MOLECULAR ANALYSIS OF GLYPHOSATE AND OSMOTIC STRESS
RESPONSIVE GENES

by

ÖZGE CEBECİ YALÇINKAYA

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

Sabancı University
February 2010

MOLECULAR ANALYSIS OF GLYPHOSATE AND OSMOTIC STRESS RESPONSIVE GENES

APPROVED BY:

Assoc. Prof. Dr. Hikmet Budak (Thesis advisor).......................................

Assoc. Prof. Dr. Levent Öztürk ....

Assist. Prof. Dr. Devrim Gözüaçık.......................................

Assist. Prof. Dr. Alpay Taralp.. ....

Assist. Prof. Dr. Ayşe Elif Erson.......................................

DATE of APPROVAL: 05.02.2010

ACKNOWLEDGEMENTS

ABSTRACT

MOLECULAR ANALYSIS OF GLYPHOSATE AND OSMOTIC STRESS RESPONSIVE GENES

Özge Cebeci Yalçınkaya
Biological Sciences and Bioengineering
PhD Thesis, 2010

Assoc. Prof. Hikmet Budak (Thesis Supervisor)

Cross species hybridization can provide a tool for elucidating biological pathways conserved among organisms. Based on sequence conservation among grass species, we selected Affymetrix GeneChip® wheat genome array as a tool to analyze changes in gene expression profiles of three Festuca species in response to varying levels of glyphosate. Differences in transcript expression upon glyphosate application at 5% and 20% of the recommended rate were recorded. Differences highlighted metabolic categories, including photosynthesis, protein synthesis, and stress responses. Expression levels of a larger number of transcripts altered with 20% glyphosate. RT-PCR analysis was conducted for experimental validation. This is the first report to analyze the potential of cross species hybridization in Festuca species and the data help extend our knowledge on the cellular processes affected by glyphosate.

Autophagy related gene, *Atg8* has been used for monitoring autophagy in various organisms. In this study, *Atg8* gene was identified in *Brachypodium distachyon* (named as *BdAtg8*) under osmotic stress. Expression profile of *BdAtg8* was examined in a variety of tissues of different ages and osmotic stress conditions. Expression level of *BdAtg8* elevated with osmotic stress, especially in the roots. *BdAtg8* complemented atg8Δ::*kan MX* yeast mutants grown under starvation conditions. Monodansylcadaverine was used to observe autophagosomes, and autophagy was shown to be constitutively active in *Brachypodium*. Autophagy was more active in plants exposed to osmotic stress. BdATG8 protein was expressed in yeast and analyzed with western blotting. In conclusion, under osmotic stress conditions, *BdAtg8* gene is required for induction of autophagy in *Brachypodium*.

ÖZET

GLİFOSAT VE OZMOTİK STRESE CEVAP VEREN GENLERİN MOLEKÜLER ANALİZİ

Özge Cebeci Yalçınkaya
Biyoloji Bilimleri ve Biyomühendislik
Doktora Tezi, 2010

Doç. Dr. Hikmet Budak (Tez Danışmanı)

Anahtar Kelimeler: çapraz tür hibridizasyonu, Festuca, glifosat, otofaji, *Atg8*, *Brachypodium*, ozmotik stres

Çapraz tür hibridizasyonu organizmalar arası korunan biyolojik yolların açıklığa kavuşturulmasında bir araç vazifesi görebilir. Çim türlerindeki sekans korunumu baz alınarak, "Affymetrix GeneChip® buğday genom array"i, üç Festuca türünün gen ekspresyon profillerinde glifosatın değişen seviyelerine karşılık meydana gelen değişiklikleri analiz etmek için bir araç olarak kullanılmıştır. Uygulanması tavsiye edilen değerin %5 ve %20'si düzeyindeki glifosat uygulamasıyla, transkript ekspresyonunda meydana gelen değişiklikler kaydedilmiştir. Bu değişiklikler, metabolik kategorilerden olan fotosentez, protein sentezi ve stres tepkilerine dikkat çekmiştir. %20 glifosat uygulamasıyla, daha fazla sayıda transkriptin ekspresyon seviyeleri değişmiştir. RT-PCR analizi, deneysel doğrulama için yürütülmüştür. Bu rapor, çapraz tür hibridizasyonu tekniğinin Festuca türündeki potansiyelini analiz eden ilk rapor olmakla birlikte, bu analizler glifosatın etkilediği hücresel süreçlerdeki bilgi dağarcığımızı genişletmekte yardımcı olacaktır

Otofajiyle ilgili bir gen olan, *Atg8* bir çok organizmada otofajiyi izlemek için kullanılmaktadır. Bu çalışmada, *Atg8* geni ozmotik stres koşullarındaki *Brachypodium distachyon* türünde tanımlanmıştır (*BdAtg8* olarak isimlendirilmiştir). *BdAtg8* geninin ifade profili farklı yaşlardaki çeşitli dokularda ve ozmotik stres koşullarında incelenmiştir. Ozmotik stresle, *BdAtg8* geninin ifade seviyesinin özellikle köklerde arttığı gözlenmiştir. *BdAtg8* geninin açlık koşullarında büyüyen atg8Δ::*kan MX* maya mutantlarını komplement ettiği gösterilmiştir. Otofagozomları gözlemlemek için "monodansylcadaverine" kullanılmış ve otofajinin *Brachypodium*'da temel olarak aktif olduğu gösterilmiştir. Otofajinin, ozmotik strese maruz kalmış bitkilerde daha aktif olduğu gösterilmiştir. BdATG8 proteini mayada eksprese edilmiş ve "western blot" tekniği ile analiz edilmiştir. Ozmotik stres koşullarında, *BdAtg8* geninin *Brachypodium*'da otofajinin indüklenmesi için gerekli olduğu sonucuna varılmıştır.

TABLE OF CONTENTS

CHAPTER I

GENE EXPRESSION PATTERNS OF THREE *FESTUCA* SPECIES EXPOSED TO VARYING LEVELS OF GLYPHOSATE USING AFFYMETRIX GENECHIP® WHEAT GENOME ARRAY

CHAPTER II

CLONING AND FUNCTIONAL ANALYSIS OF *Brachypodium Atg8*, AUTOPHAGY-RELATED GENE, UNDER OSMOTIC/DROUGHT STRESS

CHAPTER I

GENE EXPRESSION PATTERNS OF THREE *FESTUCA* SPECIES EXPOSED TO
VARYING LEVELS OF GLYPHOSATE USING AFFYMETRIX GENECHIP[®]
WHEAT GENOME ARRAY

ABSTRACT

Glyphosate is an herbicide mainly used for weed control. It has been shown to act as an inhibitor of an aromatic amino acid biosynthetic pathway, while other processes and pathways affected by glyphosate are not known. In the absence of whole genome sequences, cross species hybridization can provide a tool for elucidating biological pathways conserved among organisms. Comparative genome analyses indicated a high level of colinearity among grass species and Festuca, on which we focus here, and showed rearrangements common to the Pooideae family. Based on sequence conservation among grass species, we selected the Affymetrix GeneChip® Wheat Genome Array as a tool for the analysis of expression profiles of three Festuca (fescue) species with contrasting tolerances to varying levels of glyphosate. Differences in transcript expression upon foliar glyphosate application at 1.58 mM and 6.32 mM, representing 5% and 20% of the recommended rate for weed control, respectively, were recorded. Differences highlighted categories of metabolic processes, such as photosynthesis, protein synthesis, and stress responses. Expression levels of a larger number of transcripts altered in response to 20% glyphosate application. Differential expression of genes encoding proteins involved in the shikimic acid pathway could not be identified by cross species hybridization. RT-PCR analysis was conducted for experimental validation of result of a selected transcript. This is the first report to analyze the potential of cross species hybridization in Fescue species and the data and analyses help extend our knowledge on the cellular processes affected by glyphosate. This study is also crucial for opening the way for better understanding of the mechanisms and pathways regulated by glyphosate in Fescue species.

Keywords cross species hybridization, fescue, glyphosate

ÖZET

Glifosat yabani ot kontrolünde kullanılan başlıca herbisitlerdendir. Glifosatın aromatik aminoasit biyosentetik yolağında inhibitör olarak rol aldığı gösterilmiş olmakla birlikte, glifosatın etkileyebileceği diğer süreç ve yolaklar bilinmemektedir. Tüm genom sekansı olmadığı durumlarda, çapraz tür hibridizasyonu organizmalar arası korunan biyolojik yolakların açıklığa kavuşturulmasında bir araç vazifesi görebilir. Karşılaştırmalı genom analizleri, çim türleri ve bizim bu çalışmada odaklandığımız Festuca arasında yüksek düzeyde bir doğrudaşlık olduğuna işaret etmiş ve Pooideae familyasında ortak olan rearanjmanları göstermiştir. Çim türlerindeki sekans korunumu baz alınarak, "Affymetrix GeneChip® buğday genom array"i, glifosatın değişen seviyelerine toleransları zıtlık gösteren üç Festuca (fescue) türünün ekspresyon profillerini analiz etmek için bir araç olarak kullanılmıştır. 1.58 mM ve 6.32 mM düzeyindeki glifosatın (yabani ot kontrolünde uygulanması tavsiye edilen değerin, sırasıyla, %5 ve %20'si) yapraktan uygulanmasıyla transkript ekspresyonunda meydana gelen değişiklikler kaydedilmiştir. Bu değişiklikler, metabolik süreç kategorilerinden olan fotosentez, protein sentezi ve stres tepkilerine dikkat çekmiştir. %20 glifosat uygulamasıyla, daha fazla sayıda transkriptin ekspresyon seviyeleri değişmiştir. Ekspresyonları farklılık gösteren genlerin kodladığı proteinlerden şikimik asit yolağıyla ilgili olanlar çapraz tür hibridizasyonuyla tanımlanamamıştır. Seçilen bir transkript sonucu, RT-PCR analizi ile deneysel olarak doğrulanmıştır. Bu rapor, çapraz tür hibridizasyon tekniğinin Fescue türündeki potansiyelini analiz eden ilk rapor olmakla birlikte bu analizler glifosatın etkilediği hücresel süreçlerdeki bilgi dağarcığımızı genişletmekte yardımcı olacaktır. Bu çalışma, glifosatın Fescue türünde regüle ettiği mekanizmaların ve yolakların daha iyi anlaşılmasına yol açması bakımından da önemlidir

Anahtar kelimeler çapraz tür hibridizasyonu, fescue, glifosat

TABLE OF CONTENTS

ABBREVIATIONS

ANOVA: Analysis of variance

cDNA: Complementary DNA

CSH: Cross species hybridization

DNA: Deoxyribonucleic acid

EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase

ESC: Embryonic stem cell

EST: Expressed sequence tag

gDNA: Genomic DNA

ml: Mililiter

min: Minute

mM: Milimolar

mRNA: Messenger ribonucleic acid

PCA: Principal component analysis

POEA: Polyethoxylate tallowamine

RMA: Robust multichip average normalization

RNA: Ribonucleic acid

ROS: Reactive oxgen species

Rpm: Revolution per minute

rRNA: Ribosomal RNA

RT-PCR: Reverse transcription polymerase chain reaction

SSH: Species specific hybridization

μl: Microliter

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Glyphosate® (N-phosphonomethylglycine) is a broad spectrum herbicide that affects plants systemically after application to the leaf surface. It is phytotoxic and prevents further growth by blocking aromatic amino acid production, leading to the arrest of protein synthesis and secondary compound formation. It specifically inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a nuclear encoded chloroplast-localized enzyme in the shikimic acid pathway of plants and microorganisms (Steinrücken and Amrhein 1980).

Weed control and overall management in turf courses has emerged as a substantially important field in agriculture. Although, glyphosate is relatively inexpensive and less toxic to non-target organisms, it has not been extensively used in turfgrass weed management programs due to its possible adverse effects on turfgrass growth, like provoking injuries on desired turfgrass (Hart et al. 2005). Until now, glyphosate usage has been limited to spot treatments. However, in the presence of natural glyphosate-tolerant turfgrass species, such as cool-season perennial turfgrass, there is an increased reliance on the usage of glyphosate for weed control (Hart et al. 2005). Recently, transgenic glyphosate tolerant creeping bent grass (*Agrostic stolonifera* L.) has been developed (Reichman, et al., 2006). Under the light of these novel findings, the development of cultivars with more tolerance to glyphosate is considered to be a good alternative for weed control using this environmentally friendly herbicide in lawns, golf courses and other turf areas. Additionally, determination of the effective glyphosate rate that can be directly used on turfgrass fields to control weeds is essential for extensive usage of this herbicide. Evolution of resistance to other herbicides with different modes of action, increased reliance on the herbicide glyphosate for weed control (Baylis 2000). A better understanding of its action on turfgrass species is

essential for the development of future management strategies both to slow down the evolution of resistance and to control existing populations.

The Affymetrix GeneChip® Wheat Genome Array was selected to identify global gene expression changes in three selected fescues. The rationale for selecting the wheat genome array for the CSH experiment was based on the close relatedness of perennial ryegrass, which is relatively similar to fescues, to the Triticeae (Jones et al. 2002). In the same study, the existence of synteny and colinearity among the genetic maps of ryegrass and Triticeae cereals has been postulated. Triticeae, ryegrass, and fescues reside in the same subfamily, Pooideae of the Poaceae family (Soreng and Davis 1998). High level of similarity in terms of gene order among these families makes it feasible to consider CSH to reveal the cross species conservation of biological processes and their genetic control mechanisms. Festuca species were selected for their differential glyphosate tolerance based on dry matter production, chlorophyll content, and shoot concentration of shikimic acid (Su et al. 2009). Based on these morphological and physiological data, selected genotypes were used to analyze and understand global expression changes upon glyphosate treatment. Large-scale functional profiling of Festuca species with differential tolerance to glyphosate treatment will be a beneficial resource for future investigations concerning biochemical effects of glyphosate on turfgrasses.

2 OVERVIEW

2.1 General information on fine leaf fescues

2.1.1 Fine leaf fescues and their relation to Triticeae

Fine fescues are named as "fine", since they have very narrow (fine) leaves. Fescue is a large genus belonging to the grass family, Poaceae (Clayton and Renvoize, 1986). This genus is closely related to ryegrass (Lolium). Triticeae, ryegrass, and fescues reside in the same subfamily, Pooideae of the Poaceae family (Soreng and Davis 1998). In a previous study, close relatedness of perennial ryegrass, which is relatively similar to fescues, to the Triticeae has been reported (Jones et al. 2002). In the same study, the existence of synteny and colinearity among the genetic maps of ryegrass and Triticeae cereals has been postulated. A recent study also reported that meadow fescue (*Festuca pratensis*) genome was highly orthologous and colinear with those of ryegrass, oat, maize, and sorghum (Alm et al., 2003). These studies have documented that there is a high level of conservation at macro-syntenic scale among the members of Pooideae saubfamily of Poaceae family.

2.1.2 Fine leaf fescues as turfgrass

Five fine fescue species are commonly used as turfgrass (Turgeon, 2002). Especially, they are used for turf in northeastern U.S. lawns (Jauhar, 1993). In addition to their usage for turf, fine fescues are also used for forage and conservation purposes. Fine fescues display huge variation in terms of their morphology, growth habits, and cytology. Most commonly used fine fescues used for turf include strong creeping red

fescue (*Festuca rubra* ssp. *rubra*), slender creeping red fescue (*Festuca rubra* ssp. *littoralis*), chewing's fescue (*Festuca rubra* ssp. *commutata*), hard fescue (*Festuca longifolia*), and sheep fescue (*Festuca ovina*). Strong creeping red fescue (*Festuca rubra* ssp. *rubra*) produces long and abundant rhizomes and has 56 chromosomes. Slender creeping red fescue (*Festuca rubra* ssp. *littoralis*) produces shorter rhizomes and has 42 chromosomes. Among the fine fescues, chewing's fescue (*Festuca rubra* ssp. *commutata*) is the variety which tolerates most lower mowing height. Hard fescue (*Festuca longifolia*) is a bunch type grass and well adapted to moist soils. It has stiff leaves. Sheep fescue (*Festuca ovina*) has bluish green color and has higher adaptation to dry and gravelly soils. Fine leaf fescues are extensively used in turfgrass breeding programs, because of their ease in turf management. They generally require less water, fertility and mowing in comparison to other turfgrass varieties (Meyer and Funk, 1989). Recently, numerous genetically improved cultivars have been developed with better characteristics, such as higher tolerance to acidic and infertile soils, moderate shade or full-sun conditions. In recent years, fine leaf fescues have received attention of investors in United States and European turfgrass industry in use of fine fescues for golf turf, lawn, and road-side settings (Ruemmele et al., 1995).

2.1.3 Importance of weed management

Conservation of turfgrass settings is essential for life quality of human. Especially, in lawns and road-site settings, weed is a key problem. Herbicide use has been the most prevalently used strategy to control weeds in turfgrass areas. Although there is an ongoing debate about the challenges of herbicides in terms of health and environmental issue, herbicide use stays as the best alternative for weed control in both public and private turfgrass setting.

One of the major weeds which populate golf course turf is annual bluegrass (*Poa annua* L.). This grass cultivar has an ability to grow in putting greens and fairways in golf course areas (Gaussoin and Branham, 1989). Selective removal of this weed has not been achieved yet, since consistently effective herbicide for selective removal of this weed is not available.

Glyphosate is an herbicide which is relatively inexpensive and less toxic to non-target organisms. However, it has not been extensively used in turfgrass weed management programs due to its possible adverse effects on turfgrass, like provoking injuries (Hart et al. 2005). Until now, glyphosate usage has been limited to spot treatments. However, with the presence of natural glyphosate-tolerant turfgrass species, cool-season perennial turfgrass, and the development of transgenic glyphosate-tolerant creeping bent grass (*Agrostic stolonifera* L.) (Reichman, et al., 2006), demand for the usage of glyphosate for weed control has increased (Hart et al. 2005). Additionally, evolution of resistance to other herbicides with different modes of action, increased reliance on the herbicide glyphosate for weed control (Baylis, 2000). Use of environmentally friendly glyphosate for weed control in lawns, golf courses and other turf areas depends on the development of cultivars with greater tolerance to glyphosate. Additionally, determination of the effective glyphosate rate that can be used directly on turfgrass fields to control weeds is essential for extensive usage of this herbicide. Future biochemical studies which will shed light on the mode of action of this herbicide on turfgrass should be performed.
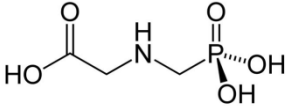
2.2 What is Glyphosate?

2.2.1 Glyphosate application

Glyphosate® (N-phosphonomethylglycine) is an herbicide which is widely used in the world. It was first introduced to the market in 1974. With novelties in agricultural practices, the usage of glyphosate has ascended in the 2000s. Additionally, its cheap price was another factor which induced its extensive usage in agriculture (Savela and Hynninen, 2004).

2.2.2 Properties of glyphosate

Glyphosate is a non-volatile substance, which is not prone to photochemical degradation. Table 2.1 summarizes the physical and chemical properties of glyphosate.

Table 2.1	
Physico-chemical properties of glyphosate	
Parameter	Glyphosate [a]
Chemical structure	
Chemical name	[(N-phosphonomethyl)glycine]
Empirical formula	$C_3H_8NO_5P$
Molar mass	169.08
$K_{OW}LogP$	< -3.2 (pH 2-5, 20°C)
Water solubility	(20°C)
Vapour pressure	7.5×10^{-8} mmHg
pKa	pKa_1 0.8 pKa_2 3.0 pKa_3 6.0 and pKa_4 10.0
Freundlich sorption coefficient (Kf)	0.6-303 L kg^{-1}[b]
Photodegradation in soil	Not substantial over 31 days
Photodegradation in water	DT_{50}< 28 days
Half-life in soil (field)	DT_{50} 3-174 days
Half life in water	DT_{50} 5-91 days
[a] Tomlin 2000 [b] Vereecken 2005	

2.2.3 Glyphosate mode of action

Glyphosate is a systemic herbicide whose foliar application is effective on plants. After absorbtion from the foliage, it is successively translocated to other body parts of the plants via phloem (Laitinen, 2009). The major mode of action of glyphosate on plant growth is inhibition of aromatic aminoacid synthesis via competitive inhibition of EPSP

synthase enzyme (Steinrücken and Amrhein 1980). EPSP synthase is an essential enzyme, which plays a key role in skimic acid pathway for the production of essential aromatic amino acid precursor, chorismate. With inhibition of synthesis of essential aromatic aminoacids, phenylalanine, tyrosine and tryptophan, protein synthesis is blocked in glyphosate sensitive species. The major determinants of efficiency of glyphosate are its ability to translocate to sensitive body parts of plants through phloem and inhibition of activity of essential enzymes in plants. Shikimic acid pathway is absent in animals, which take aromatic amino acids via their diet, so animals are not sensitive to the mode of action of glyphosate (Giesy et al., 2000; Monheit, 2000).

Previous studies reported that glyphosate is not degradable at all in plants or the degradation rate is insignificant (Eberbach and Bowner, 1995). However, metabolization of glyphosate to aminomethylphosphonicacid (AMPA) in plants was reported in FAO evaluation in 1997.

2.2.4 Glyphosate toxicity

Glyphosate has been postulated to be non-toxic to terrestrial and aquatic organisms in low doses (Giesy et al., 2000; Monheit et al., 2004). Contradictory results came from a study by Relyea (2005). In this study, application of commercial glyphosate product, Roundup® to amphibians was shown to be toxic. These results were heavily discussed by the scientific community in terms of abnormal application rates, unrealistically high aquatic exposure, and limitations in experimental set-up (Borggaard and Gimsing 2008).

Surfactants are generally added to herbicides in order to enhance penetration of the active molecule through the plant cuticle. Polyethoxylate tallowamine (POEA) is the surfactant added Roundup® for the stated purpose. Some surfactants can enhance the toxicity of herbicides or can be toxic themselves more than the active ingredient of the herbicide. For instance, it has been proposed that the major reason for acute toxicity of Roundup® to amphibians might have been caused by POEA, rather than Roundup®, itself (Giesy et al., 2000; Monheit et al., 2004).

7

2.3 Introduction to Cross Species Hybridization (CSH) microarray technology

2.3.1 Species Specific Hybridization vs Cross Species Hybridization

A microarray is a solid support (usually a glass slide or a nylon membrane) to which oligonucleotides or cDNA probes are permanently fixed and to which fluorescently labeled cDNA samples synthesized from mRNA pool of a target species are hybridized. Co-hybridization of two samples, labeled with two different fluorophores, allows the comparison of transcriptome levels of each sample. Expression differences retrieved from this hybridization are given as a set of ratio representing fold differences between the two samples for each "feature" on the microarray. In most of the microarray experiments, oligonucleotides and cDNA probes fixed to the array and fluorescently labeled cDNA samples are coming from the same species. Hybridizations in which the target and reference species are the same are named as Species-Specific Hybridization (SSH) (Bar-Or et al. 2007). SSH is a valuable tool to analyze whole genome expression changes upon any treatment, for species whose array platform is present. However, for organisms lacking genomic sequence information, no commercial array platform is available. On the contrary, this condition is no longer a limitation to perform transcriptomic analysis in such organisms. It has become more explicit that there exists sufficient sequence homology for a number of genes within phylogenetically close or distant species. This allows the fact that genomic sequence information of one species can be utilized to investigate gene expression patterns in other species. The number of studies utilizing probes from one species to investigate expression levels of another species is growing. One of the primary reasons for this fact is that fabricating a new microarray is time- consuming and costly task. Therefore, if an array platform for a closely related species is present, cross species hybridization is suggested to be an effective technique, since a few hybridizations are sufficient to get high-throughput gene expression information.

Several challenges encountered while performing a CSH study are: The level of sequence divergence between target and reference species, the nature of the probes on the array, the choice of experimental design, filters used in data analysis and validation of CSH data (Bar-Or et al., 2007; Buckley 2007).

2.3.2 The level of sequence divergence between target and reference species in CSH

Selection of a suitable array platform for a given target species is suggested to depend on phylogenetic relatedness of target and reference species. However, studies contributed contradictory evidences about this assumption. A study by Renn et al. (2004) supported this view and suggested that more consistent results could be gathered by CSH of closely related species. In another study by Rise et al. (2004), similar results were obtained. In this study, co-hybridization of RNAs from two fish species to an array comprising expressed sequence tags (EST) from two other fish species was performed. The results of this study suggested that with increasing phylogenetic closeness, hydridization performance increased. Although, efficiency of hybridization decreased with decreasing evolutionary relatedness, there remains substantial amount of hybridization. Contradictory evidence came from a study by Gilad et al. (2005) which suggested that these CSH results might be biased even in studies of species having only ~1% sequence divergence. Hybridization of human and chimpanzee RNA to an array consisting of probes of both species was performed and the results of this hybridization study demonstrated that even a small sequence divergence, in evolutionary manner, might affect hybridization performance and contribute a bias to CSH results. However, the source of this difference in hybridization efficiency might be gene-based differences rather than the genome sequence divergence which was not fully considered in this study.

