b) MDC stained 20% PEG treated root sample, (c) Unstained control root sample, (d) Unstained 20% PEG treated root sample

4.4.2 LysoTracker® red staining

LysoTracker® red (Invitrogen) is a dye, freely permeant to cell membranes and typically concentrates in spherical organelles such as lysosomes, endosomes. This probe is used to monitor autophagosomes because of their internal pH level. Previous studies indicated that LysoTracker Red specifically stains autophagosomes and can be used in plant autophagy researchs (Moriyasu et al., 2003). Control and 20% PEG treated root samples were incubated with E64d which is the membrane-permenant cysteine protease inhibitor. In previous studies, E64d was used to accumulate autophagic bodies inside vacuoles in *Arabidopsis thaliana* (Bassham, 2007) or outside the vacuoles, in smaller organelles called autolysosomes, in tobacco cells (Inoue et al., 2006).

LysoTracker® red staining indicated that autophagosomes are also present in control plant, demonstrating that autophagy is constitutively active in *T. dicoccoides* (Fig 4.9 a). However, staining results showed also that the number of autophagosomes in roots of drought stress treated plants increases in comparison to the control plant (Fig 4.9 b). Autophagosomes were not observed in LysoTracker® red stained samples without E64d treatment (Fig 4.9 c-d), indicating that E64d is necessary for the detection of autophagosomes in plants.

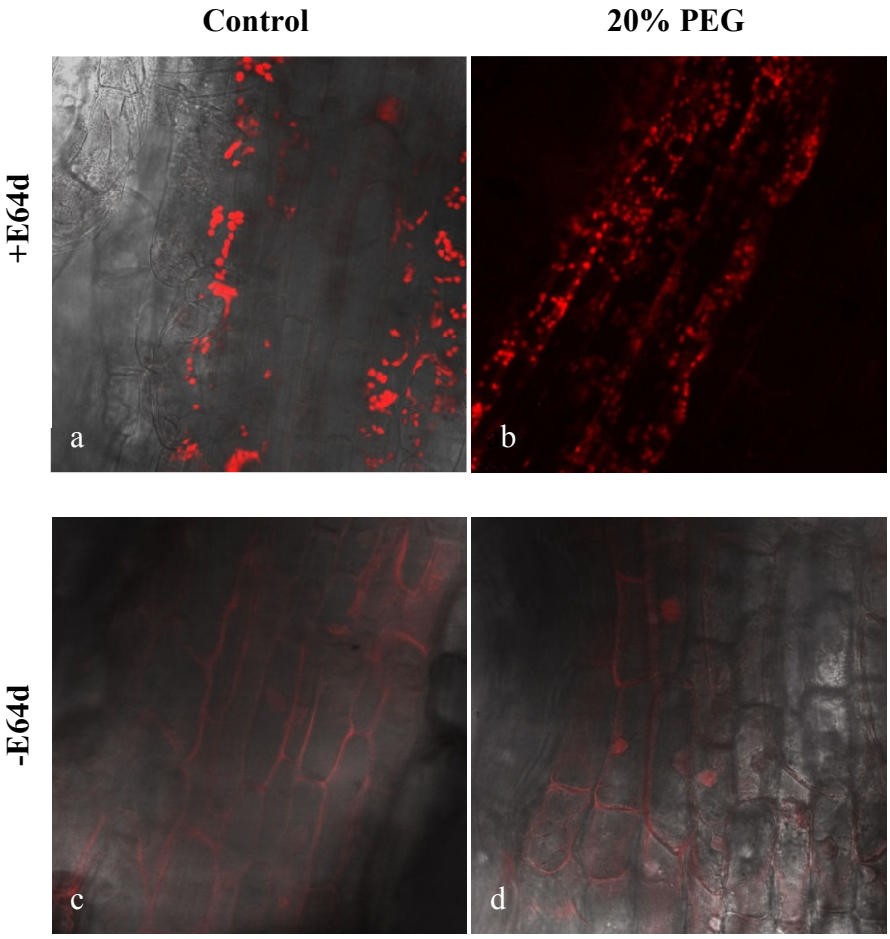

Fig 4.9 LysoTracker® red stained root tips of control and 20% PEG treated samples. Autophagosomes were observed in control (a) and PEG treated (b) root samples incubated with E64d for 1 day. Control (c) and PEG treated (d) root tips were also stained without E64d treatment, however autophagosomes were not visible in this case.

4.5 Functional analysis of *TdAtg8*

4.5.1 Yeast complementation with *TdAtg8*

Full length ORF of *TdAtg8* gene was amplified and ligated with pGEM T-easy vector. *E.coli* cells were transformed with pGEM T-easy-*TdAtg8* recombinant vector. The insertion of target gene was checked with colony PCR, as shown in Fig. 4.5. Plasmids were purified from colonies and were sequenced (Fig 4.10).

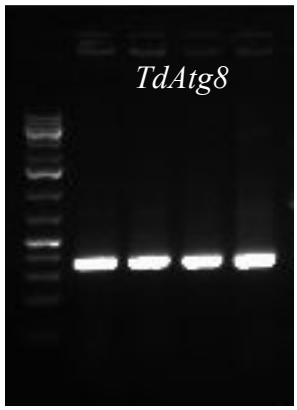

Fig 4.10 Colony PCR to check the insertion of *TdAtg8* into *E.coli* cells

AAGCTTCATGGCCAAGACTTGCTTCAAGACCGAGCACCCCCTGGAAAGGAGG
CAAGCTGAATCTGCTAGGATCCGTGAGAAGTATGCTGATAGAATTCCGGTGAT
CGTTGAGAAGGCTGATAAGTCTGATGTCCCGGAAATTGATAAGAAGAAGTAT
CTTGTCCCGGCCGACCTCACTGTTGGCCAGTTTGTCTACGTGGTGCGGAAGAG
GATCAAGCTGAGCCCAGAAAAGGCCATCTTCGTCTTTGTGAATAGCACCTTGC
CACCGACTGCTTCGTTGATGTCAGCCATCTATGAAGAAAACAAGGACGAGGA
CGGCTTCCTGTACATGACTTACAGTGGCGAGAACACTTTCGGCTCTGCCTAAC
TCGAG

Fig 4.11 CDS of *TdAtg8* inserted into pGEM T-easy vector. Forward and reverse primer sequences were underlined and restriction sites were labelled as bold characters

Purified plasmids containing full length ORF of *TdAtg8* and pYES2 expression vector were digested with *XhoI* and *HindIII* restriction enzymes. *XhoI* / *HindIII* digested pYES2 vector was ligated with *XhoI* / *HindIII* digested *TdAtg8* fragment and inserted into yeast *Atg8* mutant strain (BY4741, atg8Δ::*kanMX*, *MATa*, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) to test if TdATG8 protein is a functional homolog of yeast ATG8. Mutant strain was complemented with TdATG8 by introducing pYES2 yeast expression vector containing the target gene. The cloning was checked by colony PCR (Fig 4.12). Mutant yeast strain was also used as a negative control.

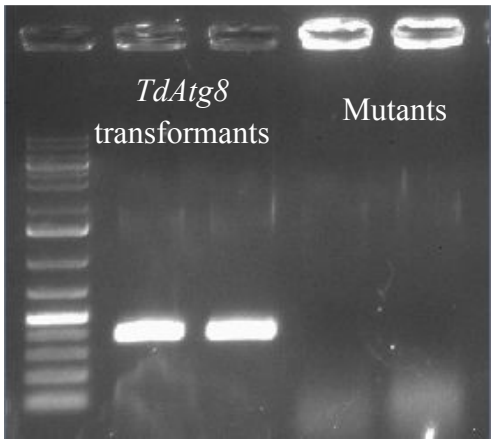

Fig 4.12 Colony PCR result of yeast transformants to check the insertion of *TdAtg8* into yeast mutant strain

Yeast transformants were grown in medium without nitrogen to induce starvation and hence autophagy in yeast. Galactose was used as carbon source since pYES2 vector has GAL1, galactose inducible promoter. URA was the auxotrophic marker in the study. pRS316 vector containing yeast ATG8 protein was also cloned to yeast mutant strain as a positive control. TdATG8 transformant yeast cells and positive control cells were grown better than mutant strain under N deficiency conditions (Fig 4.13). The similarity between *TdAtg8* transformants and yeast *Atg8* transformants indicated that *TdAtg8* functionally complements the yeast *Atg8* gene. Transformants were also grown on standard rich medium (YPD) for loading control.

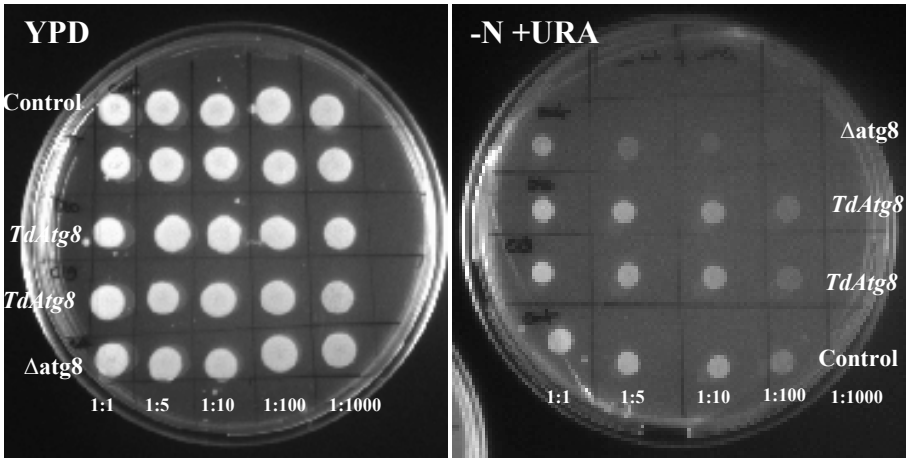

Fig 4.13 Functional complementation of yeast mutant strain with *TdAtg8* gene

4.5.2 Western blotting analysis with anti-API for functional complementation of yeast with *TdAtg8*

Functional complementation of yeast with *TdAtg8* gene was further investigated with the maturation of API enzyme which indicates that an active autophagy pathway is present in yeast. API is synthesized in the cytosol as its precursor form, prAPI (61 kDa), and is matured to mAPI (50 kDa) inside the vacuole in the presence of an active autophagy pathway. This maturation was used to check if *TdAtg8* is the homolog of yeast *Atg8*. Yeast mutant strain and transformants containing pYES-*TdAtg8* recombinant vector were grown under N deficiency conditions and their total proteins were extracted. Western blot analysis was performed with polyclonal anti-API antibody. mAPI was found in *TdAtg8* transformants and also in yeast *Atg8* transformants. The prAPI form was also present in *TdAtg8* transformants indicating that, *TdAtg8* do not complement the yeast *Atg8* totally, since in that case only mAPI should be found. However, the presence of mAPI showed also that *TdAtg8* complements functionally the yeast *Atg8* and causes the activation of autophagy in yeast.

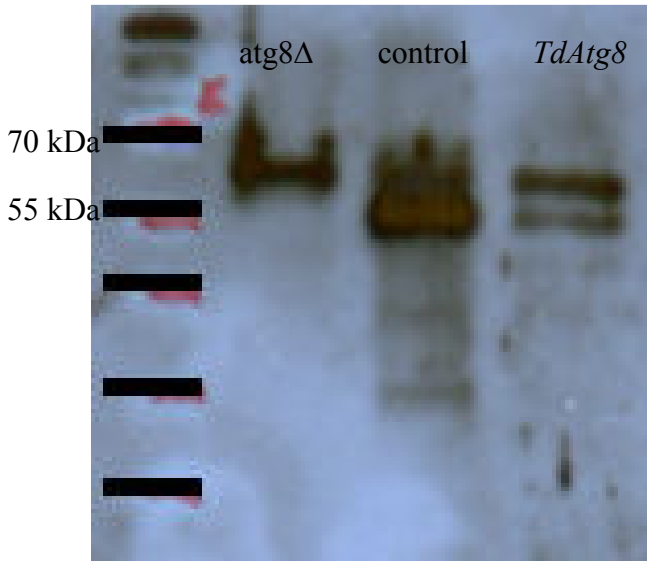

Fig 4.14 Western blotting analysis of *TdAtg8* yeast transformants with anti-API antibody

4.5.3 Western blotting analysis of TdATG8 protein

Full length ORF of *TdAtg8* gene was cloned into pACT2 yeast expression vector to construct a fusion protein containing GAL4 activation domain (AD), an HA epitope tag and TdATG8 protein, for further investigation of functional expression of TdATG8 protein in yeast. Recombinant vector was transformed into yeast mutant strain and yeast cell lysates were analyzed with western blotting using monoclonal anti-HA antibody. The expected size of the fusion protein was ~27 kDa since the additional size of GAL4 AD/HA tag (~14 kDa) to TdATG8 (~13 kDa) protein. Western blotting results indicated that TdATG8 was expressed in yeast.