2.3.3 Matching degree of target transcripts and probes

The number of hybridizations does not always reflect the quality of a CSH study. The major factor that determines the success of a CSH study is the degree of similarity between the sequences of target transcripts and the probes. In a CSH experiment, a number of probes might perfectly match with target transcripts, or make a low match, or cross hybridize to transcripts of several genes of the target transcripts, or make no match (Bar-Or et al. 2007). In other words, the degree of similarity of target and probe sequences might play an important role in the CSH quality depending on the number of

such imperfect hybridizations. Outcomes of the matching degree differences will be discussed below.

2.3.3.1 Cross hybridization

One of the main reasons for debates going on in the scientific community regarding reproducibility and reliability of microarray studies is cross hybridization (Draghici et al., 2006). Cross hybridization is defined as hybridization of a probe to more than one gene of the transcripts of the target organism. Since the sequence similarity between probe and transcripts in CSH studies is relatively low in comparison to SSH studies, the number of cross hybridizing probes in CSH is much greater. In a study by Bar-Or et al. (2006) it was postulated that ~16% of potato transcript sequences matched to more than one tomato microarray cDNA probe sequences in BLAST. Therefore, this might result in a bias in CSH studies.

2.3.3.2 Low hybridization signal

Low amount of matching between the sequences of target and probe in CSH might decrease the number of hybridizations to a single spot, and hence the signal intensity. There are a number of studies in the literature supporting this view (Ji et al., 2004; Renn et al., 2004; Moore et al., 2005; Bar-Or et al. 2006). In one study (Ji et al., 2004), hybridization of human, cattle, dog, and pig RNA to human Affymetrix microarray resulted in lower hybridization signals in CSH experiments in comparison to SSH. In another study (Renn et al., 2004), RNAs from 8 different fish species was hybridized to a brain specific cDNA array of African cichlid fish. A decrease in the number of hybridized spots was reported especially for pylogenetically distant fish species. The explanation for this observation was that the extent of sequence mismatch among target and probe sequences increases with phylogenetic distance. Therefore, extra measures should be taken in order to calculate the power of CSH in detection of expression level differences in other species, since the sensitivity of array analysis to detect differences in gene expression decreases with increasing phlylogenetic distance.

Another study supporting this conclusion reported that signal reduction might result in a bias in gene expression profiles obtained with CSH studies (Bar-Or et al. 2006).

2.3.3.3 Reproducibility

Another criterium which is crucial for high performance of a CSH study is the extent of its reproducibility. Several studies reported that CSH data are reproducible (Nuzhdin et al., 2004; Bar-Or et al., 2006; Donaldson et al., 2005; von Schalburg et al., 2005). For example, Donaldson et al. (2005) reported that hybridization of bovine and ovine RNAs to bovine innate immune microarray resulted in 56% and 52% of hybridization, respectively. Presuming same set of genes are expressed in the two species, the bovine innate immune microarray detects 94% of the transcripts from ovine, which is an indicative of high degree of reproducibility of CSH. However, reproducibility is not always sufficient for biological validity of CSH results. Draghici et al. (2006) proposes that although CSH data are reproducible, they might not be biologically meaningful and significant.

2.3.4 Criteria for a successful CSH

To overcome these challenges and enhance efficiency of CSH analyses, a number guideline should be followed. These include the compatibility of the microarray selected, experimental design, hybridization conditions, data analysis and validation.

2.3.4.1 Compatibility of microarray selected

One of the essential requirements for the high performance of CSH studies is the evaluation of compatibility of the target species to the reference microarray platform. Sequence divergence is the primary determinant in selection of suitable array platform for CSH study, since differentiation of hybridization due to imperfect matches and those due to real expression profile differences is crucial for extracting valid biological data from CSH studies. One important point which should be considered with caution is the

fact that phylogenetic relatedness does not always reflect the divergence at the gene level. If sufficient genome sequence data are not available for a target species, hybridization of genomic DNA of target species to the microarray platform of the potential reference species might be helpful to quantify the effect of sequence divergence on efficiency of hybridization. For example, in a study (Ranz et al. 2003), genomic DNAs from *Drosophila melanogaster and Drosophila simulans* were hybridized to *D. melanogaster* array. Hybridization efficiency of *D. melanogaster* DNA was 4.2% greater than that of *D. simulans*, which was in good correlation with sequence divergence among those species (3.8% sequence divergence).

If there is sufficient genomic data for the target species, it is possible to determine the sequence divergence of target and reference species directly. In a study by Saetre et al. (2004), the genomes of dog and human were compared to assess genome-wise similarity between the two organisms before hybridizing dog RNA to human Affymetrix cDNA microarray. The level of completeness of genome databases will be the determining factor in the power of this method for the determination of sequence divergence between target and reference species.

2.3.4.2   Nature of the probes

One of the factors which affect the efficiency of CSH is the nature and length of the probes on the microarray platform. There are three major probe types used in microarray studies: Affymetrix probe sets, longer oligomers (~60-mers) and even longer cDNA probes. Affymetrix probe sets are comprised of 11-20 probe pairs (~25-mers). Each probe pair consists of perfect match (PM) probe and mismatch (MM) probe. PM probes generates detectable signal when target RNA binds to it. MM probe is utilized to eliminate any false and contaminating signal within that measurement (http://www.affymetrix.com/). cDNA probes come from cDNA library of a reference organism and are generally longer than short oligonucleotide probes (affymetrix probes). Affymetrix probes are more prone to sequence mismatches than cDNA probes and might lead to cross hybridization. cDNA probes are suggested to be better alternatives for identification of differentially regulated transcripts, since cross hybridization probability is lower for these longer probes. In the literature, there are

several studies supporting this view. In one study, Enard et al. (2002) hybridized several primate species RNAs either to human Affymetrix oligonucleotide array or to cDNA microarray. The outcome of this study demonstrated that usage of longer probes and maintenance of high stringency hybridization conditions lead to less cross hybridization than the usage of shorter affymetrix probes. Other supportive evidence came from a study by Walker et al. (2006). In this study, macaque monkey (*Macaca fascicularis*) RNA was hybridized either to human Affymetrix 25-mer oligonucleotides or to human Applied Biosystems 60-mer oligonucleotide microarray. Higher number of differentially regulated transcripts was identified with longer oligonucleotide array with a lower false rate. Under the light of these results, it might be suggested that cDNA microarrays are the sole microarray platforms in CSH. However, there exist several disadvantages for this array platform. For instance, as stated in a study by Halgren et al. (2001), the presence of chimeric clones and contaminating clones in low quality cDNA libraries might contribute to an increase in false identification rate of differentially regulated transcripts.

2.3.4.3 Importance of the experimental design

Two major experimental designs are utilized in CSH studies. In the first design, a microarray platform containing probes from species 1 is utilized to compare expression profiles of two different species (2 and 3). In this type of design, there are two factors which contribute to the bias in CSH studies: the degree of sequence divergence between species 1 and 2, and the degree of sequence divergence between species 1 and 3. In some cases species 1 might be the same as species 2 or 3. This type of design is suggested to be effective in CSH studies which aim to compare transcriptomic profiles of two closely related species. For example, in one study, this type of experimental design was employed to investigate sex-dependent gene expression profiles in different Drosophila species (Meiklejohn et al., 2003; Ranz et al., 2003). In another study, Gilad et al. (2005) demonstrated that sequence divergence might have an important effect on comparison of gene expression levels of very closely related species, human and chimpanzee. A cDNA microarray comprising probes of human, chimpanzee, orangutan, and rhesus macaque monkey was utilized to compare gene expression levels of these primates. The results obtained from these hybridizations showed that even a subtle

13

sequence divergence might have an essential effect on comparison of gene expression patterns of species that diverged only ~1%, like human and chimpanzee in CSH studies.

In the second experimental design utilized in CSH experiments, two different samples from the same species are competitively hybridized to a microarray platform of another species. This type of experimental design circumvents the effect of sequence divergence between the target species. Only divergence factor, sequence divergence between target and reference species, affects the target samples equally and leads to acquisition of more reliable and valid data from CSH studies. Target samples might differ in terms of type of treatment, time point, tissue, developmental stage or location. There are several studies reporting the utilization of this type of design to study differences in gene expression profiles. For example, in a study (Renn et al., 2004), target brain or muscle samples from 8 different fish species, including African cichlid (*Astatotilapia burtoni*) were hybridized to a cDNA microarray consisting of probes from brain ESTs of African cichlid (*Astatotilapia burtoni*). Competitive hybridization of human and brain samples from African cichlid to this cDNA microarray resulted in identification of 804 differentially regulated genes between brain and muscle tissue. Hybridization of brain and muscle RNA samples from 7 other fish species lead to identification of lower number of differentially regulated genes, as the phylogenetic distance increased. However, such decrease in the hybridization was not very high, even for the most diverged species zebra fish (*Danio rerio*) (diverged from African cichlid by ~200 million years). 80% of the differentially regulated genes identified by hybridization of African cichlid brain and muscle RNA samples to the African cichlid brain array were also identified by hybridization of perciform (diverged from African cichlid by 65 million years) brain and muscle samples to the African cichlid cDNA array. Only 20% of the differentially regulated transcripts were obtained from zebra fish hybridization experiment (the most diverged species). These results supported the assumption that CSH is a powerful technique to detect differences in gene expression profiles of two target samples from the same species, especially when the sequence divergence between the target and reference species is small.

2.3.4.4 Data analysis

To obtain biologically meaningful results from CSH studies, filtration of probe sets is suggested to be essential. There are two major approaches to filter data in CSH studies. First approach is genomic-data based filtration and utilized sequence homology between probe and the target sequences. Probe pairs showing high homology to target species are selected for further data analysis. The ones having lower amount of homology are excluded during data analysis. Bar-Or et al. (2006) utilized this approach to filter data in CSHs of tomato samples to potato cDNA microarray and potato samples to tomato cDNA array. Khavitovich et al. (2004) postulated the use of this filtration approach to find out the probe pairs that are matched between human and chimpanzee.

In the second approach, probe pairs with high level of matching to the target sample are selected by hybridization of genomic DNA of target species to the reference microarray. Several studies reported the usage of this approach to filter CSH data. In a study (Ranz et al., 2003), *D. simulans* gDNA was hybridized to *D. melanogaster* cDNA array. The results of this hybridization analysis demonstrated that there is not much difference between these two species in terms of sequence. Therefore, it was deduced that CSH of *D. simulans* RNA to *D. melanogaster* cDNA array would give biologically meaningful data. In another study by Hammond et al. (2005), probe sets were filtered by hybridization of gDNA of *Brassica oleracea* to *Arabidopsis thaliana* Affymetrix microarray. Probe sets having one or more than one perfectly matching probe with hybridization levels above a certain threshold were used in data analysis. Probe selection enhanced the identification of differentially regulated transcripts in test and control samples. This gDNA hybridization based approach seems to be more advantageous than genomic-data based filtration approach, because it can also be used for probe set selection for species without an available genomic data.

2.3.4.5 Validation of CSH data

As in all microarray experiments, confirmation of expression data with techniques of mRNA quantification such as quantitative real time PCR or northern blotting is

important. Several CSH studies used these techniques to validate their data (Ji et al., 2004; Moody et al., 2002; Huang et al., 2000).

Mapping differentially regulated genes to regions on chromosome shown to be related to certain biological processes by previous studies is another approach to validate CSH data. In a study by Fang et al. (2005), after CSH of rat RNA to mouse cDNA microarray, differentially regulated genes were mapped to rat, mouse and human chromosomes. Any correlation in mapping of this gene was deduced to be meaningful and that gene was considered to be an orthologous gene.

2.3.5 CSH applications

CSH is offered as a new and useful tool to perform a large-scale functional profiling of a species without an available genome sequence. CSH is an important tool for identifying molecular mechanisms and pathways conserved among species (Hughes et al. 2000; Ihmels et al. 2005; McCaroll et al. 2004). These studies included CSH analysis of highly diverged species, *Caenorhabditis elegans* and *Drosophila melanogaster* (McCaroll et al. 2004), and of more related organisms *Candida albicans* and *Saccharomyces cerevisiae* (Ihmels et al. 2005). In a recent study (Sun et al. 2007), mechanisms controlling embryonic stem cell (ESC) pluripotency were investigated by comparing gene expression patterns of human and mouse ESC orthologous genes. Another recent study reported that usage of a multi-species cDNA array identified conserved genes expressed in oocytes. Gene sequences from three organisms, bovine, mouse and *Xenopus laevis,* diverged in their evolutionary position, have been utilized to design a multi-species cDNA array for the identification of conserved sequences playing roles in molecular mechanisms or pathways common to all species (Vallée et al. 2006). In both studies evolutionarily distant species were selected to identify common mechanisms and pathways. Additionally, a comparison of results obtained by CSH with species specific hybridization (SSH) proved that biological processes analyzed by CSH closely reflected the analysis found by SSH (Bar-Or et al. 2006).

CSH is also widely used for comparative genomics in plants to identify evolutionary conserved mechanisms and pathways. In such studies, transcripts from closely related species are hybridized to a microarray consisting of probes generated from one of those species. For instance, in a study, *Arabidopsis thaliana* GeneChips were utilized to study a complex response, zinc hyperaccumulation, in cadmium and zinc tolerant close relative, *Arabidopsis halleri* ssp. *halleri* (Becher et al., 2004). Via this transcriptome-wide analysis, several genes playing key roles in cellular metal uptake and detoxification in the shoots were identified. The results of this study confirmed the effectiveness of usage of *Arabidopsis thaliana* oligonucleotide microarrays in comparative transcriptomic profiling in *A. halleri.*

In another study, CSH to tomato cDNA array was utilized for comparative transcriptomic profiling in Solanaceae family members, tomato, pepper and eggplant (Moore et al., 2005). With this study, apart from the transcripts commonly expressed during fruit development, transcripts functioning in a variety of diverged mechanisms were also identified. Since the tomato cDNA microarray used in this study only represented a small portion of the genes in the tomato genome, acquirement of more biologically meaningful data depends on the development of a more extensive array platform.

CSH is also utilized for studying complex traits in a target plant species via utilization of an array platform of a distantly related species. For instance, in a recent study, Rice GeneChip genome array was utilized to study the response of banana (Musa ssp.) to drought stress at transcriptomic level (Davey et al., 2009). To identify differentially regulated transcripts, Musa RNA was cross hybridized to GeneChip of rice, which is distantly related to Musa. Before hybridization, genomic DNA hybridization based probe selection strategy was utilized to obtain a more biologically relevant data in the end. Of the 2910 transcripts displaying >2 fold expression difference in response to drought, many were annotated to function in abitotic stress-response pathways. In conclusion, the results of this study indicated the feasibility of CSH to perform transcriptomic profiling in Musa using an array platform from a distantly related species, rice, with a preliminary probe selection step.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

In the experiments, three different turfgrass species, Ambrose (*Festuca rubra* subsp. *falax*), Cindy Lou (*Festuca rubra* subsp. *littoralis*) and Discovery (*Festuca brevipila*) were used.

3.1.2 Chemicals

All chemicals were obtained from Merck (Germany), SIGMA (US), Fluka (Switzerland), and Riedel de Häen (Germany).

3.1.3 Growth Media, Buffers and Solutions

The growth media, buffers, and solutions used in this study were prepared according to the protocols as outlined by Sambrook *et al.*, 2001.

3.1.4 Equipment

Equipments used in this research are listed in Appendix.

3.2 Methods

3.2.1 Plant growth conditions and glyphosate treatments

Seeds of three different turfgrass species, Ambrose (*Festuca rubra* subsp. *falax*), Cindy Lou (*Festuca rubra* subsp. *littoralis*) and Discovery (*Festuca brevipila*) were directly planted on soil and grown under controlled conditions in the greenhouse with daytime and nighttime temperatures of 25°C and 20°C, respectively. Glyphosate [RoundUp Ultra; acid equivalent (a.e.): 356 g L^{-1} N-[phosphonomethyl]glycine, Monsanto Co.] treatment was performed four weeks after sowing by spraying a total volume of 100 ml of either 5% or 20% solution (1.58 mM and 6.32 mM, respectively) directly on the leaves under open air conditions. Plants at the three-leaf growth stage were sprayed with freshly prepared glyphosate solution until all leaves were fully wet (about 10 ml) but without run-off. Control plants were sprayed with distilled water. Leaf samples were collected 5 days after treatment.

3.2.2 Total RNA isolation

Total RNA isolations were carried out by Trizol reagent (Invitrogen) according to the manufacturer's instructions with a few modifications. Two hundred mg leaf tissue was ground with 1.5 ml Trizol$^{®}$ reagent (Invitrogen) (without adding liquid nitrogen). Using a wide bore pipette tip, 1 ml of liquid was taken into an eppendorf tube, which was kept on ice while processing the other samples. After processing all the samples, they were incubated at room temperature for 10 min, 0.4 ml of chloroform was added and the tubes were shaken and incubated at room temperature for 5 min. Then, samples were centrifuged at 12,000 rpm for 15 min at 4°C. The upper layer containing RNA was

transferred to a fresh tube. After chloroform extraction, 0.5 ml isopropanol was added to precipitate RNA. Samples were then incubated at room temperature for 10 min and spun at 12,000 rpm for 10 min at 4ºC. The RNA pellet was washed with 1 ml of 75% ethanol after centrifugation. Samples were mixed by vortexing and spun at 7,500 rpm for 5 min at 4ºC. The RNA pellet was dried at room temperature for 10 minutes and placed in 20-50 µl formamide, depending on the size of the pellet, and allowed to sit in the 55ºC water bath for an hour to improve suspension.

Three separate RNA isolations were performed for each glyphosate dose of each species. RNA concentrations were determined spectrophotometrically and RNA qualities were checked by denaturing gel before the microarray analysis. RNA samples were treated with Dnase I (Fermentas) according to manufacturer's instructions.

3.2.3 Gene chip analysis

Microarray analysis was performed using the Affymetrix GeneChip® Wheat Genome Array to identify the global gene expression profiles of the turfgrass species used in this study. This array contains 61,127 probe sets representing *Triticum aestivum, T. turgidum*, *T. turgidum* ssp. *durum*, *T. monococcum*, and *Aegilops tauschii* transcripts. All hybridizations were performed as biological triplicates of control and glyphosate treated samples (RNA isolation and cRNA labeling were done separately for each hybridization) of three Festuca genotypes and two glyphosate dosages, for a total of 36 Affymetrix GeneChip® Wheat Genome Arrays. Labeled cRNAs were synthesized from six micrograms of total RNA using the One-Cycle Target Labeling and Control Reagents (Affymetrix) according to the manufacturer's instructions. Synthesized cRNA samples were cleaned up, fragmented and hybridized to Affymetrix GeneChip® Wheat Genome Array for 16 hours at 45°C and 60 rpm using Hybridization Wash and Stain Kit (Affymetrix) in Fluidics Station 450 according to the manufacturer's instructions. Finally, the array was scanned with GeneChip® Scanner 3000 with GeneChip® Operating Software script.

3.2.4 Data analysis

Partek® Genomics Suite version 6.3 Beta (Partek Incorporated) was used to analyze cell intensity files using robust multichip average (RMA) normalization. Principal component analysis (PCA) and Box&Whiskers plots were utilized to check the quality of data obtained from the hybridization experiments. Analysis of variance (ANOVA) test was used for further analysis of log transformed expression values ($p<0.1$ and DE<-2 or DE>2). Raw data are submitted to ArrayExpress database (http://www.ebi.ac.uk) under E-MEXP-1647. Differentially expressed probes in all genotypes and at both glyphosate doses were subjected to cluster analysis using Cluster (Eisen et al., 1998) and Treeview programmes (Page, 1996).

3.2.5 Annotation of Affymetrix GeneChip® probes

The target sequences of differentially expressed probes were retrieved from NetAffx database. The annotation of the probe sets were performed by BLASTn hits (e value < 0.0001) to TC collections of wheat and rice from TIGR database (http://www.tigr.org) and to GenBank nr database (NCBI, Release date Feb 20, 2008, http://www.ncbi.nlm.nih.gov). Functional categorization was performed using The Munich Information Center for Protein Sequences (MIPS) annotation categories (http://mips.gsf.de/) and ExPASy proteomics server (Gasteiger et al., 2003).

3.2.6 RT-PCR Analysis

Two micrograms of total RNA isolated from control and glyphosate-treated Festuca species were used to synthesize first strand cDNA with the Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. cDNAs were quantified spectrophotometrically and diluted to 400 ngμl^{-1}. One μl of this cDNA was amplified with 0.5 μM of gene specific primers and 18S rRNA primers in a total of 20 μl volume. The primer pair selected for RT validation was specific to alternative oxidase, because it was found to be up-regulated in all three species

4 RESULTS

4.1 Cross Species Hybridization Analysis

Gene expression profiles of three Festuca species, Ambrose, Cindy Lou and Discovery, were examined in response to increasing levels of glyphosate using the Affymetrix GeneChip® Wheat Genome Array. The CSH approach led to significant differential regulation of only 1337 probe sets (231 probes from Ambrose, 767 from Cindy Lou, and 339 probes from Discovery) at the defined threshold expression values of $p<0.1$ and DE<-2 or DE>2.

4.2   Gene Expression Profiles in Festuca species

The number of differentially expressed probes increased proportionally at 20% foliar glyphosate treatment. CSH with the Cindy Lou led to detection of a larger number of probes (Fig. 4.1C), whereas CSH with the Ambrose resulted in detection of the lowest number of differentially expressed probes (Fig. 4.1A). The total number of differentially expressed probe sets in Discovery was intermediate to that of Ambrose and Cindy Lou in response to glyphosate (Fig. 4.1B). Interestingly, the number of differential expressed probes was almost constant in cultivar Ambrose irrespective of the glyphosate rate. However, the number of probes increased with 20% glyphosate treatment for both Discovery and Cindy Lou. This response was stronger in Cindy Lou cultivar (Fig. 4.1)
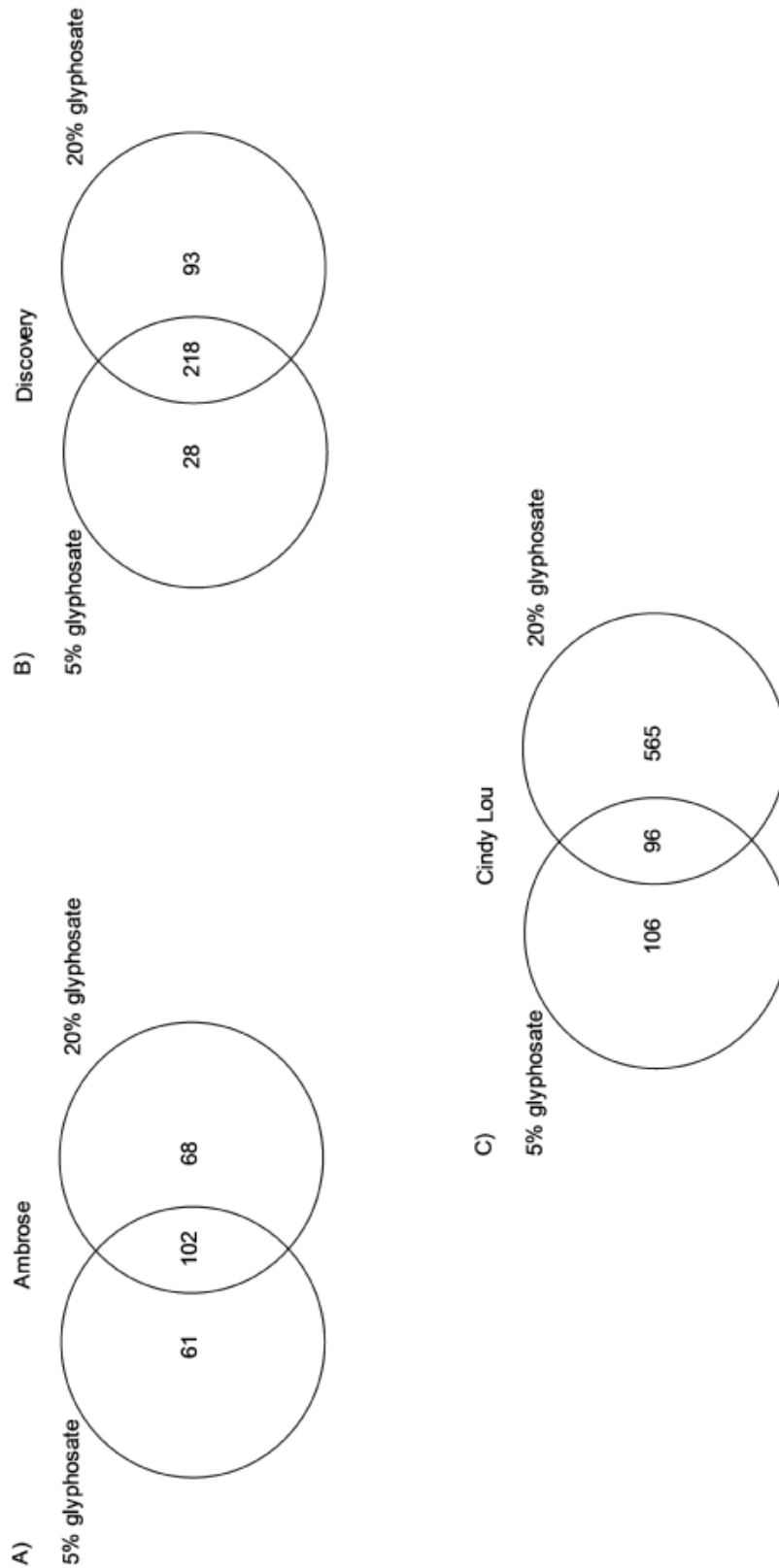
Fig. 4.1 Venn diagrams showing the total number of differentially expressed probes in three Festuca genotypes in response to two glyphosate doses, 5% and 20%. A) Ambrose; B) Discovery; C) Cindy Lou.

In Cindy Lou, most of the differentially expressed probes were found to be up-regulated at the 5% glyphosate application, but this pattern was opposite for the 20% glyphosate application (Fig. 4.2C). Transcripts altered with 20% glyphosate applications were mostly down-regulated. In contrast, for Ambrose, the number of up-regulated probes was more than the down-regulated probes for both glyphosate doses (Fig. 4.2A).
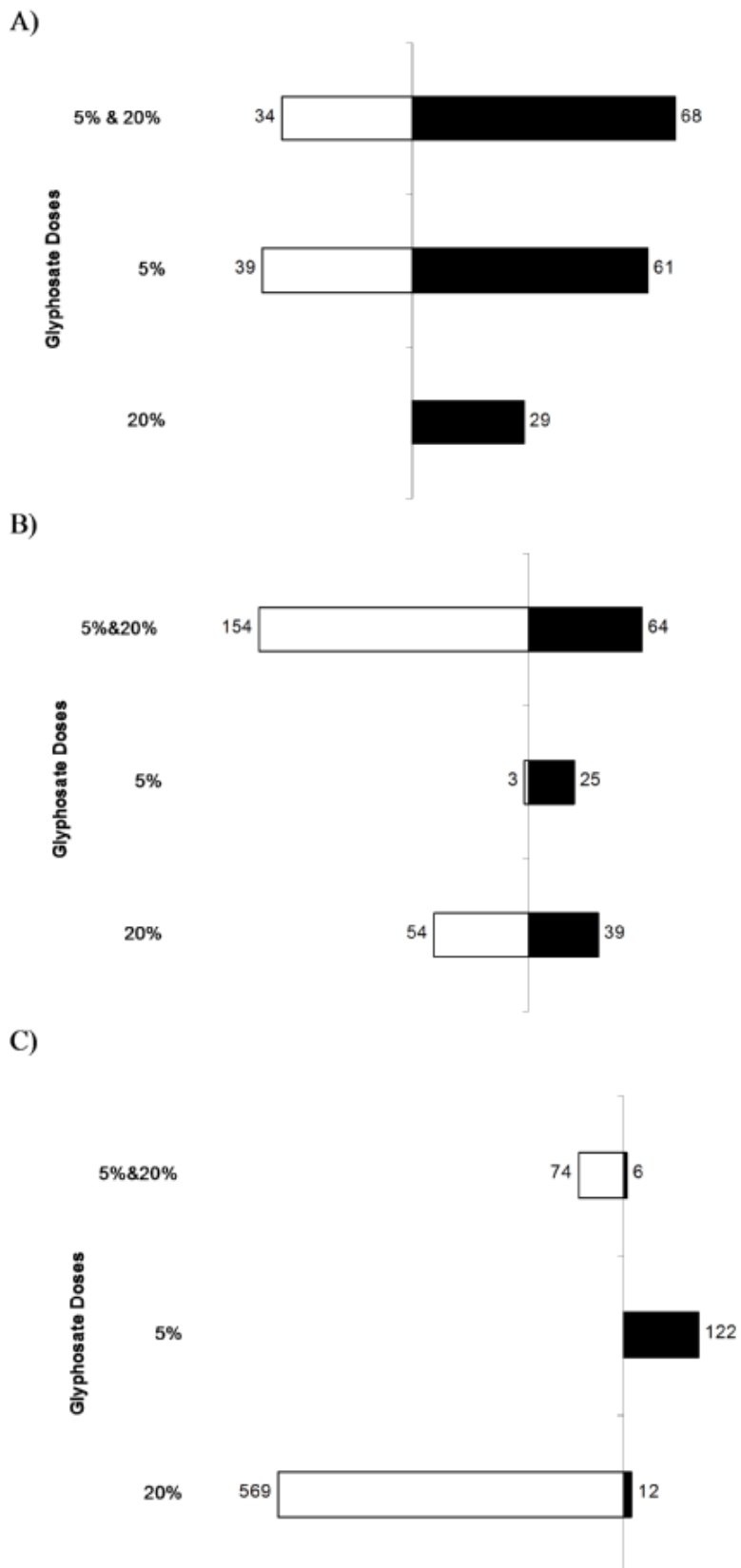
Fig. 4.2 The total number of differentially expressed probe sets (p< 0.1) in glyphosate treated plants. A) Ambrose; B) Discovery; C) Cindy Lou]. Up-regulated probes are represented by black bars, whereas down-regulated probes are represented by white bars.

The total number of common up-regulated probes in Ambrose and Discovery at both glyphosate doses was greater than the number of common probes in Discovery and Cindy Lou (Fig. 4.3). These results were opposite for down-regulated probes.
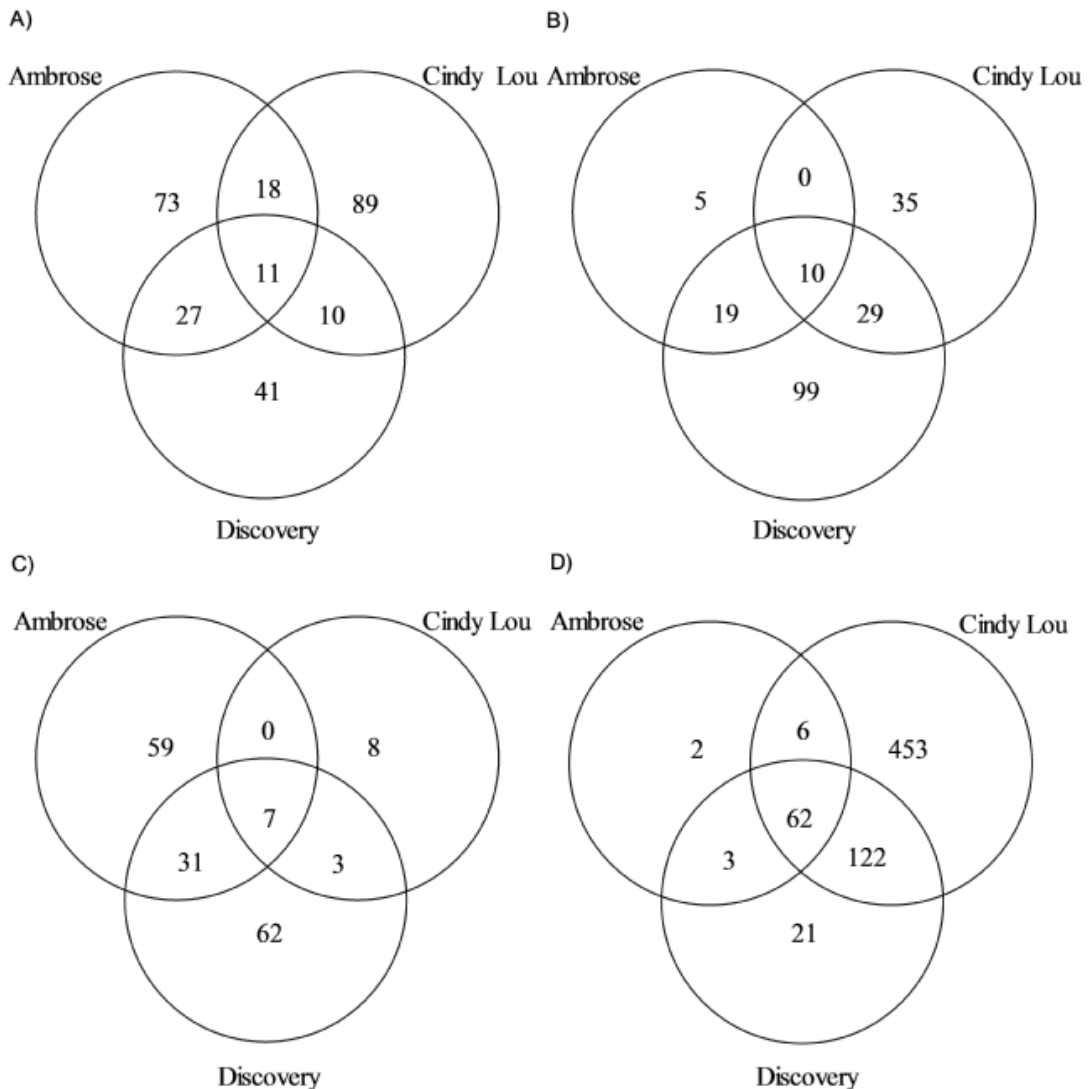
Fig. 4.3 Venn diagrams showing the total number of differentially expressed probe sets common to three Festuca species. A) Up-regulated by 5% glyphosate application B) Down-regulated by 5% glyphosate application C) Up-regulated by 20% glyphosate application D) Down-regulated by 20% glyphosate application.

4.3 Functional Analysis

Probes with differential expression were annotated by homology searches of target sequences using BLASTn in the TIGR wheat and rice genome databases (http://www.tigr.org) and the GenBank nr database (http://www.ncbi.nlm.nih.gov). Subsequently, we searched for functions using the ExPASy proteomics server (Gasteiger et al., 2003) (Fig. 4.4). Differentially expressed probes were grouped into 21 functional categories according to MIPS functional categories (http://www.mips.gsf.de/). The largest probe sets were categorized under "Photosynthesis" (~25.3%, average of three genotypes), "Metabolism" (~24.6%), "Protein Synthesis" (~19.1%), "Unclassified" (~13.7%), "Transport & Mechanisms" (~10.1%), "Energy" (~6.7%), and "Protein Fate" (~6.4%) (Fig. 4.4).
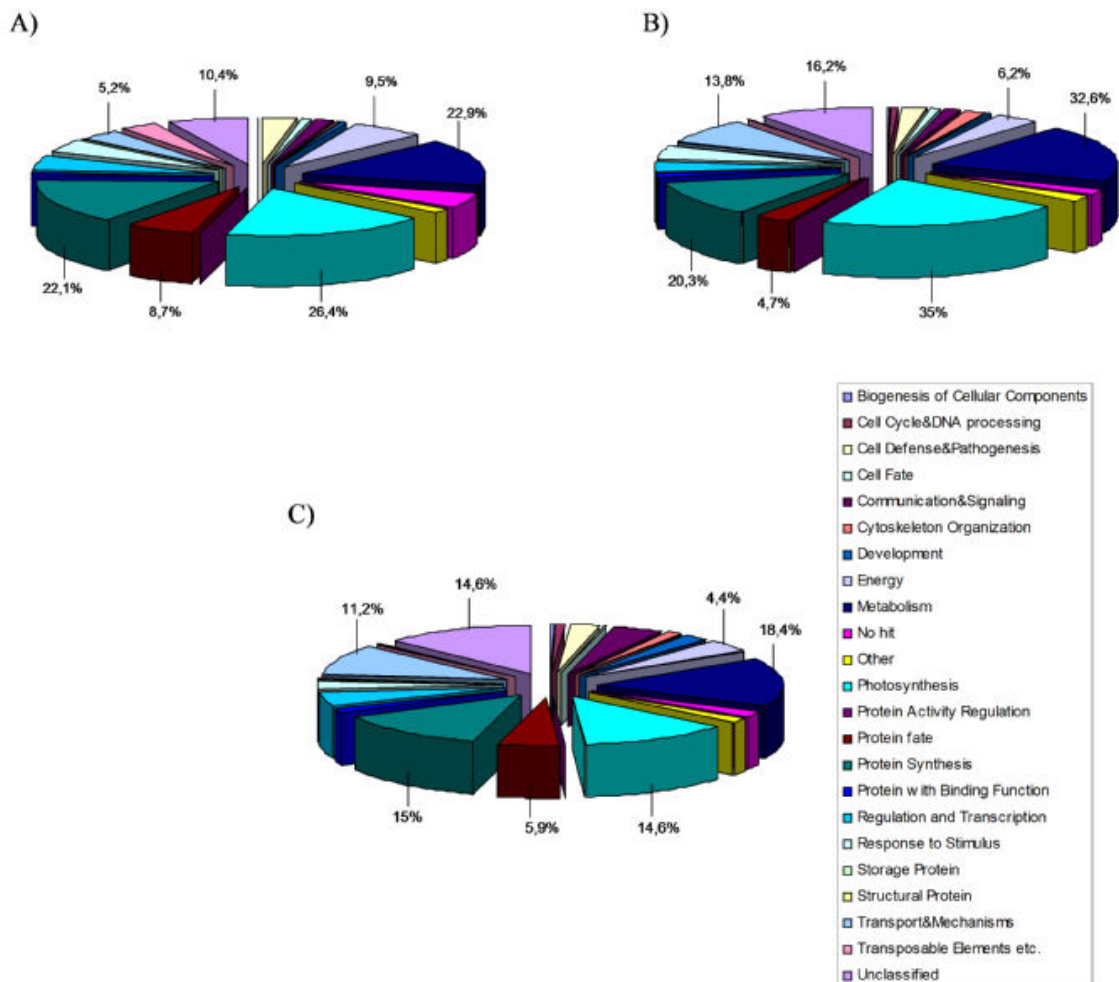
Fig. 4.4 Functional annotation of all differentially regulated probes in three Festuca species. A) Ambrose, B) Discovery, C) Cindy Lou.

The probes in the group, "Photosynthesis" were down-regulated in all three genotypes In Ambrose, all differentially expressed probes except the ones in the photosynthesis category were found to be up-regulated at both glyphosate rates (Fig. 4.5A). As for Cindy Lou, the transcript abundance pattern was found to be different from Ambrose. A major portion of the probes residing in the listed categories were up-regulated by the 5% glyphosate application. However, increasing glyphosate treatment to 20% resulted in downregulation of most probes (Fig. 4.5C). As for Discovery, most probes played roles in protein synthesis, photosynthesis, and transport mechanisms which were down-regulated (Fig. 4.5B).
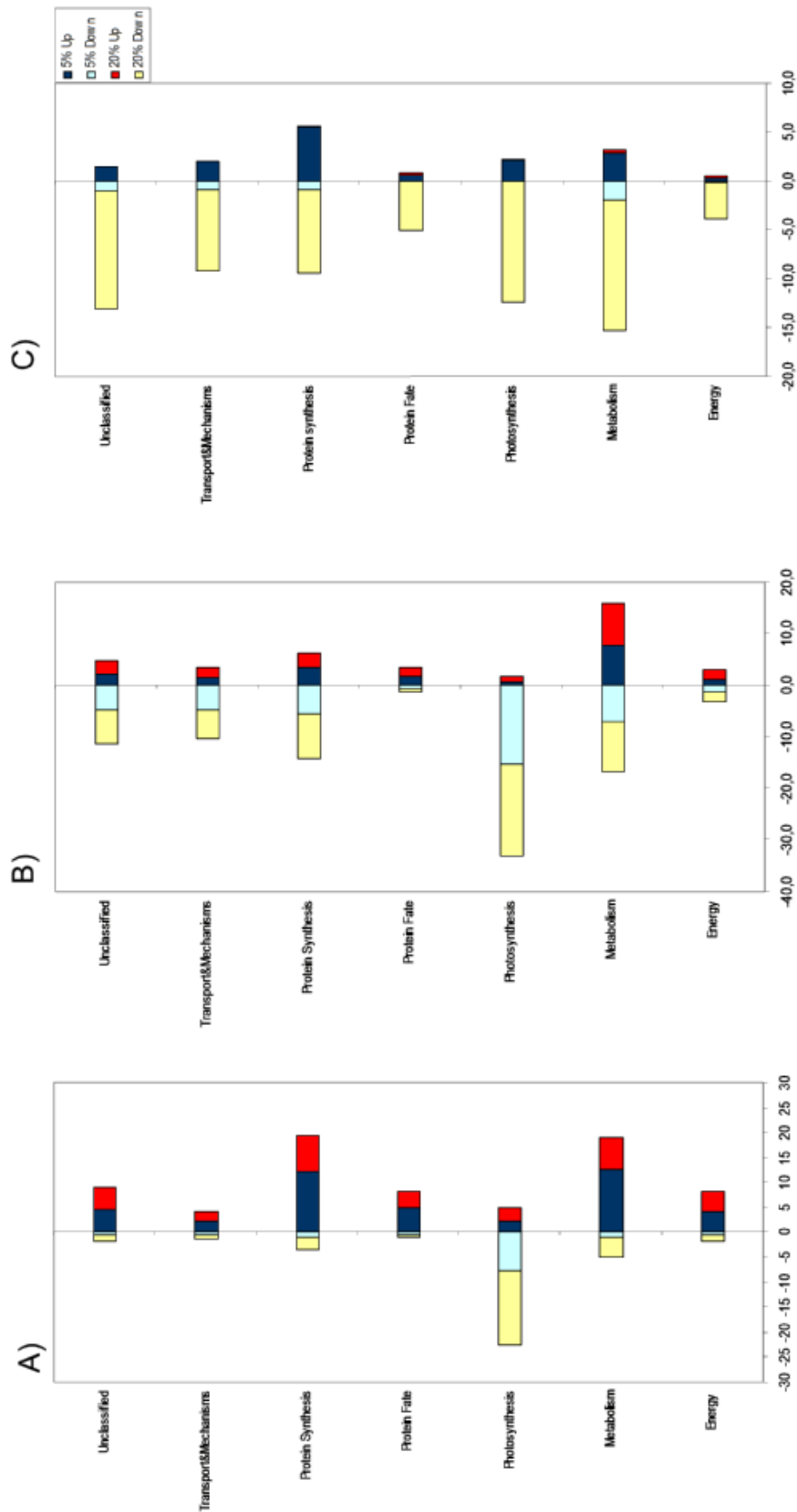
Fig. 4.5 Functional categories of largest differentially expressed probe sets in three Festuca genotypes. A) Ambrose, B) Discovery, C) Cindy Lou] exposed to 5% and 20% glyphosate.

Cluster analysis using differentially expressed probes common to all genotypes for both glyphosate rates showed that differentially expressed probes in all Festuca genotypes in response to 5% (21 probes) and 20% glyphosate (71 probes) treatment grouped separately. The Treeview results indicated that differentially expressed probes of Cindy Lou and Discovery clustered together for both glyphosate rates (Figs. 4.6A and 4.6B). Ambrose was shown to cluster separately from Cindy Lou and Discovery, which are proposed to be more tolerant to glyphosate in comparison to Ambrose (Su et al. 2009).
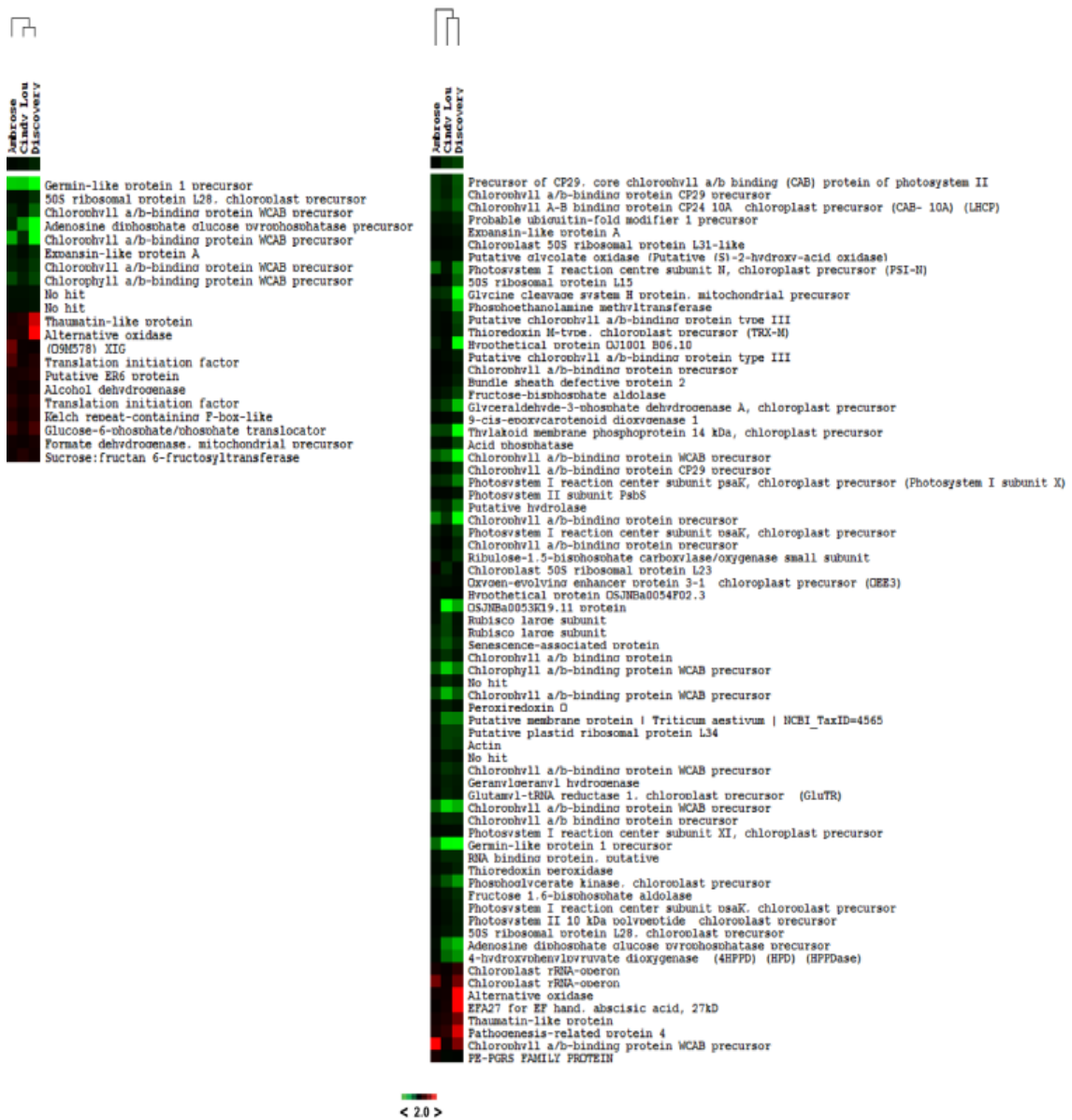
Fig. 4.6 Cluster analysis of probes with differential regulation in three Festuca genotypes. A) 5% glyphosate B) 20% glyphosate. The color saturation reflects the fold change where green is for more than 2 fold down-regulated and red is for more than 2 fold up-regulated probes with p<0.1.

4.4 Effects of glyphosate on photosynthesis and detoxification of Reactive Oxgen Species (ROS)

Analysis of the differentially expressed probes with roles in photosynthesis revealed that glyphosate led to the down-regulation of most probes related to photosynthesis in all Festuca species at both glyphosate doses. This reduction in gene expression was mostly apparent in transcripts functioning in chlorophyll biosynthesis, photosystem activities and RuBisCo, a key player in the Calvin cycle (Table 4.1A). The decline in transcript abundance was more pronounced for plants treated with the higher glyphosate dose.

Transcript profiling of Festuca species via CSH to Wheat Affymetrix Gene Chip indicated that glyphosate treatment at both rates led to down-regulation of majority of probes functioning in detoxification of ROS in all Festuca species (Table 4.1B).

Transcripts functioning in signal transduction pathways were also found to be down-regulated in Cindy Lou at both glyphosate doses. However, both glyphosate rates resulted in up-regulation of transcripts playing roles in cell signaling in Discovery (Table 4.1C)

Table 4.1 List of common or species-specific probes related to (A) photosynthesis, (B) oxidative stress, and (C) cell signaling and differentially expressed in response to two different doses of glyphosate. Given are the fold changes calculated by ANOVA. Up-regulation fold changes are given in bold letters, whereas down-regulation by italics. No significant differential expression is shown with an empty cell. Ambrose probe fold changes are highlighted with red, Discovery with blue Cindy Lou with black.