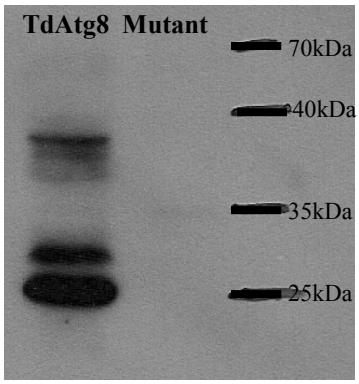

Fig 4.15 Western blotting analysis of TdATG8 protein with anti-HA antibody

TdATG8 proteins, extracted from both control and 20% PEG leaf and root tissues, were also labeled with anti-AtAtg8 antibody.

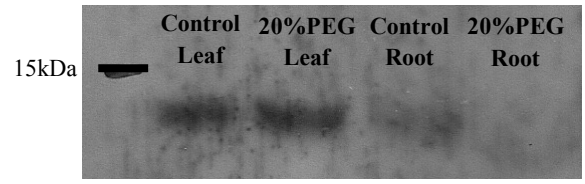

Fig 4.16 Western blotting analysis of TdATG8 protein with anti-AtAtg8 antibody

4.5.4 *PDS* Silencing

In prior to *TdAtg8* silencing for functional analysis, *PDS* gene was silenced by VIGS in *T. dicoccoides* to show the applicability of this method in wild emmer wheat.

PDS gene was used as a monitor gene in previous studies because of its photo-bleaching activity (Holzberg 2002; Scofield 2005).

4.5.4.1 BSMV:GFP analysis

Plants at third leaf stage were inoculated with BSMV:GFP vector to visualize virus spreading as a control. Leaves were collected after 5 days of inoculation. FES control and BSMV:00 which contains empty vector were used as negative controls. Samples were observed under fluorescence microscope. GFP expression was not so distinct because of the thickness of leaf sample; however some symptoms indicating GFP were observed.

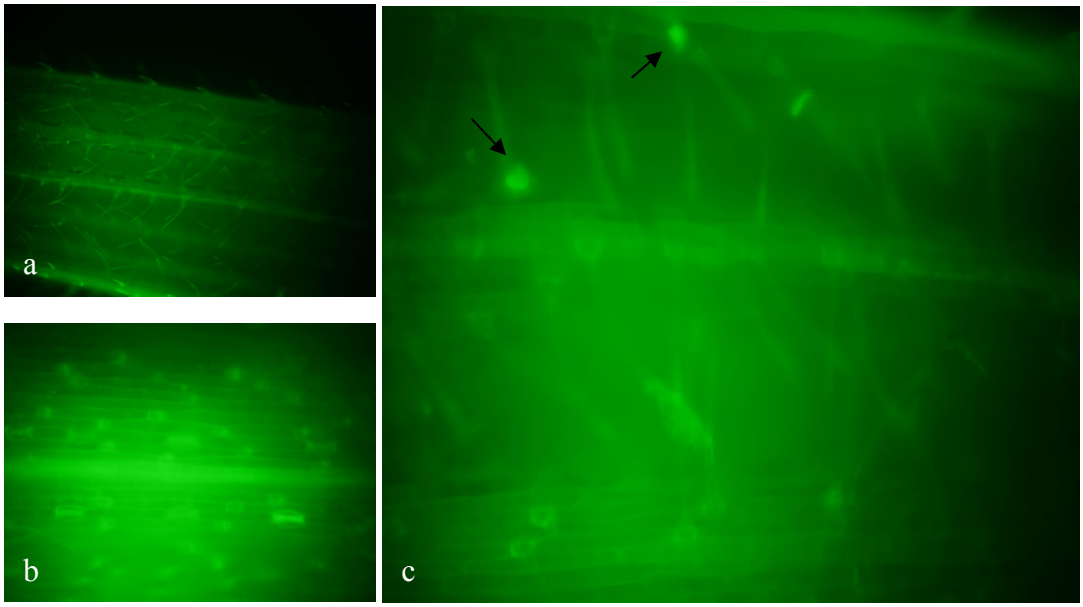

Fig 4.17 GFP visualization in BSMV:GFP inoculated plants. GFP expression was not observed in FES control (a) and empty vector (b), however spreading of the virus was demonstrated with BSMV:GFP inoculation (c).

4.5.4.2 Phenotypic analysis of *PDS* silencing

Silencing *PDS* prevents the protection of chlorophyll from photo-bleaching and causes unusual white tissues in leaves. The effect of *PDS* silencing, streaks and patches of white tissue, was observed in young leaves of plants inoculated with BSMV:PDS vector after 21 days of inoculation until 45 days (Fig 4.18). The same effect of the virus was not examined in plants infected with BSMV:00, empty vector. In previous studies, the first symptoms of silencing was visualized at 7 dpi (Holzberg et al., 2002; Scofield et al., 2006), however, in our case, white tissues were not observed until 15 dpi. This difference can be explained due to different species used in the studies.

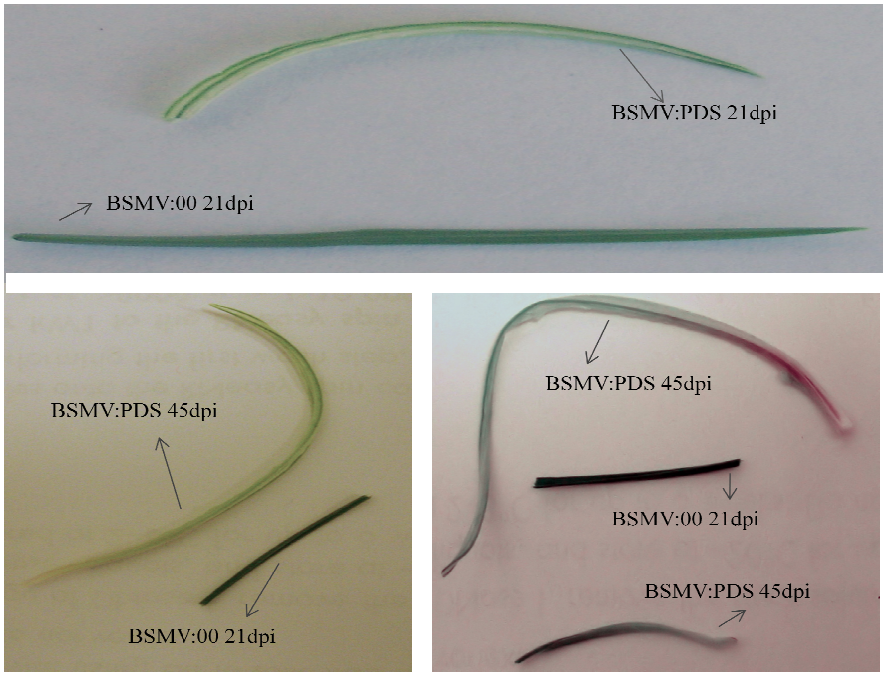

Fig 4.18 Phenotypic analysis of *PDS* silencing

4.5.4.3 Quantitative analysis of *PDS* expression level by Q-RT PCR

Total RNAs of silenced leaves were purified. Following cDNA synthesis and DNase treatment, q-RT PCR was performed. 90% decrease in *PDS* expression level

confirmed phenotypic results and indicated that VIGS is an efficient method for functional analysis of specific genes in *T. dicoccoides.*