A)

		Ambrose		Discovery		Cindy Lou	
		Fold Change					
		5%	20%	5%	20%	5%	20%
Probe Set ID	Target Sequence						
Ta.27761.1.S1_x_at	Photosystem I reaction center subunit psaK, chloroplast precursor	*-3,30122*	*-13,2487*	*-3,01112*	*-37,0513*	-	*-6,62621*
Ta.27751.3.S1_x_at	Photosystem I reaction center subunit XI, chloroplast precursor	-	*-3,53086*	*-3,03125*	*-6,11773*	-	*-5,63676*
Ta.28750.1.S1_at	Photosystem II 10 kDa polypeptide chloroplast precursor	-	*-2,6278*	*-4,16983*	*-17,2639*	-	*-8,04824*
Ta.1161.1.S1_at	Photosystem II subunit PsbS	-	*-3,47497*	-	*-11,5118*	-	*-3,58749*
Ta.1139.1.S1_at	Precursor of CP29, core chlorophyll a/b binding (CAB) protein of photosystem II	*-5,04791*	*-28,6882*	*-7,37361*	*-46,5839*	-	*-18,6875*
Ta.28265.1.S1_at	Oxygen-evolving enhancer protein 3-1, chloroplast precursor (OEE3)	-	*-8,69806*	*-3,77085*	*-5,07437*	-	*-10,209*
Ta.30702.1.S1_x_at	Chlorophyll a/b-binding protein WCAB precursor	*-40,9861*	*-58,6499*	*-64,5336*	*-98,1407*	*-10,1345*	*-119,736*
Ta.20639.3.S1_x_at	Chlorophyll a/b-binding protein precursor	*-3,23933*	*-6,25985*	*-6,19484*	*-18,7793*	-	*-2,68378*
TaAffx.128414.219.S1_x_at	Rubisco large subunit	*-9,75494*	*-16,4849*	*-3,08632*	*-10,8861*	-	*-37,5039*
Ta.2752.2.S1_x_at	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	-	*-14,2542*	-	*-27,3256*	-	*-7,39744*

B)

		Cindy Lou		Discovery	
		Fold Change			
Probe Set ID	Target Sequence	5%	20%	5%	20%
Ta.28714.1.S1_at	Thioredoxin peroxidase	-	*-10,1624*	*-2,57569*	*-17,9772*
Ta.6572.1.S1_a_at	Peroxiredoxin Q	-	*-17,3425*	*-4,85985*	*-7,80475*
Ta.18063.2.S1_at	Putative glutathione peroxidase	*-2,69247*	*-5,38618*	-	-
Ta.547.1.S1_at	Cytosolic glutathione reductase	-	-	9,12843	11,6039
Ta.14644.2.S1_x_at	Superoxide dismutase [Cu-Zn] 4A	-	-	2,52839	2,62177

C)

Probe Set ID	Target Sequence	Cindy Lou		Discovery	
		Fold Change			
		5%	20%	5%	20%
Ta.6269.1.S1_at	Putative serine/threonine kinase 38	-	*-3,59976*	-	-
Ta.991.1.S1_a_at	Serine/threonine-protein kinase SAPK8	-	*-7,70463*	-	-
TaAffx.86456.1.S1_s_at	Putative calcium/calmodulin-dependent protein kinase CaMK	-	*-6,76413*	-	-
Ta.11837.1.S1_at	Calmodulin	-	*-5,03177*	-	-
Ta.6979.1.S1_s_at	Phosphatidylinositol 3-and 4-kinase family-like,	-	*-40,9189*	-	-
Ta.1890.1.S1_x_at	Nt-iaa28 deduced protein	*-2,85957*	*-2,85957*	-	-
Ta.6968.2.S1_a_at	Ethylene-responsive small GTP-binding protein	-	*-2,60726*	-	-
Ta.25390.1.S1_s_at	(Q91W51) WASP family 1	-	-	3,49126	4,82506

4.5 Reverse transcriptase (RT) PCR analysis

RT-PCR was performed on cDNA generated from total RNAs of both control and three 5% glyphosate-treated Festuca to validate our CSH results. The candidate probe, TA.233.1.S1_AT was found to have homology to "Alternative Oxidase" by functional analysis and was differentially expressed in all three genotypes. Sequencing of this product amplified in Fescue showed a high level of similarity (*Triticum aestivum, E* value 1e−90) with wheat. Amplification of this probe was shown to be enhanced with 5% glyphosate treatment, in accordance with the CSH data (Fig. 4.7).
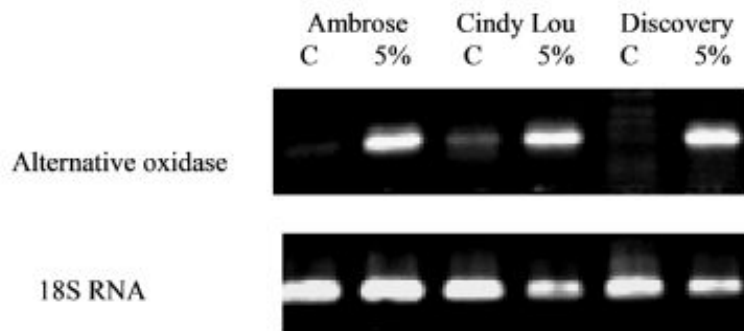
Fig. 4.7 A representative picture of validation of microarray result using RT-PCR for one common Festuca probe set.

.

5 DISCUSSION

5.1 The percentage of hybridization was low as a result of CSH to wheat genome array

A total of 1337 festuca probes with either > 2 fold or <-2 difference in expression levels were identified as a result of CSH analysis. Although only 2.2% of the probes in total stayed above the threshold, biologically meaningful information could be extracted from this data set, which could be used to elucidate conserved mechanisms responsive to glyphosate common to fescues and wheat. The low percentage of hybridization might be explained by the presence of interspecies differences between the probe and target sequences. The single nucleotide polymorphisms may result in alteration of probe-hybridization affinities and hence, generate lower hybridization signal intensities (Benovoy et al. 2008). Low percentage of hybridization, which is also detected in our CSH study, might be eliminated by probe set filtration which is based on hybridization of genomic DNA of target species to the reference microarray. This approach has been employed by several researchers for better CSH data (Ranz et al., 2003; Hammond et al., 2005). Additionally, much higher hybridization ratios have been reported in recent studies exploiting CSH with cDNA arrays (Sun et al., 2007; Vallée et al. 2006,) because cDNA platforms are likely more suitable for CSH studies owing to the longer cDNA probes. The wheat array platform chosen in our study appears to provide a benefit in that it enabled the detection and identification of highly conserved biological processes common to fescues and wheat, such as photosynthesis or reactive oxygen species scavenging.

5.2 Gene expression profiles of Festuca species differed after glyphosate treatment

The numbers of differentially expressed probes for 20% foliar glyphosate treatment were proportionally increased with previously detected glyphosate tolerance levels of Festuca species (Su et al., 2009) used in this experiment. Hybridization with the most tolerant genotype Cindy Lou (*Festuca rubra* subsp. *littoralis*) led to detection of larger number of probes, whereas the most sensitive cultivar Ambrose (*Festuca rubra* subsp. *falax*) ended up with the lowest number of differentially expressed probes. This number in Discovery (*Festuca brevipila*) was in between the number of probe sets in Ambrose and Cindy Lou in accordance with the glyphosate tolerance levels of these three genotypes. The results indicated that the higher the tolerance to glyphosate, the larger was the portion of probe sets differentially expressed in Festuca genotypes used. Interestingly, the number of probes with differential expression was almost constant in cultivar Ambrose irrespective of the glyphosate rates, whereas the number of probes increased with 20% glyphosate dosage for both Discovery and Cindy Lou with a strong response in the later. Although very speculative, this observation might represent the direct relationship between the molecular response and the tolerance capacity where better tolerance requires more effective change in gene expression profile to minimize toxic effects of glyphosate on plant metabolism.

20% glyphosate application rate was found to down-regulate the differentially expressed probes. Additionally, the number of common down-regulated probes in Cindy Lou and Discovery was greater than the number of up regulated probes. The mechanisms controlled by common down-regulated probe sets are likely more conserved among Cindy Lou and Discovery and appear to initiate a response to the higher doses of glyphosate.

5.3 The largest differentially expressed probe sets were categorized under "Photosynthesis" in Festuca

As could be expected, the probes in the largest functional category, "Photosynthesis" were down-regulated in all genotypes, since one of the secondary responses of plants to glyphosate is the inhibition of photosynthesis via several routes

(Geiger et al. 1986; Servaites et al. 1987; Tan et al. 2006; Wong 2000). Up-regulation of majority of the differentially expressed probes except the probes in "Photosynthesis" category in Ambrose suggested that most biological processes, except photosynthesis, were active in this genotype or showed enhanced expression. Majority of the differentially expressed probes with 5% glyphosate application were found to be up-regulated in Cindy Lou. However, when the level of glyphosate applied was raised to 20%, most of the probes were down-regulated. These results support our proposal that glyphosate leads to the induction of a more profound down-regulatory response when it is applied at a relatively higher dose (20%) in Cindy Lou. This result was expected based on a previous report indicating that glyphosate led to the inhibition of many biological processes, including chlorophyll synthesis, plant tissue ion fluxes, and activity of anti-oxidative enzymes (Sergiev et al. 2006). The probes residing in "Protein Synthesis", "Photosynthesis" and "Transport&Mechanisms" categories were down-regulated. Inhibition of protein synthesis was an expected response, because the major mode of action of glyphosate is inhibition of aromatic amino acid biosynthesis, and hence protein synthesis (Amrhein et al. 1980).

5.4 Differentially expressed probes of Cindy Lou and Discovery clustered together for both glyphosate rates

Similarities and differences between three Festuca cultivars with differential tolerance to glyphosate were sought by clustering of differentially expressed probes common to all genotypes for both glyphosate rates, 5% glyphosate (21 probes) and 20% glyphosate (71 probes). Clustering results indicated that differentially expressed probes of Cindy Lou and Discovery clustered together for both glyphosate rates. Glyphosate sensitive genotype, Ambrose, was shown to cluster separately from Cindy Lou and Discovery, which are proposed to be more tolerant to glyphosate with respect to Ambrose. In other words, transcriptome changes in cultivar Cindy Lou and Discovery with both glyphosate rates resembled each other. Clustering of glyphosate sensitive genotype, Ambrose from glyphosate tolerant genotypes, Cindy Lou and Discovery indicated that glyphosate responses of tolerant and sensitive genotypes are distinct.

5.5 Glyphosate treatment led to down-regulation of photosynthesis related genes

A closer look to the differentially expressed probes showed that glyphosate led to the down-regulation of probes functioning in chlorophyll biosynthesis, photosystem activities and RuBisCo, a key player in the Calvin cycle (Table 1).

A major mode of action by glyphosate affects the aminolevulinic acid (ALA) pathway, or the porphyrin biosynthesis pathway. In the ALA pathway, glyphosate interferes with the activity of aminolevulinate synthase preventing the conversion of succinyl CoA (from the tricarboxylic acid cycle) to ALA. Blockage of this step in porphyrin biosynthesis leads to a decline of compounds containing porphyrin, such as chlorophyll (Kitchen et al. 1981). Additionally, it has been reported that leaf chlorophyll content of plants exposed to sub-lethal doses of glyphosate is lower (Tan et al. 2006; Wong 2000). Hence, one of the primary reasons for the decline in expression levels of probes playing a role in photosynthesis might be related to the deleterious effect of glyphosate on chlorophyll. Glyphosate treatment was also shown to inhibit photosynthesis by blocking the allocation of carbon to starch (Geiger et al. 1986), and resulted in an immediate and rapid decline in the level of ribulose bisphosphate and associated photosynthetic carbon metabolism in sugar beet (Servaites et al. 1987). These studies are consistent with the reduction in the levels of transcripts related to photosynthetic pathways being linked to the inhibitory effect of glyphosate in Festuca species.

5.6..Regulation of detoxification of reactive oxygen species (ROS) is genotype dependent

ROS generation causes oxidative damage to membrane lipids, DNA, and proteins (Apel and Hirt 2004). CSH-based transcript profiling of Festuca species indicated that glyphosate treatment at different rates leads to the down-regulation of transcripts involved in the detoxification of ROS. The major strategy used by plants to tolerate oxidative stress is the production of anti-oxidative enzymes that convert ROS to less toxic compounds. In a previous study, it has been shown that glyphosate will exert its deleterious effects on maize plants by amplifying lipid peroxidation of biomembranes

(MDA) (Sergiev et al. 2006). Additionally, a small number of changes appeared in lipid peroxidation and antioxidative defense mechanisms in susceptible and resistant soybean cultivars exposed to sub-lethal doses of glyphosate (Moldes et al. 2008). In this study, probes with homology to antioxidative enzymes, such as putative glutathione peroxidase, thioredoxin peroxidase, and peroxiredoxin Q were down-regulated. The major reason for down-regulation of these peroxidases might be the inhibition of ALA by glyphosate action.

5.7 RT PCR validated CSH results

As shown in Fig. 4.7, RT-PCR result showed the same trend, and expression level of "Alternative Oxidase" agreed with the results of the CSH experiment.

6 CONCLUSION

Maintenance of turfgrass in landscapes has a great impact on human life from a visual, functional, and economic point of view. Despite its economic and social importance, there have been no genome-wide studies in fescues for understanding molecular mechanisms regulated by glyphosate. Additionally, availability of sequence information is limited for fescues. Furthermore, despite the growing knowledge of genes acting in responses of plants to herbicides, a big picture of key cellular processes that determine plant tolerance to herbicides has not been defined yet. Therefore, it is likely that the study of fescues from a global point of view will aid in identification of key biological mechanisms active in glyphosate responses of fescues and might constitute a rich resource for future studies in the pursuit of identification of herbicide tolerance genes and markers in plants.

This is the first report to analyze the potential of cross species hybridization in Fescue species and the data and analyses will help extend our knowledge on the cellular processes affected by glyphosate. Cross hybridisation of fescue RNAs to the Affymetrix GeneChip® Wheat Genome Array identified 1337 transcripts (probe-sets) displaying either <-2 or >2-fold difference in expression levels in response to glyphosate. Gene annotations based on wheat and rice databases indicated that many of these transcripts were involved in highly conserved biological processes common to fescues and wheat, such as photosynthesis or reactive oxygen species scavenging. Functional categorization and clustering analysis of the differentially expressed probes demonstrated that the pattern of gene expression regulation was similar in glyphosate tolerant fescues, Cindy Lou and Discovery, in contrast to glyphosate sensitive fescue, Ambrose. RT results mostly agreed with the CSH microarray data, which confirmed the proficiency of CSH in comparative transcriptome analysis.

This study is important for paving the way to better understand the mechanisms and pathways regulating glyphosate responses of Festuca species. Data presented here will also help researchers to determine the effective rate of glyphosate that should be used for weed control in turfgrass areas. In the long term, the results of this study might help in development of future turfgrass management strategies both to slow down the evolution of herbicide resistance and to control existing weed populations.

7 REFERENCES

1. Steinrücken, H.C. and Amrhein, N.T. 1980. The herbicide glyphosate is a potent inhibitor of 5- enolpyruvylshikimate-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94, 1207-1212.

2. Hart, S.E., Derr, H.F., Lycan, D.W., Rose-Fricker, C., Meyer, W.A. 2005. Increased glyphosate tolerance in 'Aurora Gold' hard fescue (*Festuca longifolia*). Weed Tech. 19, 640-646.

3. Reichman, J.R.,Watrud, L.S., Lee, E.H., Burdick, C.A., Bollman, M.A., Storm, M.J., King, G.A., Mallory-Smith, C. 2006. Establishment of transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) in nonagronomic habitats. Mol. Ecol. 15, 4243–4255.

4. Baylis, A.D. 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pest. Manag. Sci. 56, 299-308.

5. Jones, S.J. et al. 2002. An enhanced molecular marker-based map of perennial ryegrass (*Lolium perenne* L.) reveals comparative relationships with other *Poaceae* genomes. Genome. 45, 282–295.

6. Soreng, R.J. and Davis, J.I. 1998. Phylogenetics and character evolution in the grass family (Poaceae): simultaneous analysis of morphological and chloroplast DNA restriction site character sets. Bot. Rev. 64**,** 1–85.

7. Su, Y.S., Ozturk, L., Cakmak, I., Budak, H. 2009. Turfgrass species response exposed increasing rates of glyphosate application. Eur. J. Agron. 31, 120-125.

8. Clayton, W. and S. Renvoize. 1986. Genera Graminum, grasses of the world. Kew Bul. Additional Ser. 13, 1–389.

9. Alm, V., Fang, C., Busso, C.S., Devos, K.M., Vollan, K., Grieg, Z., Rognli, O.A. 2003. A linkage map of meadow fescue (*Festuca pratensis* Huds.) and comparative mapping with other Poaceae species. Theor. Appl. Genet. 108, 25–40.

10. Turgeon, A.J. 2002. Turfgrass Management, 6th ed. Prentice-Hall, Upper Saddle River, NJ.

11. Jauhar, P.P. 1993. Cytogenetics of the Festuca-Lolium complex: Relevance to breeding. Springer-Verlag, Berlin/New York.

12. Meyer, W.A., and C. R. Funk. 1989. Progress and benefits to humanity from breeding cool-season grasses for turf. p. 31-48. In D.A. SIeper, K.H. Asay, and J.F. Pedersen (ed.) Contributions from breeding forageand turf grasses. CSSA publ. 15. CSSA, Madison, WI.

13. Ruemmele, B.A., Brilman, L.A. and Huff, D.R. 1995. Fine fescue germplasm diversity and vulnerability. Crop Sci. 35, 313–316.

14. Gaussoin, R.E. and B.E. Branham. 1989. Influence of cultural factors on species dominance in a mixed stand of annual bluegrass/creeping bentgrass. Crop Sci. 29, 480-484.

15. Savela M-L. and Hynninen E-L. 2004. Slower growth in pesticide sales. Kemia Kemi. 31, 57-59.

16. Tomlin, C. (Ed.) 2000. The pesticide manual. 12th edition. Pp. 488-491. The British Crop Protection Council, Furnham, Surrey, UK.

17. Vereecken, H. 2005. Mobility and leaching of glyphosate: a review. Pest Manag. Sci. 61, 1139-1151.

18. Laitinen, P. 2009. Fate of the organophosphate herbicide glyphosate in arable soils and its relationship to soil phosphorus status. Doctoral Dissertation. Universtiy of Kuopio.

19. Monheit S. 2004. The ecotoxicology of surfactants in glyphosate based herbicides. Noxious Times. 6 (2), 6-12.

20. Giesy, J.P., Dobson, S., Solomon, K. R. 2000. Ecotoxicological risk assessment for Roundup herbicide. Rev. Environ. Contam. Toxicol. 165, 35-120.

21. Eberbach, P. and Bowmer, K. 1995. Conversion of 14C-glyphosate to carbon dioxide by alligator weed. J. Aquat. Plant Manag. 33, 27-29.

22. Relyea, R.A. 2005. The impact of insecticides and herbicides on the biodiversity and reproductivity of aquatic communities. J. Ecol. Appl. 15, 618-627.

23. Borggaard, O.K. and Gimsing, A.L. 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag. Sci. 64, 441-456.

24. Bar-Or, C. Y., Czosnek, H., Koltai, H. 2007. Cross species microarray hybridizations: a developing tool for studying species diversity. Trends Genet. 23, 200-207.

25. Buckley, B.A. 2007. Comparative environmental genomics in non-model species: using heterologous hybridization to DNA-based microarrays. J. Exp. Biol. 209, 1602-1606.

26. Renn, S.C.P., Aubin-Horth, N. and Hofmann, H.A. 2004. Biologically meaningful expression profiling across species using heterologous hybridization to a cDNA microarray. BMC Genomics. 5, 42.

27. Rise, M.L., von Schalburg, K.R., Brown, G.D., Mawer, M.A., Devlin, R.H., Kuipers, N., Busby, M., Beetz-Sargent, M., Alberto, R., Gibbs, A.R. et al. 2004. Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res. 14, 478-490.

28. Gilad, Y., Rifkin, S.A., Bertone, P., Gerstein, M. and White, K.P. 2005. Multi-species microarrays reveal the effect of sequence divergence on gene expression profiles. Genome Res. 15, 674-680.

29. Draghici, S., Khatri, P., Eklund, A.C., Szallasi, Z. 2006. Reliability and reproducibility issues in DNA microarray measurements. Trends Genet. 22, 101–109.

30. Bar-Or, C., Bar-Eyal, M., Gal, T.Z., Kapulnik, Y., Czosnek, H., Koltai, H. 2006. Derivation of species-specific hybridization-like knowledge out of cross-species hybridization results. BMC Genomics. 7, 110.

31. Ji, W., Zhou, W., Gregg, K., Yu, N., Davis, S. and Davis, S. 2004. A method for cross-species gene expression analysis with high-density oligonucleotide arrays. Nucl. Acids Res. doi: 10.1093/nar/guh084.

32. Renn, S.C.P., Aubin-Horth, N. and Hofmann, H. A. 2004. Biologically meaningful expression profiling across species using heterologous hybridization to a cDNA microarray. BMC Genomics doi: 10.1186/1471-2164-5-42.

33. Moore, S., Payton, P., Wright, M., Tanksley, S., Giovannoni, J. 2005. Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. J. Exp. Bot. 56, 2885–2895.

34. Nuzhdin, S.V., Wayne, M.L., Harmon, K.L., MacIntyre, L.M. 2004. Common pattern of evolution of gene expression level and protein sequence in Drosophila. Mol. Biol. Evol. 21, 1308–1317.

35. Donaldson, L. et al. 2005. Construction and validation of a bovine innate immune microarray. BMC Genomics. 6, 135.

36. von Schalburg, K.R., Rise, M.L., Cooper, G.A., Brown, G.D., Gibbs, A.R., Nelson, C.C., Davidson, W.S. and Koop, B.F. 2005. Fish and chips: various methodologies demonstrate utility of a 16,006-gene salmonid microarray. BMC Genomics. 6, 126.

37. Ranz, J.M., Castillo-Davis, C.I., Meiklejohn, C.D. and Hartl, D.L. 2003. Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. Science. 300, 1742-1745.

38. Saetre, P. et al. 2004. From wild wolf to domestic dog: gene expression changes in the brain. Brain Res. Mol. Brain Res. 126, 198–206.

39. Enard, W., Khaltovich, P., Klose, J., Zollner, S., Heisseg, F., Giavalisco, P., Nieselt-Struwe, K., Muchmore, E., Varkl, A., Ravid, R. et al. 2002. Intra- and interspecific variation in primate gene expression patterns. Science. 296, 340-343.

40. Walker, S.J., Wang, Y., Grant, K.A., Chan, F., Hellmann, G.M. 2006. Long versus short oligonucleotide microarrays for the study of gene expression in nonhuman primates. J. Neurosci. Methods. 152, 179–189.

41. Halgren, R.G., Fielden, M.R., Fong, C.J., Zacharewski, T.R. 2001. Assessment of clone identity and sequence fidelity for 1189 IMAGE cDNA clones. Nucleic Acids Res. 29, 582–588.

42. Meiklejohn, C. D., Parsh, J., Ranz, J.M., Hartl, D.L. 2003. Rapid evolution of male-biased gene expression in *Drosophila*. Proc. Natl. Acad. Sci. USA 100, 9894-9899.

43. Khaitovich P. et al. 2004. A neutral model of transcriptome evolution. PLOS Biol. 2, 682-689.

44. Hammond, J.P., Broadley, M.R., Craigon, D.J., Higgins, J., Emmerson, Z.F., Townsend, H.J., White, P.J., May, S.T. 2005. Using genomic DNA-based probe-selection to improve the sensitivity of high-density oligonucleotide arrays when applied to heterologous species. Plant Methods. 1, 10.

45. Moody, D.E., Zou, Z., McIntyre, L. 2002. Cross-species hybridisation of pig RNA to human nylon microarrays. BMC Genomics 3, 27.

46. Huang, G.S., Yang, S-M., Hong, M-Y, Yang, P-C., Liu, Y-C. 2000. Differential gene expression of livers from ApoE deficient mice. Life Sci. 68, 19–28.

47. Fang, H. et al. 2005. Bioinformatics approaches for cross-species liver cancer analysis based on microarray gene expression profiling. BMC Bioinformatics 6 (Suppl. 2), S6.

48. Hughes, T.R. et al 2000. Functional discovery via a compendium of expression profiles. Cell 102:109–126.

49. Ihmels, J., Bergmann, S., Berman, J., Barkai, N. 2005. Comparative gene expression analysis by differential clustering approach: application to the *Candida albicans* transcription program. PLoS Genet. Vol 1 No 3 e39.

50. McCaroll, S.A. et al. 2004. Comparing genomic expression patterns across species identifies shared transcriptional profile in aging. Nat. Genet. 36, 197–204.

51. Sun, Y., Li, H., Liu, Y., Shin, S., Mattson, M.P., Rao, M.S., Zhan, M. 2007. Cross-species transcriptional profiles establish a functional portrait of embryonic stem cells. Genomics. 89, 22-35.

52. Vallée, M., Robert, C., Méthot, S., Palin, M-F., Sirard, M-A. 2006. Cross-species hybridizations on a multi-species cDNA microarray to identify evolutionarily conserved genes expressed in oocytes. BMC Genomics. 7,113.

53. Becher, M., Talke, I.N., Krall, L., Kramer, U. 2004. Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J. 37, 251-268.

54. Davey, M.W., Graham, N.S., Vanholme, B., Swennen, R., May, S.T., Keulemans, J. 2009. Heterologous oligonucleotide microarrays for transcriptomics in a non-model species; a proof-of-concept study of drought stress in *Musa*. BMC Genomics. 10, 436.

55. Sambrook, J., Manniatis, T., Fritsch, E. F., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press. 3rd edition.

56. Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D. 1998. Cluster analysis and display of genomewide expression patterns. Proc. Natl. Acad. Sci. USA. 95,14863-14868.

57. Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers, Comput. Appl. Biosci. 12, 357-358.

58. Gasteiger, E., Gattiker, A., Hoogland C., Ivanyi, I., Appel, R.D., Bairoch, A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucl. Acids Res. 31, 3784-3788.

59. Benovoy, D., Kwan, T., Majewski, J. 2008. Effect of polymorphisms within probe target sequences on oligonucleotide microarray experiments. Nucl. Acids Res. 36, 4417-4423.

60. Geiger, D.R., Kapitan, S.W., Tucci, M.A. 1986. Glyphosate inhibits photosynthesis and allocation of carbon to to starch in sugar beet leaves Plant Physiol. 82, 468-472.

61. Servaites, J.C., Tucci, M.A., Geiger, D.R. 1987. Glyphosate effects on carbon assimilation, Ribulose Bisphosphate Carboxylase activity, and metabolite levels in sugar beet leaves. Plant Physiol. 85, 370-374.

62. Tan, S., Evans, R., Singh, B. 2006. Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. Amino Acids 30, 195–204.

63. Wong, P.K. 2000. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauda* Berb 614. Chemosphere 41, 177–182.

64. Sergiev, I.G., Alexieva, V.S., Ivanov, S.V., Moskova, I.I., Karanov, E.N. 2006 The phenylurea cytokinin 4PU-30 protects maize plants against glyphosate action. Pestic. Biochem. Physiol. 85, 139-146.

65. Amrhein, N., Deus, B., Gehrke, P., Steinrücken, H.C. 1980. The site of the inhibition of the shikimate pathway by glyphosate Plant Physiol. 66, 830–834.

66. Kitchen, L.M., Witt, W.W., Rieck, C.E. 1981. Inhibition of delta-aminolevulinic acid synthesis by glyphosate. Weed Sci. 29, 571-577.

67. Apel, K. and Hirt, H. 2004. REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. Annu. Rev. Plant Biol. 55, 373-399.

68. Moldes, C.A., Medici, L.O., Abraha, O.S., Tsai, S.M., Azevedo, R.A. 2008. Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. Acta. Physiol. Plant. 30, 469–479.

APPENDIX

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

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

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
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
Refrigerator:	+4º, Bosch, TURKEY
Spectrophotometer:	BIO-RAD, SmartSpec™ 3000, USA
	VARIAN, Cary 300 Bio Uvi-visible spec., AUSTRALIA
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

CHAPTER II

CLONING AND FUNCTIONAL ANALYSIS OF *Brachypodium Atg8*, AUTOPHAGY-RELATED GENE, UNDER OSMOTIC/DROUGHT STRESS

ABSTRACT

Autophagy, literally means self eating, is an evolutionary conserved catalytic process for vacuolar or lysosomal degradation of cytoplasmic contents. Double membraned vesicles, called autophagosomes, containing cytoplasmic components are formed and targeted to vacuole or lysosome for degradation upon induction of autophagy. As an evolutionary conserved mechanism, autophagy is widely studied in yeasts, mammals and plants. Among 30 identified autophagy genes, one of the mostly widely studied genes, *Atg8* has been also used for monitoring autophagy in a variety of organisms. In plants, autophagy has been related to abiotic stress factors, including nutrient starvation, oxidative stress, salt stress and osmotic stress. In this study, for the first time to our knowledge, *Atg8* gene was identified in *Brachypodium distachyon* (named as *BdAtg8*). Expression profile of *BdAtg8* was examined in a variety of tissues of different ages and under osmotic stress conditions. Expression level of *BdAtg8* was observed to profoundly increase with osmotic stress treatment, especially in the roots. Further functional analyses of BdATG8 were performed. *BdAtg8* gene was shown to complement atg8Δ::*kan MX* yeast mutants grown under starvation conditions in yeast complementation experiments. Monodansylcadaverine (MDC), a convenient marker to monitor autophagy in plant cells, has been used to observe autophagosomes in *Brachypodium* and autophagy was shown to be constitutively active in *Brachypodium*. Moreover, with MDC staining, autophagy was shown to be more active in plants exposed to osmotic stress in comparison to the plants grown under normal conditions. For further functional analysis, *Brachypodium* ATG8 protein was expressed in yeast and analyzed with western blotting. We conclude that, under osmotic stress conditions, *BdAtg8* gene is required for induction of autophagy in *Brachypodium*.

Keywords autophagy, *Atg8*, *Brachypodium*, osmotic stress, drought

ÖZET

Kendi kendini yeme anlamına da gelen "Otofaji", sitoplazma içeriğinin kofullarda veya lizozomlarda parçalanmasını sağlayan ve de evrimsel olarak korunan katalitik bir süreçtir. Otofajinin indüklenmesiyle, sitoplazmik bileşenleri içeren ve "otofagozom" olarak isimlendirilen çift zarlı vesiküller oluşturulurlar ve parçalanmak üzere koful veya lizozoma yönlendirilirler. Evrimsel olarak korunan bir mekanizma olarak otofaji, maya, memeli ve bitki sistemlerinde yaygın olarak çalışılmaktadır. Tanımlanmış 30 otofaji geni arasında en yaygın olarak çalışılanlardan biri olan *Atg8* geni, bir çok organizmada otofajiyi izlemek için de kullanılmaktadır. Bitkilerde otofaji; besin açlığı, oksidatif stres, tuz stresi ve ozmotik stresi de içeren çeşitli abiyotik stres faktörleriyle ilişkilendirilmiştir. Bu çalışmada, bizim bilgimiz dahilinde ilk defa, *Atg8* geni *Brachypodium distachyon* türünde tanımlanmış ve *BdAtg8* olarak isimlendirilmiştir. *BdAtg8* geninin ifade profili farklı yaşlardaki çeşitli dokularda ve ozmotik stres koşullarında incelenmiştir. Ozmotik stres uygulamasıyla *BdAtg8* geninin ifade seviyesinin özellikle köklerde ciddi miktarda arttığı gözlenmiştir. BdATG8 proteinin fonksiyonel analizleri yürütülmüştür. Maya komplementasyon deneylerinde *BdAtg8* geninin açlık koşullarında büyüyen atg8Δ::*kan MX* maya mutantlarını komplement ettiği gösterilmiştir. Bitki hücrelerinde otofajiyi izlemek için elverişli bir markör olan "monodansylcadaverine" (MDC), *Brachypodium*'da otofagozomları gözlemlemek için kullanılmış ve otofajinin *Brachypodium*'da temel olarak aktif olduğu gösterilmiştir. Ayrıca, MDC boyamasıyla, otofajinin, ozmotik strese maruz kalmış bitkilerde normal koşullarda büyüyen bitkilerdekine göre daha aktif olduğu gösterilmiştir. Fonsiyonel analizlere ilaveten *Brachypodium* ATG8 proteini mayada eksprese edilmiş ve "western blot" tekniği ile analiz edilmiştir. Ozmotik stres koşullarında, *BdAtg8* geninin *Brachypodium*'da otofajinin indüklenmesi için gerekli olduğu sonucuna varılmıştır.

Anahtar kelimeler otofaji, *Atg8*, *Brachypodium*, ozmotik stres, kuraklık

TABLE OF CONTENTS

ABBREVIATIONS

3-MA: 3-methyladenine

AD: Activation domain

API: Aminopeptidase I

Atg: Autophagy related gene

BAC: Bacterial artificial chromosome

BdATG8: *B. distachyon* autophagy related gene 8

BLAST: Basic local alignment search tool

bp: Base pair

cDNA: Complementary DNA

CDS: Coding sequence

CVT: Cytoplasm-to-vacuole transport

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

DO: Drop-out

EST: Expressed sequence tag

GFP: Green fluorescent protein

Gly: Glycine

HA: Hemaglutinin

kDa: Kilodalton

LB: Luria-Bertani

LEU: Leucine

LT Red: LysoTracker Red

Mbp: Mega base pair

mAPI: Mature aminopeptidase I

MDC: Monodansyl cadaverine

miRNA: MicroRNA

mg: Miligram

ml: Mililiter

min: Minute

mRNA: Messenger ribonucleic acid

MS: Murashige& Skoog

NADPH: Nicotinamide adenine dinucleotide phosphate

OD: Optical density

o/n: Over night

PAS: Preautophagosomal structure

PBS: Phosphate buffered saline

PCD: Programmed cell death

PCR: Polymerase chain reaction

PE: Phosphatidylethanolamine

PEG: Polyethylene glycol

prAPI: Precursor aminopeptidase I

Q-RT PCR: Quantitative Real Time PCR

RNA: Ribonucleic acid

RNAi: RNA interference

ROS: Reactive oxgen species

Rpm: Revolution per minute

rRNA: Ribosomal RNA

RT-PCR: Reverse transcriptase polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

μg: Microgram

μl: Microliter

TEM: Transmission electron microscopy

TOR: Target of rapamycin

URA: Uracil

YPD: Yeast extract-peptone-dextrose

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Plants use a variety of mechanisms to cope with biotic and abiotic stress factors. Autophagy is a mechanism utilized by plants to respond to such stress conditions. It has been shown to be active in responses of plants to oxidative stress, salt stress, drought stress and viral infection (Xiong et al., 2007a, 2007b; Liu et al., 2009, Liu et al., 2005). *Arabidopsis*, tobacco and rice are most widely studied plant species for both monitoring and understanding molecular basis of autophagy. *Arabidopsis* has been utilized as a model organism for all flowering plants for decades. However, it does not share biological features in agronomic terms with temperate grasses, since it is a dicot plant. Rice has been offered as a model species for all temperate grass species, including wheat, barley and rye. However, rice harbors more practical limitations for being a convenient model for grasses such that it does not have a rapid life cycle, inbreeding reproductive strategy, simple growth requirements, besides the transformation of rice plants is not easy. *Brachypodium distachyon* (L.) Beauv. is a temperate wild grass with more suitable characteristics for being a model system for all temperate grasses. It has agricultural traits more similar to temperate grasses than *Arabidopsis* and rice (Ozdemir et al., 2008). In addition, it has a small genome, short growth cycle, self-fertility, many diploid accessions, and simple growth requirements (Ozdemir et al., 2008; Ozdemir, 2009). Another important feature of this species is its close relationship to cereals. These characteristics of this species make it a more suitable model system for temperate grass species, especially for crop species. Hence, there is a growing interest for gathering genomics information about *B. distachyon*, including linkage and genetic linkage maps (Garvin et al., 2010), and whole genome sequencing (The International Brachypodium Initiative, 2010). In this study, *B. distachyon* will be extensively studied for understanding responses of cereals to shock drought stress which is one of the major abiotic stress factors limiting crop yields worldwide. Since autophagy has been shown to play roles in responses of other plant species to abiotic stress conditions, like

oxidative stress, salinity, and osmotic stress, the potential role of autophagy process in responses of *B. distachyon* plants to the abiotic stress factors should be investigated. The results of this study will give us a clue about the potential role of autophagy in responses of grass species to drought and pave the way to understand the molecular basis of abiotic stress responses of economically important crop species.

2 OVERVIEW

2.1 General information on *Brachypodium distachyon*

2.1.1 *Brachypodium distachyon* as a model to temperate grasses

Brachypodium distachyon (L.) Beauv, also known as "purple false broom", has recently been introduced as a model species to temperate cereals and forage grasses of high economical value (Draper et al., 2001). Temperate cereals constitute the most valuable source of food for humankind. However, cereals, such as wheat and barley, have huge and complex genomes which limit researches being conducted in genomics and molecular breeding areas. Therefore, there has been an increasing demand for a suitable model to the cereals in plant molecular biology research. *B. distachyon* has several desirable attributes and genome characteristics to be offered as a potential model representative of temperate cereals and forage grasses, which will be discussed here.

2.1.2 Diploid species of *Brachypodium* genus has the simplest genome in grasses

Haploid genome size of *B. distachyon* is around 355 Mbp, which is among the smallest genome size in the Poaceae family. A number ploidy levels for *B. distachyon* is available which has the basic chromosome number of 5 (n=5) (Filiz et al., 2009; Vogel et al., 2009; Filiz et al., 2008). Diploid accesion of *B. distachyon* has 10 chromosomes (2n=10). Small genome size of diploid *B. distachyon* is a valuable attribute for proposal of this species as model representative of cereals such as wheat and barley with huge genome size.

2.1.3 Diploid *B. distachyon* has suitable physical, growth and life cycle characteristics

B. distachyon is a self fertile inbreeding annual with a small size (~20 cm) and lacks seed shattering (Draper et al., 2001). In addition to their small size, diploid *B. distachyon* ecotypes have suitable growth and life cyle characteristics. *B. distachyon* ecotypes, with undemanding growth requirements, can be grown in tissue culture and have short life cycles which range from 11 to 18 weeks (Filiz et al., 2009). Under optimal conditions, they can be grown in a much shorter time, 8 weeks (Vogel et al., 2006). The ease of genetic transformation of *B. distachyon* has been reported in several studies (Christiansen et al., 2005; Vogel et al., 2006)

2.1.4 *Brachypodium* is a more suitable model to cereals in comparison to *Arabidopsis* and rice

Arabidopsis thaliana has been the most widely studied model plant species so far. It possesses several attributes that should be present in a model species: Small size, short life cycle, simple growth requirements which make it suitable for high-throughput screening studies. *Arabidopsis* is also easily transformable with the present transformation methods. Additionally, The *Arabidopsis* Genome Initiative has announced the completion of whole genome sequence of *Arabidopsis* genome, which is stated to be 125 Mbp (The *Arabidopsis* Genome Initiative, 2000). These characteristics of *Arabidopsis* have accelerated the discoveries which contributed a substantial amount of information to cereal genomics studies. However, as a dicot species, it is phylogenetically distant to the Poaceae, which harbours temperate cereals and forage grasses (Keller and Feuillet, 2000). Additionally, most of the essential agronomic traits of cereals are not shared by *Arabidopsis*. Several studies demonstrated that *Arabidopsis* genome could not be used as an "anchor" genome for chromosomal mapping in cereals (Bennetzen et al. 1998; Devos et al., 1999). Under the light of these data, *Oryza sativa* (Rice) as a monocot has been introduced to plant genomics area as the model representative of cereals. It has a number of useful properties to be proposed as a model species, including compact genome (~441 Mbp; Bennett et al., 2000) and complete

genome sequence (Dickson and Cyransoski; 2001). Additionally, rice genetic maps, EST databases and considerable germplasm collections are available. The possibility of the use of rice genome as a reference genome for chromosomal mapping studies in cereals has been investigated in several studies (Gale and Devos, 1998). However, rice is phylogentically distant from the Poaceace, including temprate crops, such as wheat, barley and forage grasses (Kellogg, 2001). Additionally, microsynteny is not always the case while locating genic region in cereal genomes taking rice genetic map as a reference. There are also technical handicaps of rice as a model species, including large pyhical size, long life cycle with demanding growth requirements. Rice also has outbreeding reproductive strategy and is not easily transformed with routine transformation methods. Besides these technical difficulties, several agronomic traits specific to temperate cereals such as resistance to specific pathogens, freezing tolerance, vernalization, perenniality, wear and injury tolerance, sward behaviour, and post-harvest biochemistry of silage are not present in rice. Under the light of such facts, the need for identification of a grass species that posseses the desired attributes to be developed and offered as model representative of cereals is evident. Phylogenetic studies have shown that the genus *Brachypodium* has diverged from ancestral Pooideae subfamily just prior to the radiation of modern "core pooids", including temperate cereals and forage grasses (Shi, 1991; Shi et al., 1993; Catalan et al., 1995, 1997; Catalan and Olmstead, 2000). Screening conserved repetitive DNA sequences in the pursuit of identification of archetypal centrome sequences among *Triticum aestivum* (bread wheat), rice, maize, and *Brachypodium* has been performed using *Brachypodium sylvaticum* (Aragon-Alcaide et al., 1996). Members of the genus *Brachypodium* differs from the other members of Poaceae with their atypical chromosome base number (n=5, 7, 8 or 9) (Shi et al., 1993). Other genome characteristics of *Brachypodium* that make it a suitable model for cereals are its small genome size, low percentage of highly repetitive DNA regions, small 5S rDNA spacer region (150 bp), simple rDNA repeat unit with a low degree of methylation (Shi, 1991; Shi et al., 1993; Catalan et al., 1995).

2.1.5 Advances on *Brachypodium* genomics

The draft genome sequence of *B. distachyon* genotype Bd21 has been completed in 2007, by whole-genome shotgun sequencing project funded by US Department of Energy (DOE). This project was coupled to a project aiming to generate 250.000 ESTs. Data from these projects are available in BrachyBase (http://www.brachybase.org). Data collected in this database will allow the researchers to identify novel genes linked to essential traits in *B. distachyon* and to carry out comparative genomics studies in other grass species, including cereals of high economical value. 8X coverage of new *B. distachyon* sequence is now available to community (The International Brachypodium Initiative, 2010).

Bacterial Artificial Chromosome (BAC) based physical maps of *B. distachyon* are being developed by John Innes Center (Norwich, UK) (Bevan, 2006) and University of California and US Department of Agriculture (USDA) (Luo et al., 2007). It has been proposed that establishment of physical map of *B. distachyon* will accelerate the efforts for developing BAC-based physical map of Chinese Spring bread wheat genome.

Presence and distribution of plant transposable elements have been searched in *B. distachyon* genome (Kalendar and Schulman, 2006). Since transposable elements, especially retrotransposons, constitute the intergenic regions in the huge genomes of cereals, determination of retrotransposon sequences will aid in development of retrotransposon-based molecular markers, like IRAP, REMAP; SSAP, and RBIP markers (Ozdemir et al., 2008). It has been found that there exists a few retrotransposons in the small genome of *B. distachyon*.

MicroRNAs (miRNAs) are small RNAs which are known to play crucial roles in regulation of genes acting in physiological and developmental processes in plants (Bartel and Bartel, 2003; Bartel, 2004; Rhoades et al., 2006). 12 putative *Brachypodium* miRNAs have been recently identified in small RNA transcriptomes obtained by the usage of deep sequencing technology (Wei et al., 2009). In another study, 26 novel *B. distachyon* miRNAs belonging to 19 miRNA families have been predicted in expressed sequence tag (EST) and genomic survey sequence databases using computational

approaches (Unver and Budak, 2009). By high-throughput sequencing of *Brachypodium* small RNA libraries, 27 conserved miRNAs and 129 predicted miRNAs have been identified in response to cold stress. Additionally, 3 conserved and 25 predicted miRNAs have been found to be differentially regulated in response to cold stress (Zhang et al., 2009).

A genetic linkage map of *B. distachyon* genotype Bd21 has also being developed by the International *Brachypodium* Initiative (Bevan et al., 2007). Anchor points on genetic linkage map of *B. distachyon* genotype Bd21 will aid the researchers to link the *Brachypodium* genome to the genomes of important cereals and biofuel crops.

371-kb region in *B. sylvaticum* genome has also been sequenced. Comparison of orthologous regions of *B. sylvaticum* genome with those of wheat and rice has indicated that there exists a perfect macrocolinearity among *Brachypodium* and wheat genomes, whereas this deduction was not valid for the comparison of wheat and rice genomes (Bossolini et al., 2007). Phylogenetically, *Brachypodium* and wheat have been proposed to diverge from each around 35-40 million years ago. However, divergence of wheat and rice has been estimated to be earlier (~50 million years) (Paterson et al., 2004). Microcolinearity has been also shown to be conserved between orthologous regions of wheat and *B. sylvaticum* genomes (Charles et al., 2006; Foote et al., 2004).

All these efforts going on in *Brachypodium* genomics will accelerate gene discovery studies in economically valuable cereals, like wheat, barley, and rye, and hence present novel opportunities for improvement of cereal production. Molecular genetic analyses of this model species have great potential to understand molecular processes occuring in cereals with huge and complex genomes which hinder gene discoveries in those species.

2.2 General introduction to drought stress

World population is increasing at an enormous rate and it is estimated to be around 6 billion by 2050. On the other hand, average yield of crop plants is declined by

limitations in water resources worldwide. It has been estimated that, drought stress and salinity, being the major abiotic stress factors, decline productivity of major crop plants by more than 50 percent (Bray et al., 2000). Plants have developed several strategies to survive and reproduce under these abiotic stress conditions. Understanding the plant tolerance to drought stress is one the major concerns of the scientific community. Primary aim of many plant breeding programs has been the development of drought tolerant crops.

Plant responses to drought can be divided into escape, avoidance, and tolerance strategies (Chaves, et al., 2003). Escape strategies of plants involve shortening life cycle, increasing growth rate, effective use of storage and use of reserves for seed production. Avoidance strategies involve minimizing water loss (stomatal closure, reduction in leaf area, senescence in old leaves) or maximizing water uptake (enhanced root growth). Tolerance to water scarcity involves osmotic adjustments or more rigid cell walls. Plants adjust the osmotic potential decreased by drought via accumulation of osmolytes such as proline, glycine betaine, mannitol, trehalose or myo-inositol (Bartels and Sunkar, 2005; Valliyodan and Nguyen 2006; Barnabas et al., 2008). Drought tolerance has been also related to the reactive oxgen species (ROS) scavenging (Sairam and Saxena, 2000). Enzymes like superoxide dismutase are synthesized and antioxidants like glutathione and ascorbic acid are produced in order to prevent or alleviate the toxic effects of ROS (Shinozaki and Yamaguchi-Shinozaki, 2007). Glutathione S-transferase and superoxide dismutase enzymes, key enzymes in ROS detoxification, have been identified in subtractive cDNA library screening performed in drought-tolerant wild emmer wheat genotype, TR39477, drought-sensitive wild emmer wheat genotype, TTD-22 and modern wheat variety, Kiziltan grown under slow drought conditions (Ergen and Budak, 2009). Comparative transcriptome analysis of drought-tolerant and sensitive wild emmer wheat genotypes grown under shock drought stress using the Affymetrix GeneChip® Wheat Genome Array has showed that antioxidative-enzyme glutathione S-transferase is differentially regulated in drought tolerant wild emmer wheat genotype (Ergen et al., 2009).

When plants are exposed to drought stress, they develop responses at physiological, biochemical and molecular levels. The response to drought stress at cellular level begins with perception of the water deficiency by specific receptors. After

plants sense the presence of water loss, they induce several signal transduction pathways inside the cell. Signaling of water deficient in plants is a complex process. Signal transduction pathways, induced by water deficieny signal, involve several phosphatases and kinases which carry out dephosphorylation and phosphorylation events, respectively (Kaur and Gupta, 2005; Mishra et al., 2006; Ergen et al., 2009). Transcripts coding calcium or calmodulin binding proteins, and protein kinases were found to be similarly regulated in drought responses of wild emmer wheat genotypes and modern wheat cultivar with subtractive cDNA library screening approach. These findings indicated that protein phosphorylation or dephosphorylation and other signal transduction elements are conserved in wild emmer wheat genotypes with differential drought tolerance and modern wheat cultivar (Ergen and Budak, 2009).

Activation of protein kinases and dephosphotases cause further activation of transcription factors (Kaur and Gupta, 2005; Nakashima and Yamaguchi-Shinozaki, 2005). Complex expression patterns of the drought-activated transcription factors are indicators for very intricate regulation of drought stress responses of plants at transcriptional level.

Many genes responsive to drought stress have been identified from different plant species involved in stress response mechanisms (Zhu, 2001; Ergen and Budak, 2009). One of the major consequences of drought stress is the increase in the production of reactive oxygen species (ROS) in plant cells (Tsugane et al., 1999). Autophagy has been suggested to play roles in degradation of oxidized proteins in *Arabidopsis* in response to oxidative stress (Xiong et al., 2007a). However, the potential role of autophagy in responses of plants to drought stress has not been studied extensively. Recently, induction of autophagy by salinity and osmotic stress has been reported in *Arabidopsis* (Liu et al., 2009). Cereals, including wheat, rice and maize are the major components of human diet worldwide. However, the molecular responses of these economically valuable plants to drought stress have not been fully elucidated yet. Investigation of roles of autophagy in *B. distachyon*; a suitable model for cereals, would be beneficial for the studies in the pursuit of enlightnment of complex molecular responses of cereals to drought stress.

Polyethylene glycol (PEG) has become a popular agent for reduction of water potential of nutrient solutions of plants without being taken up by the plants. In other words, PEG stimulates osmotic stress in the growth medium acting as a non-penetrating osmotic agent. It has been widely used to induce water stress in several plants (Kerepesi and Galiba, 2000; Murillo-Amador et al., 2002; Abebe et al., 2003; Liu et al., 2004; Turkan et al., 2005).

2.3 General introduction to plant autophagy

Autophagy (self-eating) is a cellular content degradation process which aims either recycling of cytoplasmic components or elimination of damaged or toxic molecules inside the cell. In autophagy, cytoplasmic components, including long-lived proteins and organelles are taken up either by vacuoles or lysosomes/endosomes and degraded inside these compartments (Klionsky and Ohsumi, 1999; Klionsky, 2005). Autophagy is an evolutionarily conserved mechanism among mammalian, yeast and plant cells (Bassham, 2009; Mitou et al., 2009).

Plant cells have two different types of vacuoles with different fuctions: storage and lytic vacuoles. Autophagy takes place in lytic vacuoles inside plant cells, corresponding to lysosomes where autophagy takes place in mammalians and vacuoles where autophagy takes place in yeast. When plants are exposed to adverse environmental conditions, they develop responses to cope with such stress conditions and survive. One of the major processes exploited by plant cells for this purpose is autophagy. Stress conditions inducing autophagy include sucrose, nitrogen and carbon starvation, oxidative stress, drought and salt stress, and pathogen infection (Aubert et al., 1996; Bassham et al., 2006; Rose et al., 2006; Xiong et al, 2007a, 2007b; Shin et al., 2009, Liu et al., 2009). Autophagy is also constitutively active during several developmental processes such as vacuole biogenesis in plant cells, senescence and innate immune response (Yano et al, 2007; Thompson and Vierstra, 2005; Seay et al, 2006; Yoshimoto et al., 2009).