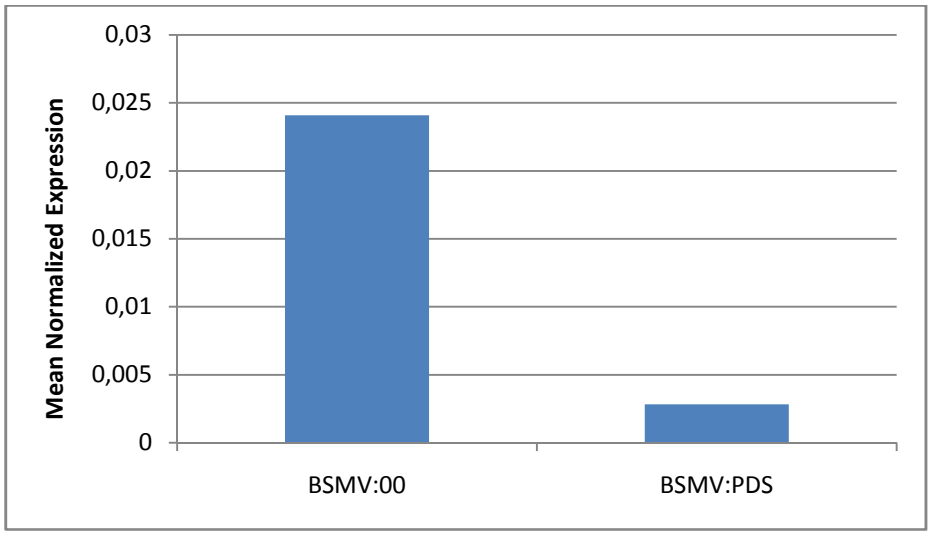

Fig 4.19 Quantitative analysis of *PDS* expression level

4.5.5 *Atg8* silencing

4.5.5.1 *TdAtg8* cloning into BSMV pγ vector

Primers were designed to amplify the fragment (133 bp) found in the 3' end of the *TdAtg8* CDS and restriction enzyme sites were added at 5' end of the primers. The fragment was amplified and transformed into *E.coli* cells with the pGEM T-easy vector. The insertion was confirmed by sequencing (Fig 4.20).

GCGGCCGCTGTTCTCGCCACTGTAAGTCATGTACAGGAAGCCGTCCTCGT
CCTTGTTTTCTTCATAGATCGCTGACATCAACGAAGCAGTCGGTGGCAAG
GTGCTATTCACAAAGACGAAGATGGCCTTTTCTGGGCTCAG**TTAATTAA**

Fig 4.20 Sequence of *TdAtg8* inserted into pGEM T-easy vector for *Atg8* silencing. Forward and reverse primer sequences were underlined and restriction sites were labelled as bold characters

pGEM-T easy vector containing *TdAtg8* fragment and pγ vector were digested with *NotI* and *PacI* restriction enzymes. Digested fragment was ligated with digested vector and pγTdAtg8 recombinant vector was transformed into *E.coli* cells. The ligation and the insertion were confirmed by colony PCR.

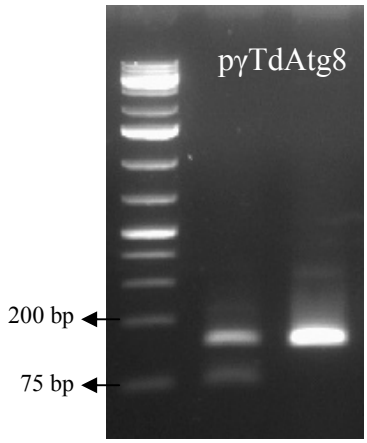

Fig 4.21 Colony PCR to confirm the insertion of *TdAtg8* fragment into pγ vector

pα, pβΔβa and pγTdAtg8 recombinant vector were digested with *MluI, BcuI* and *BsshII* restriction enzymes, respectively. Linerarized vectors were transcribed, *in vitro.* Plants were inoculated with pα, pβΔβa and pγTdAtg8 transcripts (BSMV:TdAtg8) mixed with FES. Plants were also inoculated with only FES and with empty vector BSMV:00, as negative controls.

4.5.5.2 *Fusarium culmorum* treatment and quantitative analysis of *TdAtg8* expression level

The plants were infected with *Fusarium culmorum,* a plant pathogen, at 14th day of BSMV inoculation. Samples were collected at 19th day of BSMV inoculation and 5th day of fungi inoculation.

Leaf tips were stained with Trypan blue and observed under light microscope. Results showed that the number of spores decreases in BSMV:TdAtg8 inoculated plants indicating that silencing *Atg8* make the plant more resistant to *F. culmorum.* 3 leaves of 3 different plants were examined and number of spores was counted (Fig 4.22).

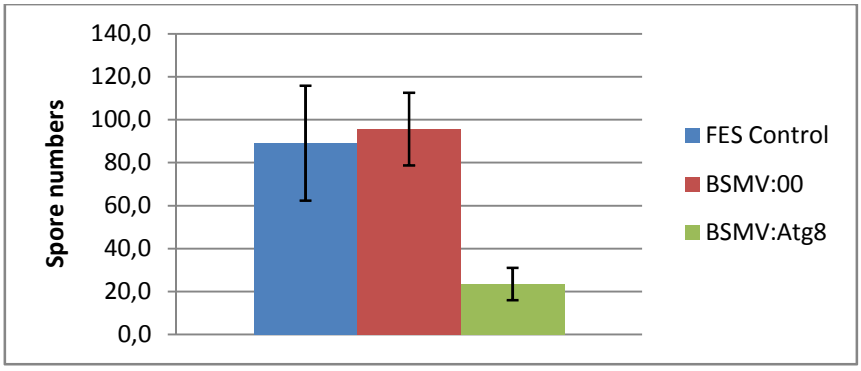

Fig 4.22 Spore numbers counted from control and BSMV:TdAtg8 inoculated leaf samples

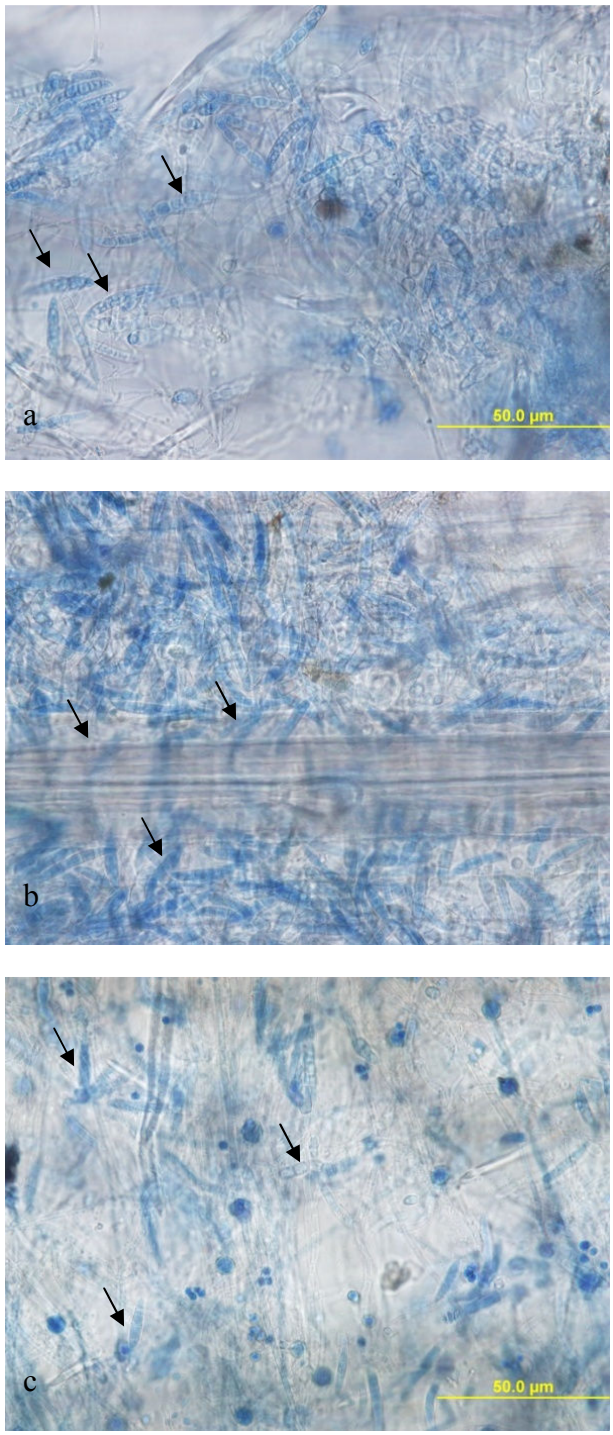

Fig 4.23 Spores (shown by arrows) stained with Trypan blue, in FES control (a), BSMV:00 (b) and BSMV:TdAtg8 (c)

Total RNA was also isolated from the remainder parts of leaf samples stained with Trypan blue. Following cDNA synthesis, Q-RT PCR was performed for quantitative analysis. Real-time PCR results indicated that *TdAtg8* expression level decreases in comparison to FES and BSMV:00 control. The increase in *TdAtg8*

expression level in BSMV:00 was explained by virus infection; since the vector used in the study is a virus, the presence of the vector induced the Atg8 expression, as expected.

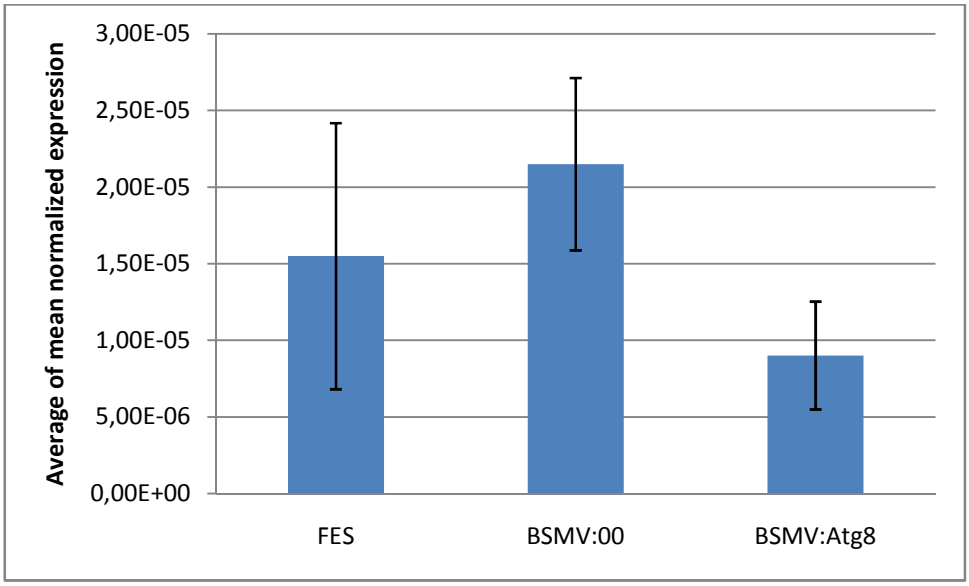

Fig 4.24 Quantitative analysis of *TdAtg8* expression level in pathogen treated leaf samples

5 DISCUSSION

Previous studies have reported that autophagy is active in plants and is induced under abiotic stress conditions including nutrient deficiency (Chen et al., 1994; Doelling et al., 2002; Hanaoka et al., 2002; Surpin et al., 2003; Yoshimoto et al., 2004; Xiong et al., 2005; Fujiki et al., 2007), drought stress, salt stress (Cebeci, 2010; Liu et al, 2009) and also oxidative stress (Xiong et al., 2007b). In this study, we cloned an autophagy related gene, *Atg8*, for the first time, in *Triticum dicoccoides* and its role in plant autophagy was identified under drought stress condition. Firstly, *Atg8* expression pattern was analyzed indicating that *Atg8* is constitutively active in roots. Measuring *Atg8* expression level in control and 20% PEG treated leaf and root tissues showed that drought stress induces autophagy in both tissues (Fig 4.2). Secondly, MDC stained-root tissues were observed under fluorescence microscope and the number of autophagosomes was found to be higher in 20% PEG treated root tissues in comparison to control ones (Fig 4.8). Thirdly, Lysotracker-stained root tissues were analyzed by confocal microscopy and it was in well-agreement with MDC staining results indicating that autophagy is induced under drought stress. The Lysotracker staining was performed in the presence and absence of E64d, a cysteine protease inhibitor; and it was reported that, autophagosomes were accumulated in the presence of E64d, as expected (Inoue et al., 2006; Moriyasu et al., 2003). These results showed that autophagy is constitutively active in *T. dicoccoides* root and leaf tissues and is induced under drought stress conditions.