2.3.1 Autophagy pathways in plants

There are two major autophagy pathways in plant cells: macroautophagy and microautophagy. This classification is based on the mechanism of transport and degradation of cytosolic constituents. In macroautophagy, in addition to cytoplasmic portions, organelles such as mitochondria, peroxisome, plastid, endoplasmic reticulum and golgi stacks are sequestered into double-membrane bounded vesicles called "autophagosomes" or "autophagic vesicles". Cellular content to be degraded is then released into vacuoles upon fusion of outer membranes of autophagosomes to tonoplast. The cytoplasmic content to be degraded is surrounded by inner membrane inside the vacuole and called "autophagic body". Vacuolar hydrolases degrade these bodies and the degradation products are recycled back to cytoplasm. Macroautophagy has been shown to be the major process for cytoplasmic content degradation during starvation and senescence conditions in several plant species. Sucrose starvation has been reported to induce autophagy in rice (Chen et al., 1994), sycamore (Aubert et al., 1996), and tobacco-cultured cells (Moriyasu and Hillmer, 2000). Carbon starvation also has been shown to induce autophagy in maize plants (Brouquisse et al., 1998).

In microautophagy, cytoplasmic content to be degraded is directly engulfed by vacuole and degraded after disintegration of membrane (Mortimore et al., 1988; Dunn et al., 2005). Microautophagy has been observed in wheat plants during the accumulation of storage proteins in seeds (Levanony et al., 1992; Shy et al., 2001). Microautophagy has been also shown to be active in cotyledon cells of vigna mungo seedlings for degradation of starch granules and other cellular components (Toyooka et al., 2001).

Autophagy pathways differ in different plant species (Figure 2.1). In *Arabidopsis*, outer membranes of autophagosomes fuse with tonoplast and autophagic bodies are released into the vacuole for subsequent degradation by vacuolar hydrolases. However, in tobacco cells, autophagy occurs in two different patways. Either the autophagosomes are directed to vacuoles or can fuse with lysosomes/endosomes for subsequent degradation of their contents. When cysteine protease inhibitor, E64 was used to inhibit autophagy, autophagic bodies accumulated inside vacuoles in *Arabidopsis* cells (Bassham, 2007). Growth of tobacco cells in the presence of E64 lead to accumulation

of autophagic bodies in smaller organelles called autolysosomes outside the vacuoles (Inoue et al, 2006).
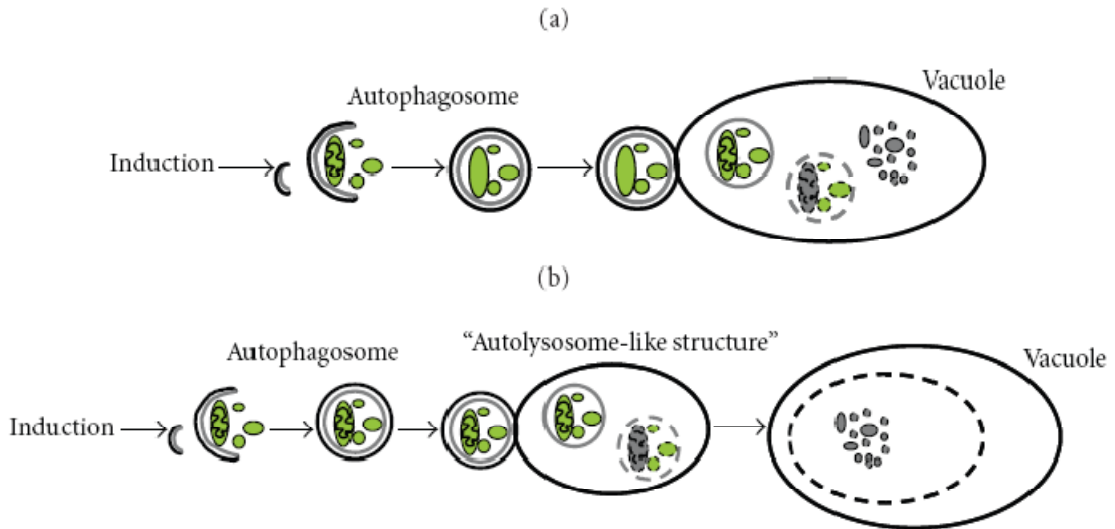
Figure 2.1 Different autophagy pathways inside plant cells (Modified from Mitou et al., 2009)

2.3.2 Autophagy machinery in plants

With the advances in yeast genetics, proteins having roles in autophagy mechanism have been identified. A group of autophagy defective yeast mutants have been isolated as a result of extensive studies on yeast autophagy (Harding et al., 1995; Thumm et al., 1994; Tsukada and Ohsumi, 1993; Wang and Klionsky 2003; Noda et al., 2002). Study of these mutants contributed to understanding of molecular mechanism of autophagy (Klionsky et al., 2003). Until now, ~30 autophagy related genes (nomenclatured as *Atg*) have been identified in yeast investigating these mutants (Klionsky et al, 2003). Conservation of autophagy pathways across species helped researchers to identify orthologs of yeast *Atg* genes in plants. Based on sequence similarity, homologs of yeast proteins have been identified in *Arabidopsis*. Knockout mutants of *Atg* genes have been studied in *Arabidopsis* and showed increased sensitivity to nitrogen deficiency and displayed early senescence symptoms (Doelling et al., 2002; Hanaoka et al., 2002; Surpin et al., 2003). In *Arabidopsis*, 25 *Atg* genes which are orthologs of 12 of yeast *Atg* genes were identified (Hanaoka et al., 2002; Doelling et al.,

2002, Yoshimoto et al., 2004; Thompson et al., 2005). Different from yeast, many *Arabidopsis* ATG proteins are encoded by small gene families (Doelling et al., 2002; Hanaoka et al., 2002). Residual differences are present within each ATG protein family in terms of aminoacid sequence. These findings suggest that autophagy mechanism is more complex in plant systems in comparison to yeast and ATG isoforms might have distinct expression patterns and functions.

Functional domains are very well conserved in corresponding ATG proteins in plants, suggesting that autophagy takes place in a similar fashion to mammalian and yeast autophagy in plants from mechanistic point of view. Autophagy mechanism can be divided into five phases: induction, nucleation, vesicle expansion and completion, fusion and degradation, and recycling (Thompson and Vierstra, 2005).

2.3.2.1 Induction

In this phase of autophagy, signaling pathways acting upstream of autophagy machinery induce autophagy. Target of rapamycin (TOR) protein (a serine/threonine kinase) is stated to be the key protein acting in this phase and downregulation of this protein has been shown to stimulate autophagy (Noda and Ohsumi, 1998). Under nutrient rich growth conditions, TOR protein is active and hyperphosphorylates ATG1 and ATG13 proteins. Upon hyperphosphorylation, ATG1-ATG13 protein complex is dissociated and became inactive. However, under starvation conditions, ATG13 is dephosphorylated and ATG1-ATG13 complex is reactivated with increasing affinity of ATG13 for ATG1. Reformation of the complex leads to autophosphorylation of ATG1 resulting in induction of autophagy (Matsuura et al., 1997; Kamada et al., 2000; Abeliovich et al., 2003). TOR protein is conserved in plants. Recently, a gene that is homologous to *TOR* in yeast and mammals has been discovered in *Arabidopsis* (Menand et al., 2002). This discovery supported the assumption that TOR pathway exists in plants. However, there is still little evidence for direct relation of TOR pathway existing in plants to autophagy. Only evidence for action of TOR on autophagy in photosynthetic organisms comes from a study on green algae, *Chlamydomonas reinhardtii*. Rapamycin (inhibitor of TOR) was found to inhibit growth of this

organism, proposed to be due to inhibition of TOR pathway (Crespo et al., 2005). Additionally, localization studies indicated that TOR localizes to endoplasmic reticulum or other microsomal structures. This finding also confirmed the view that TOR plays a role in autophagy induction (Diaz-Troya et al., 2008).

2.3.2.2 Nucleation

Building up of autophagomal membranes starts at autophagy organization site named as preautophagosomal structure (PAS). However, the lipid donors of the autophagosomal membranes could not been clarified yet. PAS is located next to the vacuole in yeast. A protein complex involving VPS34, a class III phosphatidylinositol 3-OH kinase (PI3K), and Atg6/Vps30 plays a role in initiation of nucleation. Atg6 containing complex together with other regulatory proteins regulates VPS34 protein activity, which is accumulation of phopshatidyl inositol 3-phosphate (PI3P). Accumulation of this molecule induces recruitment of proteins such as Atg18 and Atg2 to PAS for activation of autophagosome formation (Kihara et al. 2001; Xie and Klionsky 2007).

2.3.2.3 Vesicle expansion and completion

There are two major protein conjugation systems which are offered to function in autophagy of yeast, plant and mammals: Atg8 and Atg12 conjugation systems (Mizushima et al., 1998; Ichimura et al., 2000). Both systems are similar to ubiquitin-conjugation system. In the first system, ATG12 (ubiquitin-like protein) is conjugated to ATG5 protein. C-terminal glycine residue of ATG12 is conjugated to Lys-149 of ATG5 by sequential action of ATG7 (E1[2]-like enzyme) and ATG10 (E2-like enzyme) via an isopeptide bond. This conjugate is further linked to ATG16 to form ATG12-ATG5-ATG16 complex (Mizushima et al. 1999; Suzuki et al. 2001; Kuma et al. 2002). Assembly of this complex precedes ATG8 processing and ATG8-PE conjugate formation and might enhance lipidation of ATG8. With concerted action of both

conjugation systems and remaining ATG components, autophagic bodies are deposited in vacuoles in yeast and plants.

In the second conjugation system, ATG8 (a ubiquitin fold protein) is primarily processed by the cysteine protease, ATG4 (Kirisako et al., 2000). C-terminal arginine residue of ATG8 is removed and a glycine residue is exposed. ATG7 (E1-like enzyme) and ATG3 (E2-like enzyme) catalyze the conjugation of phosphatidylethanolamine (PE), a lipid moiety, to the C-terminal glycine residue of ATG8 via an amide bond, in a sequential manner (Klionsky et al., 2003). *Arabidopsis* was shown to possess the genes encoding for the proteins functioning in Atg8 conjugation system. Two different forms of ATG8 proteins were identified in *Arabidopsis*, namely, form I and form II. Faster migration of form II than form I in SDS-PAGE suggested that form II is the PE-conjugated form of ATG8 (Yoshimoto et al., 2004). *AtATG4* double mutants, *AtATG5* and *AtATG7* single mutants of *Arabidopsis* showed enhanced chlorosis, accelerated bolting, enhanced dark-induced senescence of detached leaves, and reduced seed yield under starvation conditions (Thompson, 2005). The *AtATG7^{C558S}* mutant showed hypersensitive phenotype to starvation conditions and premature leaf senescence (Doelling et al., 2002). Autophagosomes were not detected in *AtATG4* double mutants and AtATG8s were not delivered to the vacuole under nitrogen deficiency conditions (Yoshimoto et al., 2004) *AtAtg* genes encoding for Atg8 conjugation system proteins were reported to be upregulated transiently by sucrose deficiency (Rose et al., 2006). Atg8 conjugation system has also been identified in other plant species. *OsAtg8* and *OsAtg4* genes have been identified in rice (Wei et al., 2006). This study suggested that Atg8 conjugation system is conserved in rice too. In yeast, the ATG8-PE conjugate binds to the autophagic membrane via the lipid moiety and appears to help membrane expansion during vesicle formation (Kirisako et al. 1999). However, the processes acting downstream of the conjugate formation has not been clarified yet, in plants. Results of a recent study by Ketelaar et al. (2004) offered a relation between ATG-PE conjugate and microtubules in *Arabidopsis*. It has been postulated that ATG-PE conjugate functions in transport of autophagosomes upon interacting with microtubules.

2.3.2.4 Fusion

In yeast, a vesicular trafficking system is in charge of docking of autophagosomes to tonoplast. This system is comprised of proteins such as vesicle-soluble n-ethylmaleimide-sensitive factor adaptor protein receptor (v-SNARE) VTI1, the VAM3 syntaxin, and YKT7, a small GTP-binding protein of the Rab family (Fischer von Mollard and Stevens, 1999). Orthologs of VTI1 were found in *Arabidopsis* with similar functions (Sanmartin et al., 2007), and VTI12 is suggested to be the ortholog functioning in autophagy. Alterations in morphology of vacuoles were observed in *vti12* mutants, suggesting a role for VTI12 in the formation of autophagosomes and their fusion to tonoplast (Surpin et al., 2003).

In mammals, autophagosomes can fuse with lysosomes/endosomes for subsequent degradation of their contents. A Vps complex and Rab GTPases play roles in this phase. It has been postulated that formation of SNARE protein complex is the next step (Darsow et al., 1997) This complex then acts as a connector between lysosome/endosome and vacuole (Ungermann and Langosch, 2005). Presence of fusion with lysosomes before targeting to vacuole in tobacco cells offered that there might be a similar fusion mechanism in plant systems from molecular point of view (Inoue et al., 2006).

2.3.2.5 Degradation and recycling

After fusion of autophagosome outer membrane to the lysosomes or vacuoles, lipases such as ATG15 catalyzes degradation of the autophagic body membrane. Degradation of contents of autophagosomes by lytic enzymes is the next step (Kim et al., 2007). Plant vacuoles contain various enzymes for degradation of autophagic body including proteases and peptidases, nucleases and gluconases (Marty, 1999). One of the proteases active in this phase is vacuolar processing enzyme (VPE)-γ. A recent study showed that this enzyme is synthesized in an inactive form and it should be translocated to vacuole for further maturation possibly via a cytoplasm-to-vacuole (CVT) pathway

(Rojo et al., 2004). Similar to PEP4 in yeast, it might aid in proteolytic processing of other hydrolases which play roles in degradation of other constituents in autophagic bodies.

Building blocks of degraded components are recycled to cytosol for future use. Lysosome associated membrane proteins LAMP-1 and LAMP-2 are implicated in this phase (Eskelinen 2006).

Some autophagy proteins in plants are not specific for autophagy pathway and have more broad functions. For instance, AtATG6 protein has a role in vacuolar trafficking and *Arabidopsis AtATG6* knockout mutant was found to be defective in pollen tube germination (Qin et al., 2007; Fujiki et al., 2007).

2.3.3 Roles of autophagy in plants

Autophagy is suggested to play a variety of roles in plant metabolism and development. These include seed development, vacuole biogenesis, nutrient recycling during starvation conditions, senescence, apoptotic processes, hypersensitive response of plants to pathogen infection, and responses of plants to abiotic stress conditions (Thompson and Vierstra, 2005; Xiong et al., 2007a, 2007b; Liu et al., 2009).

2.3.3.1 Autophagy in plant development

Early studies suggested that autophagy does not play a significant role in plant developmental processes, since *Arabidopsis ATG* mutants did not show developmental phenotypes. However, recent studies suggested role for autophagy in development of several plant species. For instance, results of a recent study indicated that autophagy plays a role in seed development in wheat (Ghiglione et al., 2008). Number of fertile florets decreases in wheat plants grown under long-day conditions. Analysis of those aborted florets with electron microscopy indicated that there were autophagosomes in enlarged vacuoles. Supporting evidence came from expression studies which

demonstrated that expression levels of several ATG genes, in concert with some proteases and cell death-associated genes were higher in aborted florets. All these results indicated that autophagy takes place in aborted florets.

Autophagy also has a role during vacuole biogenesis. In a recent study by Yano et al. (2007), it has been postulated that the formation of vacuoles from tobacco BY-2 protoplasts involves an autophagy like process. Newly formed vacuoles were found to possess cytoplasm in the presence of cysteine protease inhibitor which indicated that vacuole reformation depends on autophagy. However, this process could not be inhibited via autophagy inhibitors, 3-methyladenine (3-MA) and wortmannin, suggesting that autophagy taking place during vacuole reformation differs from constitutive autophagy taking place under normal conditions and autophagy induced by stress conditions.

2.3.3.2 Autophagy as a response to abiotic and biotic stress

Autophagy has been shown to be induced by a variety of stress conditions in plants. Plants utilize autophagy to recycle nutrients during nitrogen and carbon deficiency (Hanaoka et al. 2002; Surpin et al. 2003; Yoshimoto et al. 2004; Xiong et al. 2005; Fujiki et al. 2007; Qin et al. 2007). In addition to the role of autophagy in nutrient recycling, plants also utilize this process to respond to both biotic and abiotic stress factors. Induction of oxidative stress via H_2O_2 addition or methyl violagen application led to development of a rapid and strong autophagy response in *Arabidopsis* seedlings (Xiong et al., 2007a). Transgenic *Arabidopsis* plants, whose *AtAtg18a* gene was silenced, were found to be defective in autophagosome formation and displayed an enhanced sensitivity to oxidative stress. Since oxidized proteins could not be degraded via autophagy pathway, they accumulated inside the cells of transgenic plants. These data suggested that autophagy is essential for degradation of damaged proteins inside the cells. When concanamycin A was used to inhibit vacuolar degradation in *Arabidopsis* plants, it was shown that oxidized proteins accumulated in wild-type plants. However, oxidized proteins could not be transferred to vacuole for degradation and stayed in cytoplasm of transgenic plants. This result supported the view that oxidized

proteins are transferred to vacuole for degradation in the presence of oxidative stress. However, the role of autophagy in degrading oxidized proteins during abiotic stress conditions remains to be elucidated. In a recent study, it has been postulated that autophagy is induced by high salinity and drought (Liu et al., 2009) in *Arabidopsis*. *Arabidopsis* plants having silenced *ATG18a* gene were shown to be more sensitive to salt and osmotic treatments. Retardation in growth of RNAi-*ATG18a* plants was greater than wild type plants after NaCl and mannitol treatments. Silenced plants were more chlorotic in comparison to wild type plants, which were still green, after salt and mannitol treatment. Chlorophyll and anthocyanin contents were higher in RNAi-*ATG18a* plants than wild type plants, after salt and mannitol treatments, respectively. The results of the same study suggested that autophagy is differentially regulated in responses of plants to salt and osmotic stress. Autophagy was found to be regulated by NADPH-oxidase-dependent pathway in response to salt stress. However, regulation of autophagy in osmotic stressed plants was found to be independent of NADPH-pathway. All these results indicated that autophagy is involved in responses of plants to salt and osmotic stress. However, exact function of autophagy in salt and and osmotic stress responses of plants should be further investigated.

Autophagy plays a role in response of plants to pathogen infection. From the study of tobacco *atg6* (also called *BECLIN1*) mutant plants infected with tobacco mosaic virus, it was found that autophagy has a role in restricting programmed cel death (PCD) triggered by hypersensitive response close to the site of pathogen infection (Liu et al., 2005). *atg6* mutants showed enhanced senescence phenotype, similarly to other autophagy mutants. Additionally, tobacco *BECLIN1* gene was shown to complement yeast *atg6* mutants, implementing a role for tobacco *BECLIN1* gene in autophagy. *atg6* mutants having a silenced *BECLIN1* gene were found to possess expanded lesions throughout the plant, even on the uninfected upper leaves. This observation suggested an important role for BECLIN1 for restriction of PCD to the pathogen infection site. However, the mechanism by which autophagy restricts spread of cell death beyond infection site still remains to be elucidated.

2.3.3.3 Autophagy as a programmed cell death mechanism

Although, autophagy seems to contribute to survival of cells during abiotic stress conditions, autophagy also acts in PCD in plant cells. Since plants have a rigid cell wall, apoptosis is not the mechanism utilized by plants to degrade cellular components before cell death. During PCD in plants, vacuole and cell size increase, organelles are taken up by vacuole and subsequently degraded, and finally vacuole lyses resulting in cell death. These events overlap with the major characteristics of autophagy in plants. These events were observed by electron microscopic analysis of cells of soybean and foxglove nectaries (Horner et al., 2003; Gaffal et al., 2007) in response to termination of nectar production.

2.3.3.4 Constitutive autophagy in plants

In addition to the role of autophagy in responses of plants to both abiotic and biotic stress factors, there exists constitutive autophagy in plants grown under normal conditions. As a result of incubation of *Arabidopsis* or barley roots in nutrient rich media containing protease inhibitor, E64d (to be able to detect accumulation of autophagic bodies), accumulation of cytoplasmic inclusions inside vacuoles of meristematic cells could be detected. This was an indication for constitutive autophagy taking place under nutrient-rich conditions (Moriyasu et al., 2003). Incubation of *Arabidopsis* and barley root tips in a growth medium containing autophagy inhibitor, 3-MA led to partial inhibition of accumulation of cytoplasmic components inside vacuole (Inoue et al., 2006). Expression of GFP (green fluorescent protein) fusion construct of *Atg8f*, autophagy marker in *Arabidopsis* plants resulted in accumulation of the fusion protein inside vacuole under normal conditions and this fusion protein was detected in autophagic bodies in the presence of concanamycin A, blocker of vacuolar degradation (Slavikova et al., 2005) The role of constitutive autophagy in degradation of damaged or oxidized molecules was confirmed by the study of *Arabidopsis* mutants, *AtAtg18a*. These mutants were found to produce greater amounts of oxidized proteins and lipids in comparison to wild-type plants. Plants can generate reactive oxygen species through

photosynthetic electron transport chain and substantial amount of oxidative damage can take place under normal conditions. Increased amount of oxidized protein and lipid generation in *Atg18a* silenced plants indicates the important role of autophagy in degradation of oxidized molecules inside plant cells (Xiong et al., 2007b).

2.3.4 Monitoring plant autophagy

To detect autophagy in plant cells, a variety of techniques are utilized. These techniques include the use of earliest tools such as transmission electron microscopy (TEM) and the novel molecular tools which have been introduced recently. Use of these tools for monitoring autophagy in plant systems will be explained below.

2.3.4.1 GFP-Atg8 dot formation

Markers have been developed to specifically label autophagosomes. Fusion of GFP with *Atg8*, autophagy marker gene has being used by many researchers to label autophagosomes. GFP-ATG8 fusion protein was expressed in *Arabidopsis* root cells and observed by confocal laser scanning microscopy. Fusion proteins were observed as many ring-shaped and punctuate structures corresponding to autophagosomes and intermediates, respectively in wild-type *Arabidopsis* root cells under normal growth conditions. These structures could not been observed in *Atatg4a4b-1* double-mutant plants expressing GFP-AtATG8, since autophagy is defective in these mutants. Fusion protein was also detected in vacuolar lumen of root cells under nitrogen starvation conditions. However, fusion protein could not be delivered to vacuoles in *Atatg4a4b-1* double-mutants. These results indicated that ATG4 is essential for processing and possibly conjugation of ATG8 to PE, and GFP: AtATG8 is a convenient marker for monitoring autophagy in plant cells (Yoshimoto et al., 2004). In another study, accumulation of GFP-ATG8 labelled autophagic bodies has been demonstrated to be enhanced after nitrogen starvation in *Arabidopsis* wild type cells, but blocked in *Arabidopsis atg7-1* mutants (Thompson et al., 2005). In *Arabidopsis atg7-1* mutants,

punctuate structures representing GFP-ATG8 labelled autophagic vesicles could not be detected after nitrogen starvation concomitantly performed with concanamycin A treatment, by fluorescence confocal microscopy. In a similar study, *atg5-1* and *atg10-1* mutants have been shown to fail accumulation of ATG8-GFP labelled autophagic vesicles after nitrogen starvation and concurrent concanamycin A treatment. GFP signal was detected as a diffused signal in cytoplasm in hypocotyl cells of mutant *Arabidopsis* plants whereas GFP-ATG8 labelled vesicles appeared as punctuate structures in nitrogen starved and concanamycin A treated wild type *Arabidopsis* cells (Phillips et al., 2008). These results confirmed the assumption that GFP-ATG8 labelled vesicles were actually autophagic vesicles and, hence can be used to monitor autophagy in plant systems.

2.3.4.2 Electron microscopy

TEM is one of the first tools utilized to monitor autophagy in cells (Ashford and Porter, 1962). Although TEM is offered as the most reliable tool to monitor autophagy, special precaution is required for interpretation of TEM data. Use of TEM to detect autophagic bodies in plant cells has been reported in several studies (Liu et al., 2005; Rose et al., 2006; Ghiglione et al., 2008). In one study, it has been postulated that autophagosomes and autolysosomes fusing with vacuoles were observed in uninfected tissue surrounding hypersensitive response programmed cell death area of *Nicotiana benthamiana* using electron microscopy (Liu et al., 2005). In another study, electron microscopy has been used to detect autophagy in *Arabidopsis* cells. Electron micrographs of cells of *Arabidopsis* plants grown in sucrose replete and sucrose starved media showed that the number of autophagic vesicles was greater in cells of sucrose starved plants in comparison to the ones grown in complete medium (Rose et al., 2006). In another recent study, autophagic vesicles in aborted floret cells of wheat have been observed by TEM (Ghiglione et al., 2008). Double membraned dense globular bodies have been detected in cells of aborted florets under electron microscopy, suggesting early phase of autophagy. However, it was also shown that when these bodies moved towards the tonoplast and fused with it, dense bodies were observed as single membraned or no membrane surrounded indicating later phases of autophagy. All these

results indicated that TEM is a reliable and successful tool to monitor autophagy in plant cells.