Coding sequence alignment of *TdAtg8* gene with full length open reading frame of *TdAtg8* was demonstrated that *TdAtg8* gene contains 5 exons and 4 introns. The genomic organization of *Atg8* was also analyzed in different *Triticum* species, *T. monococcum, T. durum* and *T. aestivum* and it was found the same exon-intron organization in these species. CDS of *Atg8* was obtained from these species and

translated to proteins. Phylogenetic tree of different *Triticum* species was constructed based on the coding sequences of ATG8. The tree showed that *T. aestivum, T. durum* and *T. dicoccoides* are evolutionary more closer to each other than the *T. monococcum* (Fig 4.5). This result was also in well-aggrement with previous studies which have demonstrated that the *T. dicoccoides* is the progenitor of modern cultivated wheat, *T.durum* and *T.aestivum* and diverged from *T.monococcum* (Fig 2.1). Alignment was also showed that all *Triticum* ATG8 proteins contain Gly residue in their C-terminal suggesting that these proteins are posttranscriptionally modifies as in yeast and other plants such as *Arabidopsis thaliana* (Hanaoka et al., 2002) and rice (Su et al., 2006).

Chromosomal localization analysis of *Atg8* was demonstrated that three and two different copies were found in cultivated wheat and wild emmer, respectively. This data was expected since more than one copy number of a gene was common in polyploide wheat. *Atg8* was found to be located on 2A, 2B, 2D and also 1B in *Triticum aestivum* by using nullisomic-tetrasomic wheat lines.

TdATG8 protein was also functionally analyzed by yeast complementation assays. First, *TdAtg8* was introduced into *Atg8* yeast mutant to detect whether or not *TdAtg8* is the ortholog of yeast *Atg8*. Drop test assay results showed that TdATG8 transformant yeast cells and positive control cells containing yeast *Atg8* were grown better than mutant strain under N deficiency conditions (Fig 4.13), indicating that *TdAtg8* is the orthologous gene of yeast *Atg8*. Second, western blot analysis of yeast transformants containing TdATG8 and mutant yeast strains was performed by anti-API antibody. Since maturation of API inside the vacuole only takes place when there is an active autophagy pathway (Ketelaar et al., 2004), obtaining two different forms of API in transformed yeast demonstrated that *TdAtg8* complemented yeast *Atg8* mutant and also activated autophagy pathway in yeast (Fig 4.14). Third, a fusion protein was constructed containing *TdAtg8* and HA tag. TdATG8-HA fusion protein was expressed in yeast and its expression was detected by western blot analysis using anti-HA antibody. This result supported the assumption that TdATG8 could function in yeast (Fig 4.15). The western blotting analysis performed with anti-AtATG8 antibody showed that this antibody can be used to determine ATG8 protein expression level under different stress conditions (Fig 4.16).

The expression pattern of *TdAtg8* was also analyzed in plants. VIGS was performed to obtain *Atg8* mutant *T.dicoccoides*. First, the efficiency of this technique was checked by *Pds* silencing. Since silencing *Pds* prevents the protection of chlorophyll from photo-bleaching and causes unusual white tissues in leaves, phenotypic results demonstrated that VIGS is efficiently working in wild emmer wheat (Fig 4.18). In addition to phenotypic observation, the expression level of *Pds* was also measured and 90% decrease was observed in the expression level of silenced plants (Fig 4.19). GFP expression was also observed by using BSMV:GFP vector to visualize virus spreading in leaf tissues (4.17). Following the determination of VIGS in wild emmer as an efficient tool, *TdAtg8* was silenced to detect the role of autophagy in the presence of a plant pathogen, *Fusarium culmorum*. Silenced plants were infected with *F. culmorum* and the number of pathogen was counted in control and silenced leaf tissues. Previous studies have reported that autophagy is involved in plant immune systems (Deretic, 2005; Levine, 2005; Liu et al., 2005) and controls HR-PCD (Liu et al., 2005). Specially, studies on tobacco showed that an autophagy related protein, ATG6, is required for the restriction of HR-PCD in the infected sites (Seay & Dinesh-Kumar, 2005; Patel et al., 2006). Our data showed that in the presence of a plant pathogen, the expression level of *Atg8* is highly upregulated, as expected, since the autophagy is induced by the immune system. However, the decrease in the number of spores in silenced plants was not expected. In comparison to control tissues, ~80% decrease in the number of spores was observed (Fig 4.22). This data suggested that, the inhibition of autophagy may induce other resistance mechanisms in plants and may result to obtain more resistant plants to viral infection.

6 CONCLUSION

The autophagy mechanism was studied in model organism such as *Arabidopsis* and tobacco; however there are a few studies investigating the role of autophagy in economically important cereals, such as wheat, barley. In this study, an autophagy related gene, *Atg8,* was identified for the first time in wild emmer wheat. Its role was analyzed in plant autophagy under drought stress conditions. Therefore the expression level of T*dAtg8* and the formation of autophagosomes were observed. The upregulated expression level and high number of autophagosomes stained by MDC and LysoTracker demonstrated that autophagy is induced in plants exposed to drought stress conditions. Yeast complementation assays have reported that *TdAtg8,* as an orthologous gene of yeast *Atg8,* complements yeast *Atg8* mutant and functionally expressed in yeast cells. The role of autophagy in the presence of a plant pathogen was also analyzed. For this purpose, VIGS was performed, for the first time, in wild emmer wheat. The functional analysis in wild emmer wheat has demonstrated that *TdAtg8* expression is upregulated in the presence of a plant pathogen and silencing *TdAtg8* decreases the fungi sporulation indicating that plant become more resistant. In conclusion, in this study, autophagy mechanism was investigated for the first time, in wild emmer wheat demonstrating that it is induced under drought stress conditions and in the presence of plant pathogen.

For future prospects, *TdAtg8* silenced plants should be exposed to drought stress to analyze the role of autophagy under drought stress conditions in wild emmer. Additionally, the relation between the autophagy and the plant immune system should also be investigated to understand the resistance mechanism of plants which plays an important role in wheat breeding studies.

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APPENDIX A

BSMV α genome sequence:

GTATGTAAGTTGCCTTTGGGTGTAAAATTTCTTGCATGCACATAATCGTAATCGATTCTTC
TTGATCTCTAAACAACACTTTCCCGTTAGCATGGCTAGCGATGAGATTGTCCGCAATCTGA
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BSMV β genome sequence

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BSMV γ genome sequence

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pγPDS sequence

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APPENDIX B

Coding sequence of *Triticum aestivum Atg8*

GenBank: FJ750848.1

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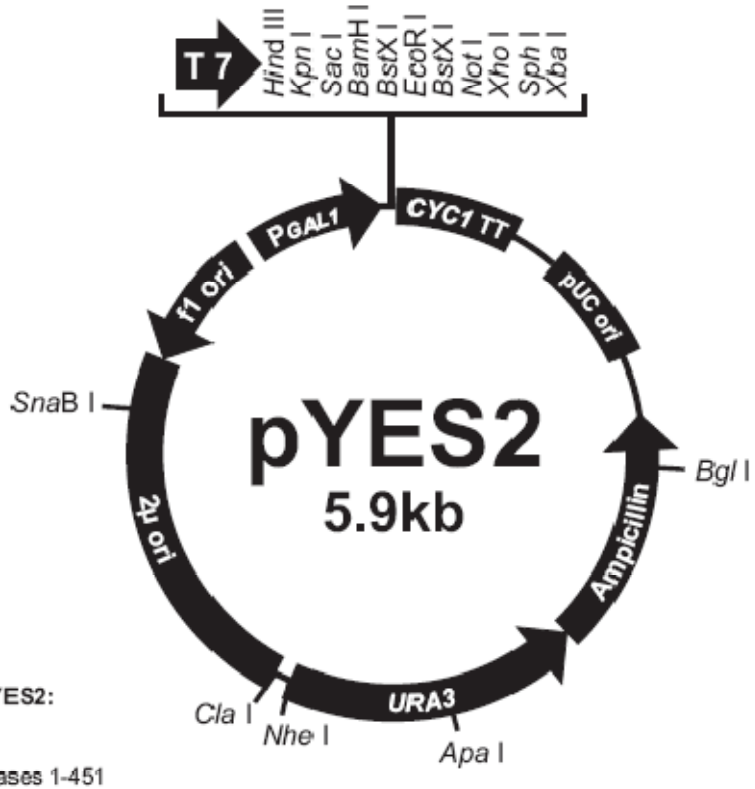
APPENDIX C

Map of pYES2

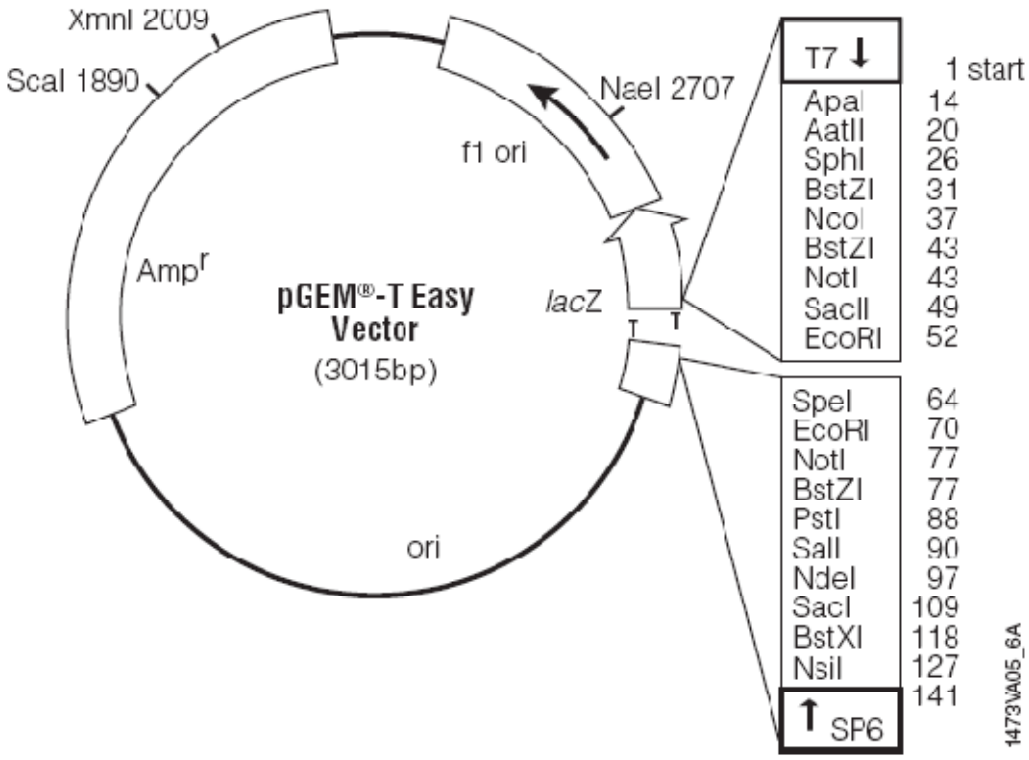


Comments for pYES2:
5856 nucleotides

GAL1 promoter: bases 1-451
T7 promoter/priming site: bases 475-494
Multiple cloning site: bases 501-600
CYC1 transcription terminator: bases 608-856
pUC origin: bases 1038-1711
Ampicillin resistance gene: bases 1856-2716 (C)
URA3 gene: bases 2734-3841 (C)
2 micron (µ) origin: bases 3845-5316
f1 origin: bases 5384-5839 (C)
(C) = complementary strand

APPENDIX D

Map of pGEM-T easy

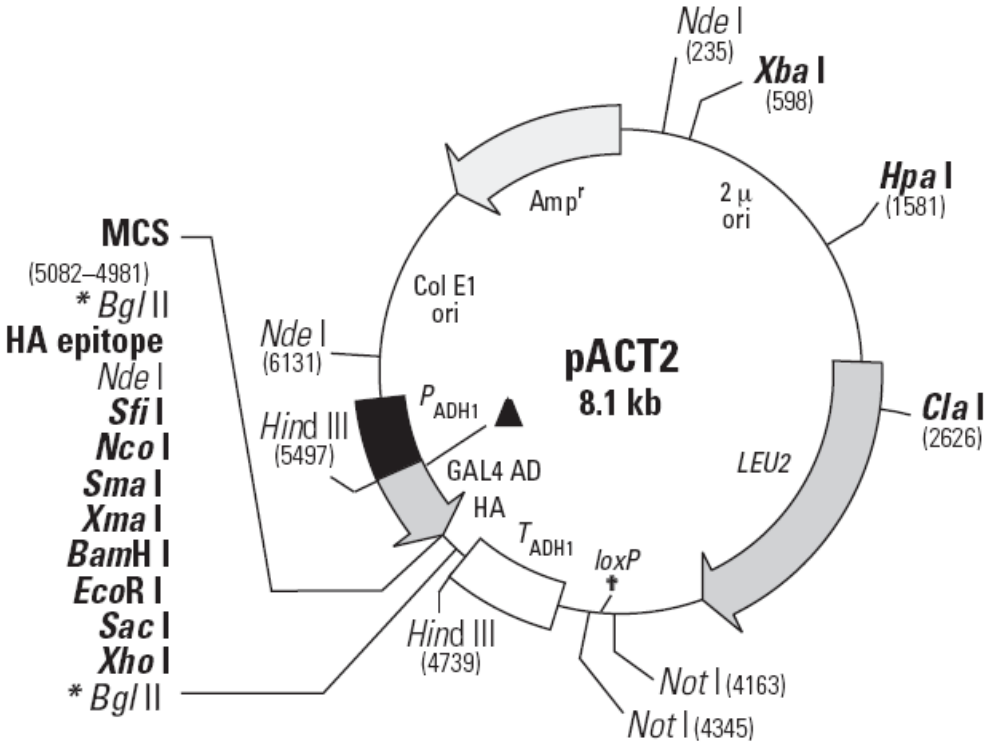


APPENDIX E

Map of pACT-2

pACT2 AD Vector Information

GenBank Accession No.: U29899.