2.3.4.3 Visualization of autophagy by fluorescent dyes

Autophagosomes can also be labeled with the acidotrophic fluorescent dyes, LysoTracker Red (Moriyasu et al., 2003) and monodansylcadaverine (MDC) (Contento et al., 2005).

2.3.4.3.1 LysoTracker Red

Weakly basic amines have the ability to accumulate in acidic compartments inside the cell. LysoTracker Red (LT Red) is an acidotrophic fluorecent dye, which has a fluorophore conjugated to a weak base in its structure. In previous studies, LT Red has been shown to be concentrated in mammalian lysosomes, which have low pH (Lepperdinger et al., 1998; Tarasova et al., 1997). This property of this dye makes it a convenient probe to label acidic organelles, like lysosome and vacuole and hence, detect autophagy inside a cell. In one study, LT red staining was performed in order to visualize autolysosomes accumulating in tobacco cells in the presence of sucrose starvation (Moriyasu et al., 2003). In the same study, LT red has been shown to be concentrated in several cytoplasmic organelles in sucrose starved and E-64d-treated barley root tip cells, which indicated the presence of active autophagy in these cells. In another study, occurance of autophagy in *Arabidopsis* root tip cells has been confirmed by LT Red staining (Inoue et al., 2006).

2.3.4.3.2 Monodansylcadaverine

Monodansylcadaverine (MDC) is a fluorescent dye that is extensively used to monitor autophagy in plant systems. It is an amine which has the capability to pass through membranes and accumulate in acidic organelles (Biederbick et al. 1995). It also

has the ability to interact with lipid-rich compartments (Niemann et al., 2000). In a previous study, MDC staining has been utilized to label autophagosomes in *Arabidopsis* suspension culture cells in the presence and absence of sucrose starvation (Contento et al., 2005). The number of stained moving, spherical structures has been shown to be greater in sucrose starved suspension culture cells in comparison to non-starved cells. The results of this study suggested that MDC stains autophagosomes in *Arabidopsis*. Specificity of MDC staining of autophagosomes was further confirmed by the results of a study by Contento et al. (2005). In the same study, sucrose starved *Arabidopsis* protoplasts expressing GFP-*AtAtg8e* were stained with MDC. Colocalization of GFP-*AtAtg8e* and MDC in sucrose starved *Arabidopsis* protoplasts indicated that MDC stained vesicles were likely to be autophagosomes. In another study by Xiong et al. (2007a), MDC staining of autophagic vesicles has been performed in order detect autophagy induced for degradation of oxidized proteins during oxidative stress in *Arabidopsis*. A study by Patel et al. (2008) also reported the use of MDC to stain autophagosomes in *Arabidopsis* root cells. MDC-stained punctuate structures were observed both in the cytoplasm of nitrogen and carbon starved wild type *Arabidopsis* root cells. However, very few MDC stained structures were detected in the starved root cells of *AtAtg6-AS* plants. These results indicated that *AtAtg6* is required for induction of autophagy in *Arabidopsis*. MDC staining of autophagosomes was also utilized to detect autophagy in rice cells. The number of MDC-stained punctuate structures has been found to be lower in *OsAtg10b* mutant cells when compared to wild-type plants after induction of oxidative stress via methyl viologen (Shin et al. 2009). In another study, MDC staining of autophagosomes has been performed in order to show that AtATG18a is required induction of autophagy by salt and osmotic stress. In RNAi-*AtAtg18a* plants grown in MS and treated with NaCl and mannitol to induce salt stress and osmotic stress respectively, the number of MDC stained autophagosomes reduced significantly, in comparison to wild type plants grown under normal conditions. These results indicated that salt and osmotic stress induced autophagy also requires AtATG18a (Liu et al., 2009).

2.3.4.4 Use of inhibitors to monitor autophagy

Since the degradation of cytoplasmic components is very rapid after fusion of autophagosomes to vacuole, cysteine protease inhibitors like E64c are used to inhibit vacuolar degradation of autophagic bodies. Accumulation of autophagosomes could be detected by light microscopy in tobacco cell after treatment with E64c (Moriyasu and Ohsumi, 1996). Similar to tobacco cells, accumulation of autolysosomes has been detected in E64d treated barley root tip cells (Moriyasu et al., 2003). The results of this study suggested that E64d stabilized autolysosome membrane and allowed concentration of acidotrophic dye, LT Red, in acidic compartments inside the cells. In another study, E64c treatment resulted in detection of higher fluorescence in autolysosomes in addition to the fluorescence in central vacuoles of tobacco cells (Yano et al., 2004). In a study by Inoue et al. (2006), it has been shown that E64d treatment allowed the accumulation of parts of cytoplasm in vacuoles of *Arabidopsis* root tip cells. Same treatment led to the accumulation of parts of cytoplasm in autolysosomes and pre-existing central vacuoles in barley root tip cells.

Bafilomycin A_1 and concanamycin A are inhibitors of vacuolar hydrolases since they can inhibit V type ATPases. These inhibitors are known to increase vacuolar pH and under such high pH conditions, vacuolar hydrolases can not function. Consequently, autophagic bodies accumulate in vacuoles. Autophagy induced by starvation in BY-2 cells can be inhibited by bafilomycin A_1 and concanamycin A. Autophagic bodies have been shown to accumulate in BY-2 cells treated with such inhibitors (Robinson et al., 2004). Similarly, treatment of *Arabidopsis* roots with concanamycin A lead to accumulation of spherical bodies in vacuolar lumen, which correspond to autophagic bodies. These structures could be observed with conventional light microscopy. When vacuolar lumen of these root cells were observed with electron microscopy, these spherical bodies were found to possess cytoplasmic structures such as endoplasmic reticulum, golgi body and mitochodria indicating that these spherical structures are autophagic bodies (Yoshimoto et al., 2004). In another study, concanamycin A treatment led to an increase in GFP fluorescence in *GFP-AtAtg8-HA* expressing *Arabidopsis* root tip cells. Additionally, a large number of fluorescing spots were also observed in vacuoles of these cells, indicating that AtATG8 containing structures are

succesfully internalized into the vacuoles in concanamycin A treated *Arabidopsis* root tip cells (Slavikova et al., 2005). In a study by Thompson et al. (2005), accumulation of autophagic bodies has been observed inside the vacuoles of nitrogen starved *Arabidopsis* cells using *GFP-Atg8a* fusion in combination with concanamycin A treatment.

Recently, 3-MA has been suggested to block autophagy in cultured tobacco cells under starvation conditions (Takatsuka et al., 2004; Inoue and Moriyasu, 2006). In another study, it has been shown that 3-MA treatment blocks autophagy by inhibiting accumulation of cytoplasm parts in vacuoles of *Arabidopsis* root tip cells (Inoue et al., 2006). In summary, treatment of cells with these inhibitors is an easy way to monitor autophagy in plant cells.

2.3.4.5 ATG5/ATG12 conjugate as a marker of autophagy

Usage of anti-AtATG12b antibodies that recognize both AtATG12a and AtATG12b, and hence AtATG12-AtATG5 conjugate is another way to detect occurance of autophagy in plant cells. Conjugates could be detected in wild-type and AtATG10/*Atatg10-1* (heterozygous) plants. However, they could not be detected in *Atatg5-1* and *Atatg10-1* mutant plants, since AtATG5 and AtATG10 are involved in AtATG12/AtATG5 conjugate formation. Conjugate was detected in *Atatg4a4b-1* double mutants, since AtATG4 does not function in formation of this conjugate. Treatment of wild-type and AtATG10/Atatg10-1 (heterozygous) roots with concanamycin A led to detection of autophagic bodied in vacuoles. However, autophagic bodies could not been detected in *Atatg5-1* and *Atatg10-1* roots treated with concanamycin A, suggesting that formation of AtATG5/AtATG12 conjugate is necessary for autophagy (Suzuki et al., 2005; Thompson et al., 2005). In summary, ATG5/ATG12 conjugate can be used as an autophagy marker in plant

2.3.4.6 Test of aminopeptidase I maturation

Cellular processes in yeast might also help understand the autophagy in plants. *S. cerevisiae* has evolved a mechanim similar to autophagy to constitutively deliver functional vacuolar proteins to lumen. This pathway is called CVT and has been proposed to play role in import of functional vacuolar proteins that can not cross the tonoplast (Thompson and Vierstra, 2005). One of the proteins in yeast, which is synthesized in cytosol as a precursor enzyme (prAPI, 61kDa) and targeted to vacuole for maturation via CVT pathway, is aminopeptidase I. Only under starvation conditions, prAPI is targeted to vacuole for conversion into mature API (mAPI, 50 kDa) (Klionsky and Ohsumi, 1999). It has been also suggested that mAPI appears in the vacuole only when autophagic pathway is functional (Ketelaar et al., 2004). Test of API maturation has been utilized in several complementation studies of yeast mutants with plant *Atg* genes for assaying functionality of these genes in yeast (Hanaoka et al., 2002; Ketelaar et al., 2004).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

In the experiments, *B. distachyon* genotype, Bd21 was used (Filiz et al., 2009).

3.1.2 Yeast strain and plasmid

Yeast *Atg8* mutant strain (BY4741, atg8Δ::*kanMX, MATa; his3Δ1; leu2Δ0; met15Δ0; ura3ΔO*) and pRS316 plasmid with yeast *Atg8* gene used in this study was kindly provided by Nakatogawa lab.

3.1.3 Antibodies

Polyclonal anti-API antibody used in this study was a kind gift of Klionsky lab.

3.1.4 Chemicals

All chemicals were obtained from Merck (Germany), SIGMA (US), Fluka (Switzerland), and Riedel de Häen (Germany).

3.1.5 Growth Media, Buffers and Solutions

The growth media, buffers, and solutions used in this study were prepared according to the protocols as outlined by Sambrook et al., 2001.

3.1.6 Equipment

Equipments used in this research are listed in Appendix D.

3.2 Methods

3.2.1 Plant growth conditions and PEG application

Seeds of *B. distachyon* genotype were planted and grown on solid medium [Murashige–Skoog Vitamin and Salt Mixture (Gibco), 2% (w/v) sucrose (Sigma), 0.8% phytagar (Gibco BRL)]. All plants were grown under 24 h light for 4 weeks including germination period. For induction of osmotic stress, plants will be transferred to solid medium containing 20% PEG. When the first symptoms became apparent on plants (~3-4 days), leaf and root samples were collected and frozen in liquid nitrogen, and stored at -80°C until further use.

3.2.2 Total RNA isolation

Total RNA isolations were carried out by TRIzol® reagent (Invitrogen) according to the manufacturer's instructions with a few modifications. Two hundred miligram (mg) of leaf tissue was ground with 1.5 ml TRIzol® reagent (Invitrogen). Using a wide bore pipette tip, 1 ml of liquid was taken into an eppendorf tube, which was kept on ice while processing the other samples. After processing all the samples, they were incubated at room temperature for 10 minutes, 0.4 ml chloroform was added and the

tubes were shaken and incubated at room temperature for 5 min. Then, samples were centrifuged at 12,000 rpm for 15 min at 4ºC. The upper layer containing RNA was transferred to a fresh tube. After chloroform extraction, 0.5 ml isopropanol was added to precipitate RNA. Samples were then incubated at room temperature for 10 min and spun at 12,000 rpm for 10 min at 4ºC. The RNA pellet was washed with 1 ml 75% ethanol after centrifugation. Samples were mixed by vortexing and spun at 7,500 rpm for 5 min at 4ºC. The RNA pellet was dried at room temperature for 10 min and placed in 20-50 µl formamide, depending on the size of the pellet, and allowed to sit in the 55ºC water bath for an hour to improve suspension.

Three separate RNA isolations were performed for each tissue type (leaf tissue and root tissue) and treatment (control and 20% PEG application). RNA concentrations were determined spectrophotometrically and RNA qualities were checked by denaturing gel. RNA samples were treated with Dnase I (Fermentas) according to manufacturer's instructions.

3.2.3 cDNA synthesis and degenerate PCR reaction

To synthesize the first-strand cDNA, 2 µg total RNA from Bd21 with and without PEG application was independently reverse transcribed using the Omniscript reverse transcription kit (Qiagen). OligodT primers used in reverse transcription reaction were purchased from Invitrogen (0.5 µg /µl).

Amplification of cDNA fragments were performed in 20 µl PCR reactions using degenerate primers designed based on well conserved regions of ATG8 proteins previously identified in yeast, *Arabidopsis*, rice and bread wheat. Multiple alignment result of ATG8 proteins is given in Fig. 4.1. Sequence information of the degenerate BdATG8 primers, named degBdATG8_F and degBdATG8_R is given in Table 3.1. Each reaction mixture contained 1 µl (1:5 diluted) first strand cDNA, 2 µl 10X PCR buffer (without MgCl$_2$), 2 mM MgCl$_2$, 0.25 mM dNTP mix, 0.5 µM of degBdATG8_F, 0.5 µM of degBdATG8_R, 1 unit Taq DNA polymerase (Fermentas). The templates were amplified at 94°C for 4 min, followed by 40 cycles of amplification (94°C for 45

s, 46.5°C for 1min 30s, 72°C for 45 s), then 72°C for 7 min. PCR products were analyzed on 1% agarose gels.

Table 3.1 Nucleotide sequences of primers used for the cloning and expression analysis of *BdAtg8* gene

Primer	Sequence
degBdATG8_F	5' GGN CAR TTY GTN TAY GTN GT 3'
degBdATG8_R	5' CAT RAA NAR RAA NCC RTC YTC 3'
HindIII-BdATG8_F	5'**AAGCTT**CATGGCCAAGACTTCGTTCAAG 3'
SacI-BdATG8_R	5' **GAGCTC**TTAGGCCAACAAGCCAAATGT 3'
SpeI-BdATG8_F	5'**ACTAGT**GGATGGCCAAGACTTCGTTCAAGC 3'
SmaI-BdATG8_R	5' **CCCGGG**AAGGCCAACAAGCCAA 3'
SmaIF-BdATG8	5' **CCCGGG**AGCCAAGACTTCGTTCAA 3'
SacIR-BdATG8	5' **GAGCTC**GGCCAACAAGCCAAATGT 3'
18S rRNA_F	5' GTGACGGGTGACGGAGAATT 3'
18S rRNA_R	5' GACACTAATGCGCCCGGTAT 3'

3.2.4 DNA extraction from agarose gels

Amplicons reamplified in a greater volume (50 μl) were run on 1% agarose gel, excised from the gels and then purified using Qiaquick® gel-extraction kit (Qiagen).

3.2.5 TA cloning

PGEM®-T Easy Vector System I (Promega) was used to clone the reamplified cDNA fragments bands. *E. coli* (strain DH5α) competent cells were transformed with the recombinant plasmids. Positive clones were selected and used for plasmid purification.

3.2.5.1 Ligation

Reamplified cDNA fragment bands were ligated to pGEM$^{®}$-T Easy vector (Promega) according to the specified amount (3:1 insert/vector ratio) in the pGEM$^{®}$–T Easy kit protocol. Ligation reaction was incubated at room temperature for 1 hour. Positive and negative controls of ligations were also performed.

3.2.5.2 Chemically competent cell preparation

Single colony of *Escherichia coli (E.coli)* DH5α strain was inoculated in 50 ml Luria-Bertani (LB) broth and grown overnight (o/n) in a shaking incubator at 37ºC, 250 rpm. 4 ml from overnight culture was transferred into 400 ml LB in sterile 2L flask and grown at similar conditions. When the OD_{590} of the cells reached ~0.375, culture was taken into 50 ml prechilled, sterile falcon tubes and left on ice for 5-10 minutes. The tubes were spun at 2700 rpm for 7 minutes at 4ºC. Supernatants were discarded and the pellets were gently resuspended in 10 ml ice-cold $CaCl_2$ solution, containing 60 mM $CaCl_2$, 15% glycerol and 10 mM PIPES (pH 7.0). The tubes were spun at 1800 rpm for 5 minutes at 4ºC. Supernatants were discarded and the pellets were resuspended in 10 ml ice-cold $CaCl_2$ solution, and kept on ice for 30 minutes. Again, the tubes were spun at 1800 rpm for 5 minutes at 4ºC. Supernatants were discarded and the pellets were resuspended in 2 ml ice-cold $CaCl_2$ solution. 200 μl aliquots were dispensed into prechilled, sterile eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80ºC. Competency of the cells was checked by transforming 5-10 ng plasmids.

3.2.5.3 Transformation

DH5α strain of *E. coli* was used for transformation. 2 μl of ligation reaction was mixed with 100 μl chemically competent cells according to the manufacturer's protocol. Since the vector contains Ampicillin resistance and *LacZ* genes, 100 μl of transformation culture was plated on LB agar/Amp/IPTG/X-Gal plate and incubated

(o/n) at 37ºC. The *pUC 18* was transformed to competent cells as a positive control. Uncut plasmid was transformed as a negative control.

3.2.5.4 Colony selection

On the basis of blue/white selection, positive clones were selected and replicas of these clones were prepared.

3.2.5.5 Colony PCR

Degenerate primers, that were previously used to detect *BdATG8* gene, was used in colony PCR reaction to confirm that the transformation is correct positive.

3.2.5.6 Preparation of glycerol stocks of transformants

Glycerol stocks of transformants were prepared in 15% glycerol and kept at -80ºC.

3.2.5.7 Plasmid isolation

Colonies of positive transformant were inoculated in 5ml (for mini preps) and 200 ml (for midi preps) of LB broth containing 100 μg/ml of ampicillin in sterile culture tubes. Cells were incubated in a shaker incubator (270 rpm) at 37ºC (o/n) (12-16 hours). QIAprep® spin miniprep kit (Qiagen) and Genopure plasmid midi kit (Roche) were used for plasmid isolation from the (o/n) culture. Isolated plasmids were checked by agarose gel electrophoresis and their concentrations were determined by absorption spectroscopy.

3.2.5.8 Restriction enzyme digestion

Purified plasmids containing the differentially expressed cDNAs were digested with EcoRI restriction enzyme (Fermentas) for a double check of the transformation and results were analysed by agarose gel electrophoresis.

3.2.5.9 DNA sequence analysis

Sequence analyses of isolated plasmids were commercially provided by MCLAB (CA, U.S.A.) using M13F or M13R primers.

Sequences were first exposed to 'VecScreen' algorithm (http://www.ncbi.nlmn.nih.gov/) to remove vector contamination. The BLAST algorithm (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov) was used homologs of *BdATG8* EST sequence.

Clustalw of *BdAtg8* translated sequence to *B. distachyon* protein sequence database helped us to obtain full coding sequence of *BdATG8* gene.

3.2.6 RT-PCR analysis

Total RNA was isolated from roots and leaves of control and 20% PEG applied Bd21 seedlings using the TRIzol reagent (Invitrogen). First strand cDNA synthesis was done using the Omniscript reverse transcription kit (Qiagen) as described previously. Amplification of cDNA fragments were performed in 20 µl PCR reactions using SpeI-BdATG8_F and SmaI-BdATG8_R primers. Sequence information of this primer set is given in Table 3.1. Each reaction mixture contained 1 µl (1:5 diluted) first strand cDNA, 2 µl 10X PCR buffer (without $MgCl_2$), 2 mM $MgCl_2$, 0.25 mM dNTP mix, 0.5 µM of SpeI-BdATG8_F, 0.5 µM of SmaI-BdATG8_R, 1 unit Taq DNA polymerase (Fermentas). The templates were amplified at 94°C for 4 min, followed by 30 cycles of amplification (94°C for 45 s, 60°C for 1 min 30 s, 72°C for 45 s), then 72°C for 7 min. cDNA fragments amplified with 18S rRNA primers were used as internal control to

show the consistent amount of beginning RNA for RT-PCR (18S rRNA primers are given in Table 3.1). PCR products were run on 1% agarose gels.

3.2.7 Quantitative real time PCR analysis

2 μg of total RNA were used for first strand cDNA synthesis with the Omniscript reverse transcription kit (Qiagen) according to the manufacturer's instructions. cDNA was 1:5 diluted and one 1 μl of this cDNA was amplified with 0.35 μM of SpeI-BdATG8_F and SmaI-BdATG8_R primers in a total volume of 20 μl using FastStart Universal SYBR green PCR master mix (ROCHE) with Icycler Multicolor Realtime PCR Detection Systems (BioRad Laboratories).

The templates were amplified at 95°C for 10 min, followed by 40 cycles of amplification (95°C for 45 s, 60°C for 1 min). The quantification was performed based on previous studies (Muller et al. 2002; Ergen and Budak, 2009) using 18S rRNA as an internal reference and two independent PCR results were averaged. Sequences of 18S rRNA primers are given in Table 3.1. Quantitative real time PCR analysis was performed for *BdAtg8* to detect tissue, age and treatment specific differential *Atg8* expression in *B. distachyon*.

3.2.8 MDC staining

Root tips (~1 cm) of control and 20% PEG treated Bd21 seedlings were excised and incubated in 0.05 mM MDC (Sigma) in phosphate buffered saline (PBS) for 20 min. To get rid of excess stain, root tips were washed two times with PBS (Contento et al., 2005). MDC-stained root tips were observed under fluorescence microscopy (Olympus, BX-60).

3.2.9 Amplification of full CDS of *BdAtg8* gene

Sequence information of the primers, with restriction sites, used to amplify CDS of *BdAtg8* gene, named HindIII-BdATG8_F and SacI-BdATG8_R is given in Table 3.1. Those primers have been designed to be in frame with GAL1 promoter of pYES2 (Invitrogen) yeast expression vector. The map of this vector is provided in Appendix B. Each reaction mixture contained 1 μl (1:5 diluted) first strand cDNA, 2 μl 10X PCR buffer (without $MgSO_4$), 5mM $MgSO_4$, 0.25 mM dNTP mix, 0.5 μM of HindIII-BdATG8_F, 0.5 μM of SacI-BdATG8_R, 1 unit Platinum® *Pfx*DNA polymerase (Invitrogen). The templates were amplified at 94°C for 4 min, followed by 30 cycles of amplification (94°C for 45 s, 57°C for 1 min 30 sec, 68°C for 45 s), then 68°C for 7 min. PCR products were analyzed on 1% agarose gels. PCR products were polyadenylated using Taq DNA polymerase for 15 min at 72°C, and run on 1% agarose gel. Band of interest was excised and extracted from gel.

3.2.10 Complementation assay of yeast atg8Δ mutant with *BdAtg8* gene

Before expression of BdATG8 in yeast, amplicons amplified with HindIII-BdATG8F and SacI-BdATG8R were ligated into pGEM®-T Easy vector and transformed into *E. coli* DH5α strain as described previously. Positive colonies, selected on the basis of blue-white selection strategy, were further confirmed with colony PCR and restriction digestion with EcoRI enzyme. Plasmids were isolated from the transformants as stated before. Sequence analyses of isolated plasmids were commercially provided by MCLAB (www.mclab.com, CA, US) using M13F or M13R primers.

The isolated plasmids were double digested with HindIII (Fermentas) and SacI (Fermentas) restriction enzymes according to the manufacturer's instructions. pYES2 vector (Invitrogen) was also double digested with the same set of restriction enzymes. To insert the *BdAtg8* CDS under the GAL1 promoter of pYES2 vector, ligation reaction was carried out overnight at 16°C following the instuctions of the manufacturer (T4 DNA ligase, Fermentas). Yeast *Atg8* mutant strain (BY4741, atg8Δ::*kanMX*, *MATa;*

his3Δ1; leu2Δ0; met15Δ0; ura3ΔO).was used in this study. Detailed information about the yeast mutant strain used in this study is given in a study by Kawamata et al. (2005).

pYES2-*BdAtg8* recombinant vector was transformed into yeast atg8Δ strain according to LiAc/SS-DNA/PEG TRAFO protocol (Gietz and Woods, 2002). As a positive control, pRS316 vector with yeast *Atg8* gene was transformed to atg8Δ yeast mutant strain. Uracil (URA) was used as the auxotrophic marker. 50 μl and 200 μl of transformation mixtures were spread on SC–URA selective medium (0.67% yeast nitrogen base, drop-out (DO) supplement without URA, 2% glucose, 2% agar). After 3-4 days, positive colonies, growing on SC–URA plates, were screened for the presence of pYES2-*BdAtg8* by colony PCR using HindIII-BdATG8_F and SacI-BdATG8_R primers. Positive colonies were further grown in SC-URA medium containing 2% galactose and 1% raffinose o/n. Glycerol stocks of transformants were prepared in 15% glycerol and kept at -80ºC.

5 μl of yeast transformants grown in SC-URA medium was spotted in serial dilutions (1, 1:5, 1:10, 1:100) on standard rich growth medium (yeast extract, peptone, dextrose: YPD) plates for control of equal loading and viability, on YPD with 20% PEG plates for detecting the effect of BdATG8 on growth of yeast in the presence of osmotic stress, and on synthetic minimal medium without nitrogen (0.67% yeast nitrogen base without ammonium sulfate and aminoacids, 2% galactose and 1% raffinose) plates for assessment of the complementation potential of BdATG8 in yeast mutants growing under starvation conditions.