APPENDIX F

Equipments

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve, OT 032, TURKEY
Balance:	Sartorius, BP 221 S, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Beckman Coultier ™ Microfuge® 18 Centrifuge, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Cassette:	Kodak Biomax MS casette, USA
Confocal microscope	Leica Microsystems Heidelberg GmbH, GERMANY
Deep-freeze:	-80ºC, Thermo Electron Corporation, USA
	-20ºC, Bosch, TURKEY
Deionized water:	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Biogen Inc., USA
	Biorad Inc., USA
	SCIE-PLAS, TURKEY

Fluorescence microscope:	OLYMPUS, BX-60, JAPAN
Gel documentatiton:	UVITEC, UVIdoc Gel Documentation System,UK
	BIO-RAD, UV-Transilluminator 2000, USA
Heating block:	Bioblock Scientific, FRANCE
	Bio TDB-100 Dry Block Heating Thermostat, HVD Life Sciences
	AUSTRIA
Ice machine:	Scotsman Inc., AF20, USA
Incubator:	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
	Nüve EN 120, TURKEY
Laminar flow:	Kendro Lab. Prod., Heraeus, Herasafe HS12, GERMANY
Magnetic stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Micropipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microwave Oven:	Bosch, TURKEY
pH meter:	WTW, pH540 GLP Multical®, GERMANY
	HANNA, pH213 microprocessor pH meter, GERMANY

Power Supply:	Wealtec, Elite 300, USA
	Biogen, AELEX, USA
Real-Time detection system:	BIO-RAD, iCyclerİQ™ Multicolor Real-Time Detection System, USA
Refrigerator:	+4º, Bosch, TURKEY
Shaker:	Excella E24 Shaker Series, New Brunswick Sci., USA
	GFL, Shaker 3011, USA
	Innova™ 4330, New Brunswick Sci., USA
Spectrophotometer:	BIO-RAD, SmartSpec™ 3000, USA
	VARIAN, Cary 300 Bio Uvi-visible spec., AUSTRALIA
Speed vacuum:	Savant, Refrigerated Vapor Trap RVT 400, USA
Thermocycler:	PE Applied biosystems, GeneAmp PCR System 9700, USA
	MJ Research, PTC-100, USA
	TECHNE, TC 512, UK
Water bath:	TECHNE, Refrigerated Bath RB-5A, UK
	JULABO, TW 20, USA

APPENDIX G

Chemicals & Enzymes

2-Mercaptoethanol	Biotechnology	A2008
2-propanol	Riedel-deHaën	34486
Acrylamide/Bisacrylamide 1:29	Sigma	A2792
Agar	Applichem	A2112
Agarose	Prona	084543PR
Ampicillin sodium	Duchefa	A0104
APS	Bio-Rad	161-0700
BcuI	Fermentas	ER1251
Bentonit	Applichem	A6918
Boric acid	Merck	1.00165
Bradford reagent	Sigma	B6916
BsshII	NEB	R0199L
Celite	Fluka	22145
Chloral hydrate	Sigma-Aldrich	15307
Chloroform	Amresco	0757
dNTP	Fermentas	R1121
EcoRI	Fermentas	ER0271
EDTA	Riedel-deHaën	34540
Ethanol	Riedel-deHaën	32221
Ethidium Bromide	Sigma	E7637
Galactose	Applichem	A2112

D-Glucose	Gibco	15023
Glycerol	Riedel-deHaën	15524
H_2O_2	Sigma-Aldrich	18312
Hepes Buffer	Sigma	H0887
HindIII	Fermentas	ER0501
IPTG	PeqLab	37-2020
LB Agar	Applichem	A0927
LB Broth	Applichem	L3027
Luminol	Fluka	09253
Magnesium sulfate	Merck	1.05882
Methanol	Sigma-Aldrich	24229
$MgCl_2$	Fluka	63063
MluI	Fermentas	ER0561
Murashige and Skoog medium	Duchefa	M0222
NaCl	Merck	1.06404
NaOH	Merck	106482
NotI	NEB	R 0189S
OligodT	Fermentas	S0131
PacI	NEB	R0547L
PageRuler™ Prestained Protein Ladder	Fermentas	SM0671
p-Coumaric acid	Sigma	C9008
PEG 6000	Duchefa	P0805
Pfu Taq DNA polymerase	Fermentas	EP0502
PIPES	Applichem	A1079
RevertAid™ H Minus M-MuLV Reverse	Fermentas	EP0451

Transcriptase		
SacI	Fermentas	ER1131
SDS	Molekula	M82553115
Sodium azide	Sigma-Aldrich	S2002
SyberGreen Master Mix	Roche	04 913 850 001
T4 DNA ligase	Fermentas	EL0334
Taq DNA polymerase	Fermentas	EP0402
TEMED	Sigma	T7024
Tris hydroxmethyl aminomethan	Merck	1.08387
Trizol Reagent	Invitrogen	15596-026
Trypan Blue cell culture tested	Sigma-Aldrich	T6146
Urea	Fluka	51461
X-Gal	PeqLab	37-2630
Yeast nitrogen base without amino acids	Sigma	Y0626
YPD	Sigma	Y1500
YPD Broth	Sigma	Y1375
Plant Agar	Duchefa	P1001
Sucrose	Duchefa	S0809
Pfx Platinium® DNA Polymerase	Invitrogen	11708-021
GeneRuler^{TM} 100 bp DNA Ladder Plus	Fermentas	SM0321
RNaseout Recombinant Ribonuclease Inhibitor	Invitrogen	479940

APPENDIX H

Commercial Kits & Vector Systems

DNA extraction kit	Qiagen	28706
High Pure Plasmid Isolation Kit	Roche	11754785001
mMessage mMachine T7 in vitro transcription kit	Ambion	AM1345
pACT2	Clontech	PT3022-5
PCR purification kit	Qiagen	28104
pGEM®-T easy vector system	Promega	A1360
pYES2	Invitrogen	V825-20
RNA plant mini kit	Qiagen	74904
Wizard® DNA purification kit	Promega	A1125