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

Transformants were selected after 48 hs growth in SC-URA medium. O/N cultures were diluted to have an OD_{600} of 0.4 in 50 ml induction medium without nitrogen for mimicking starvation conditions (0.67% yeast nitrogen base without ammonium sulfate and aminoacids, 2% galactose and 1% raffinose). BdATG8 expression was induced by galactose according to the manufacturer's instructions (pYES2 manual, Invitrogen), except few modificatons, such as the expansion of the time for galactose induction to 24 and 48 hours. After protein expression, cell lysates

were prepared according to the manufacturer's instructions (pYES2 manual, Invitrogen). Lysates were assayed for protein concentration via 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 protein was used in western blot analysis.

The proteins were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane using wet transfer method. After blocking with phosphate-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 h, the membrane was probed with a specific rabbit anti-API antibody (1:1000) o/n at 4°C and then washed and exposed to horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:10000) for 1 h. The bound antibodies were visualized using ECL western blotting substrate and enhancer according to the manufacturer's instructions (PIERCE, USA).

3.2.12 Western blot analysis using monoclonal anti-HA antibody

BdAtg8 CDS was amplified using SmaIF-BdATG8 and SacIR-BdATG8 primers. Sequences of these primers are presented in Table 3.1. These primers have been designed to be in frame with truncated ADH1 promoter of pACT2 yeast two-hybrid vector (Clontech) pACT2 vector also has a hemaglutinin (HA) epitope tag for western analysis. The map of this vector is provided in Appendix C. Each reaction mixture contained 1 μl (1:5 diluted) first strand cDNA, 2 μl 10X PCR buffer (without $MgSO_4$), 5mM $MgSO_4$, 0.25 mM dNTP mix, 0.5 μM of SmaIF-BdATG8, 0.5 μM of SacIR-BdATG8, 1 unit Pfu DNA polymerase (Fermentas). The templates were amplified at 94°C for 4 min, followed by 30 cycles of amplification (94°C for 45 s, 60°C for 1 min, 72°C for 45 s), then 72°C for 7 min. PCR products were analyzed on 1% agarose gels. The PCR was reperformed in a greater volume. PCR products were polyadenylated using Taq DNA polymerase for 15 min at 72°C, and run on 1% agarose gel. Band of interest was excised and extracted from gel.

Before expression of BdATG8 in yeast, amplicons were ligated into pGEM®-T Easy vector and transformed into *E. coli* DH5α strain as described previously. Positive colonies, selected on the basis of blue-white selection strategy, were further confirmed with colony PCR and restriction digestion with EcoRI enzyme. Plasmids were isolated

from the transformants as stated before. Sequence analyses of isolated plasmids were commercially provided by MCLAB (CA, US) using M13F or M13R primers.

The isolated plasmids were double digested with SmaI (Fermentas) and SacI (Fermentas) restriction enzymes according to the manufacturer's instructions. pACT2 vector (Clontech) was also double digested with the same set of restriction enzymes. To insert the *BdAtg8* CDS under the truncated ADH1 promoter of pACT2 vector, ligation reaction was carried out overnight at 16°C following the instuctions of the manufacturer (T4 DNA ligase, Fermentas).

pACT2-*BdAtg8* recombinant vector was transformed into yeast atg8Δ strain according to LiAc/SS-DNA/PEG TRAFO protocol (Gietz and Woods, 2002). Leucine (LEU) was used as the auxotrophic marker. 50 μl and 200 μl of transformation mixtures were spread on SC–LEU selective medium (0.67% yeast nitrogen base, drop-out (DO) supplement without LEU, 2% glucose, 2% agar). After 3 days, positive colonies, growing on SC–LEU plates, were screened for the presence of pACT2-*BdAtg8* by colony PCR using SmaIF-BdATG8 and SacIR-BdATG8 primers. Positive colonies were further grown in SC-LEU medium containing 2% glucose o/n. Glycerol stocks of transformants were prepared in 15% glycerol and kept at -80ºC.

5ml O/N cultures were transferred to 45ml YPD medium and grown until the OD_{600} reaches 0.4-0.6. Yeast pellets were prepared by centrifuging the cultures at 1000 g for 5 min at 4°C. Yeast total protein extracts were prepared according to the manufacturer's instructions (pYES2 manual, Invitrogen). Protein isolates were assayed for protein concentration via 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 protein was used in western blot analysis.

The proteins were resolved by 15% SDS-PAGE and transferred to a nitrocellulose membrane using wet transfer method. After blocking with phosphate-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk for 1 h, the membrane was probed with a specific mouse monoclonal anti-HA antibody (Origene) (1:1000) o/n at 4°C and then washed and exposed to horseradish peroxidase-conjugated anti-mouse IgG antibodies (1:10000) for 1 h. The bound antibodies were visualized using ECL western

blotting substrate and enhancer according to the manufacturer's instructions (PIERCE, USA).

4 RESULTS

4.1 Plant growth

Four week-old *B. distachyon* seedlings were transffered to normal MS and 20% PEG treated MS. PEG treatment resulted in retardation in growth of *B. distachyon* seedlings and development of necrotic patches on the bases and sheaths of the old leaves (Figs. 4.1 and 4.2) When first symptoms appeared on the leaves of the PEG treated *B. distachyon* plants, leaf and root samples were collected from the plants for further molecular analysis. Root samples were also collected for MDC staining experiment.
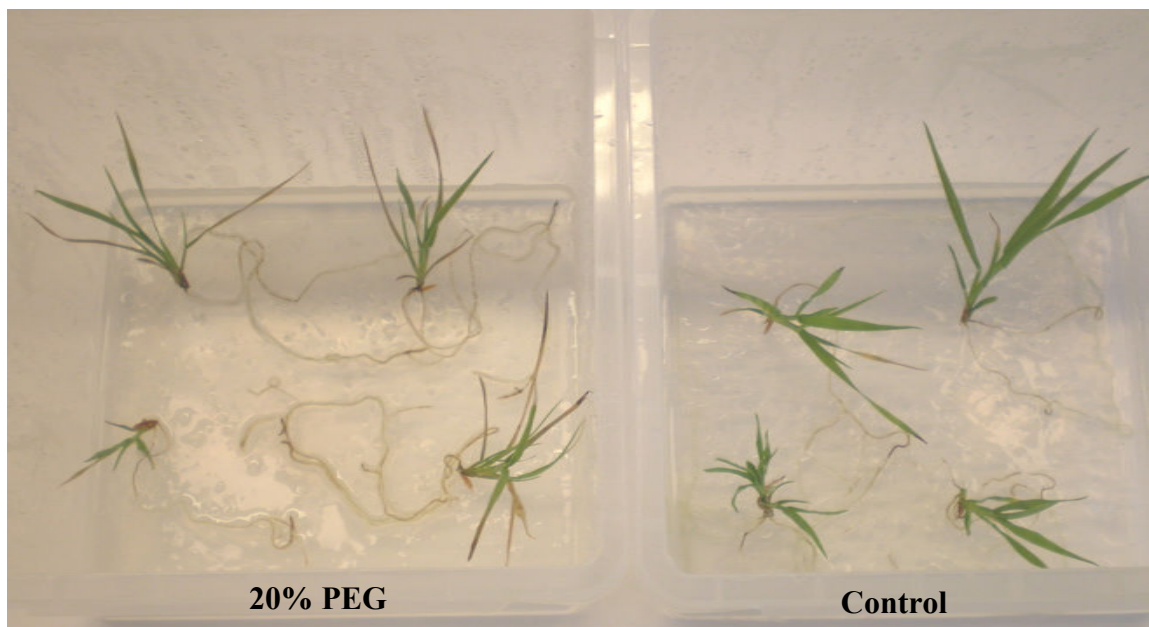
Fig. 4.1 Growth of *B. distachyon* as affected by osmotic stress. Four-week old plants were transferred to normal MS and MS treated with 20% PEG until the first symptoms appeared on the leaves (3-4 days).
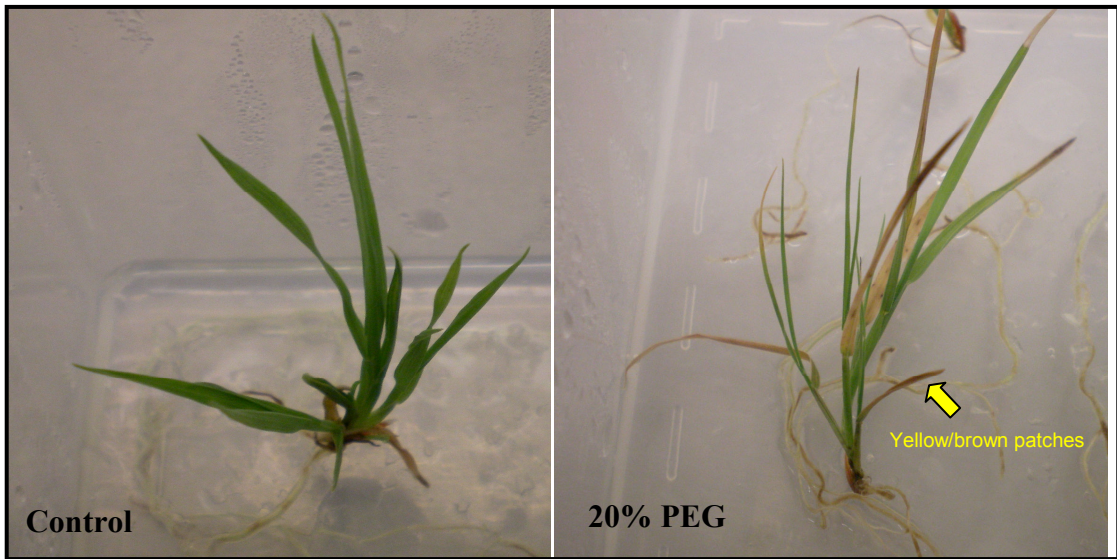
Fig. 4.2 A closer look to the stress symptoms on *B. distachyon* plants treated with PEG. Yellow/brown patches became apparent on the older leaves of *B.distachyon* plants after PEG treatment.

4.2 Identification and isolation of *B. distachyon Atg8* gene

The amino acid sequences of *A. thaliana* ATG8a (GenBank acc. No.: NM118319), *T. aestivum* ATG8 (GenBank acc. No.: AB073171), *O. sativa* ATG8, and *S. cerevisiae* ATG8 were aligned using alignment tool of Vector NTI Advance 9 (Invitrogen) and two well conserved regions were determined (Fig. 4.3)
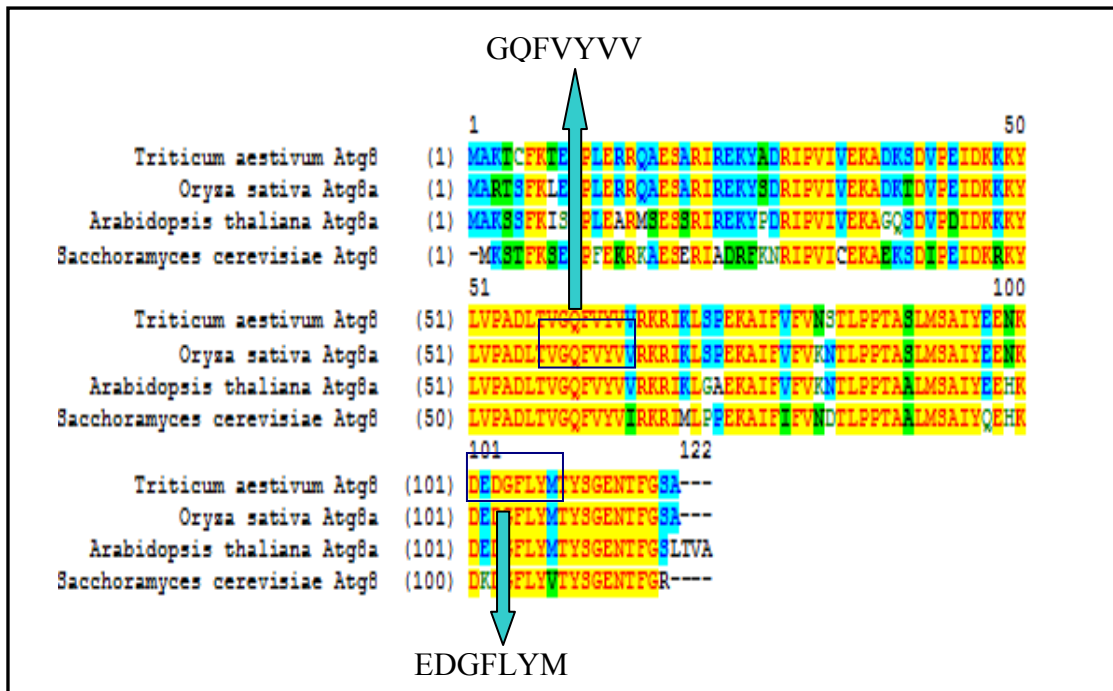
Fig. 4.3 Sequence comparison of TaATG8, OsATG8, AtATG8a, and ScATG8 proteins. Conserved residues are boxed.

Two primer pairs (named degBdATG8_F and degBdATG8_R); see Table 3.1) were designed based on these conserved regions and were used for PCR amplification. cDNAs reverse transcribed from *B. distachyon* total RNAs [leaf control-L(C), leaf stress-L(S), root control- R(C), root stress-R(S)] were used as templates. Size of the amplificon was ~250 bp (Fig. 4.4).

1% agarose gel, 100V, ~40 min

Fig. 4.4 PCR with *BdAtg8* degenerate primers

The PCR products (~250bp) were excised, isolated from the gel, and cloned into the pGEM®–T Easy vector (Promega) and sequencing analysis was commercially provided by MCLAB (www.mclab.com, CA, USA) using M13F or M13R primers. Obtained sequence was then used as a query in Blast search of nucleotide sequence databases. The result of the Blastn is given in Fig. 4.5.

FJ445008.1 Zea mays autophagy-related 8c variant 1 (*Atg8*c) mRNA, complete cds, alternatively spliced   e-value: 3e⁻²⁰ similarity: 80%

```
                     1                                                50
        Atg8 EST    (1)  --------------------------------------------------
   Zea mays atg8c   (1)  ATGGCCAAGACGAGCTCGTTCAAGCTGGAGCACCCCCTTGAGAAGAGGCA
                     51                                              100
        Atg8 EST    (1)  --------------------------------------------------
   Zea mays atg8c  (51)  GTCTGAGGCTAATCGGATCAGAGAGAAGTACCCTGACAGAATTCCAGTGA
                     101                                             150
        Atg8 EST    (1)  --------------------------------------------------
   Zea mays atg8c (101)  TTGTTGAGAAGGCTGAGAGGAGTGACATTCCTGACATCGACAAGAAAAAG
                     151                                             200
        Atg8 EST    (1)  -------------------------GGGCAGTTTGTCTATGTTGTGCG
   Zea mays atg8c (151)  TACCTCGTCCCTGCTGATCTTACGGTTGGACAATTCGTGTATGTTGTTAG
                     201                                             250
        Atg8 EST   (24)  GAAGAGGATTAAGCTGAGCCCAGAAAAGGCCATCTTTGTGTTTGTGAACA
   Zea mays atg8c (201)  GAAGAGAATCAAGCTCAGTGCTGAGAAAGCTATCTTCATCTTTGTGAAGA
                     251                                             300
        Atg8 EST   (74)  GCACCTTGCCACCAACTGCATCTTTGATGTCTGCGATCTATGAAGAGAAC
   Zea mays atg8c (251)  ACACCCTTCCACCAACAGCTGCTCTGATGTCTTCAATCTATGAAGAGAAC
                     301                                             350
        Atg8 EST  (124)  AAGGATGAGGATGGCTTCCTATTCATG-----------------------
   Zea mays atg8c (301)  AAGGATGAGGATGGATTCCTCTACATGACCTACAGTGGTGAGAACACCTT
                     351           363
        Atg8 EST  (151)  -------------
   Zea mays atg8c (351)  CGGGGCTCCTCTAG
```
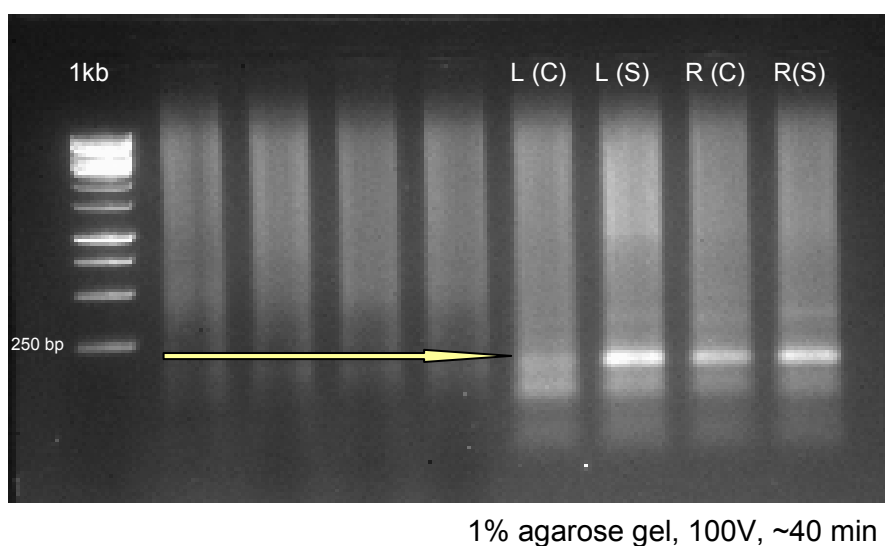
Fig. 4.5 Blastn result of *BdAtg8* EST as a query

Translated sequence of *BdAtg8* EST was aligned to *B. distachyon* protein sequence database to obtain coding sequence (CDS) of *BdAtg8* gene. Sequence of *BdAtg8* CDS is given in Appendix A. Sequence alignment of *BdAtg8* CDS with *Brachypodium* genome sequence revealed that *BdAtg8* consists of 5 exons and 4 introns (Fig. 4.6). Sequence comparison indicated that BdATG8 shows 97% homology with the *T. aestivum* ATG8, in terms of aminoacid identity. BdATG8 also has high homology to rice, *Arabidopsis*, and yeast ATG8s (90,8% *O. Sativa*, 82% *A. thaliana*, 71,7% *S. cerevisiae)* BdATG8 also contains highly conserved C-terminal glycine (Gly) residue (Fig. 4.7).

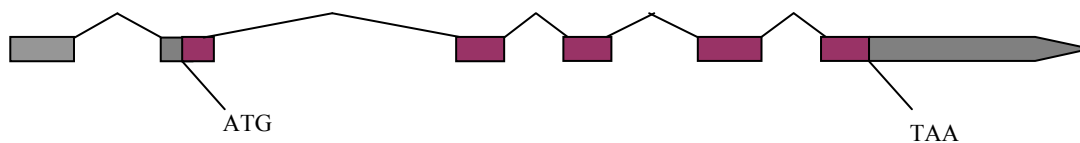ATG
TAA

Fig. 4.6 *In silico* structural analysis of *BdAtg8*. Colored and gray boxes represent coding and untranslated regions, respectively. Solid lines represent introns.

Fig. 4.7 Sequence comparison of *Brachypodium*, bread wheat, rice, *Arabidopsis*, and yeast ATG8s. Conserved glycine (G) residue is boxed.

These results demonstrated that autophagy associated gene *Atg8* is conserved in *B. distachyon*.

4.3 Expression pattern analysis

4.3.1 RT-PCR analysis

RT-PCR was performed in order to examine the expression pattern of *BdAtg8* in control and 20 %PEG treated plants and in different tissues, including leaves of different ages [L1 (oldest), L2, L3, L4 (youngest)] and root. As shown in Fig. 4.8, *BdAtg8* expression was detected in mature leaves, young leaves, and roots in both control and 20 PEG% treated plants, indicating that *BdAtg8* is constitutively expressed in whole plant. However, it was found that *BdAtg8* is abundantly expressed in the roots.

Additionally, expression of this gene was found to be higher in younger leaves in comparison to the older leaves in the presence of osmotic stress.
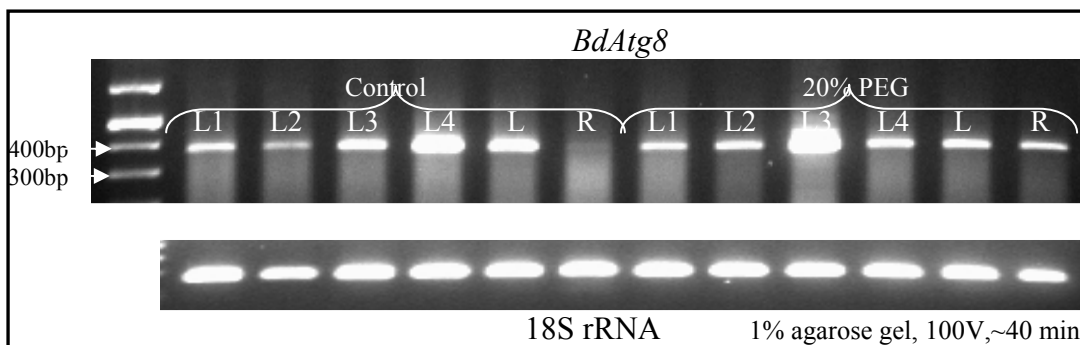
Fig. 4.8   RT-PCR analysis of *BdAtg8* in various tissues [L1 (oldest), L2, L3, L4 (youngest), leaf and root] of control and osmotic stress (20% PEG) applied Bd21 seedling. L stands for leaf and R stands for root. 18 rRNA was used as a loading control.

4.3.2 Quantitative real-time PCR

Quantitative real time PCR was performed in order to analyze tissue, age and treatment specific expression of *Atg8* in *B. distachyon*. 18S rRNA was used as an internal reference for normalization of data. The quantification was performed based on our previous studies (Cebeci et al., 2008; Ergen and Budak, 2009). Q-RT PCR results were in good aggrement with RT-PCR data. *BdAtg8* expression was detected in all tissues analyzed and under both control and osmotic stress conditions. These results revealed that *BdAtg8* is constitutively expressed in whole plant. It was found that level

of expression of *BdAtg8* increases profoundly with osmotic stress treatment, especially in the roots. Additionally, expression of this gene was found to be slightly higher in younger leaves in comparison to the oldest leaves in the presence of osmotic stress. However fold increase in expression of *BdAtg8* gene in younger leaves was lower in comparison to the *BdAtg8* expression changes in roots after osmotic stress treatment. When the leaves of Bd21 seedlings were pooled and analyzed by Q-RT PCR, no fold change in *BdAtg8* gene expression was detected (Fig. 4.9).
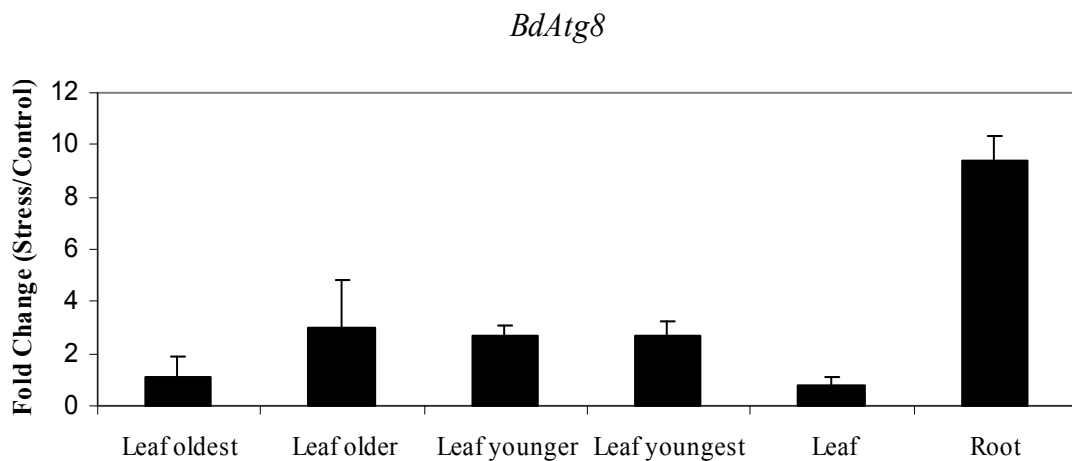
BdAtg8

Fig. 4.9 Fold change in expression level of *BdAtg8* in various tissues of Bd21 seedlings anayzed by quantitative real-time PCR (18S rRNA was used as an internal reference). *Q-Gene* software was used for calculating mean normalized expression values and fold changes.

4.4 Monitoring autophagy in *B. distachyon* roots using the fluorescent dye, monodansylcadaverine

MDC is a fluorescent dye extensively used in plant autophagy monitoring studies to observe autophagosomes.To observe autophagosomes in *B. distachyon* seedlings, roots of control and 20% PEG treated plants were stained with MDC. Roots (~10mm from the tip) were excised and observed using fluorescence microscopy. Weak staining of cell wall and plasma membrane was observed in control roots, in addition to small number of MDC stained spherical structures. MDC fluorescence signal was found to be higher in roots of plants treated with 20% PEG for four days and the number of MDC stained autophagosomes increased in the roots of these plants (Fig 4.10). MDC stained

autophagosomes were mainly located in vicinity of the root vascular system (Fig 4.11). Additionally, numerous moving MDC stained spherical structures were detected in the cytoplasm of the root cells of plants treated with PEG (Fig 4.12). These data indicate that autophagy can be induced by osmotic stress in *B. distachyon* roots.
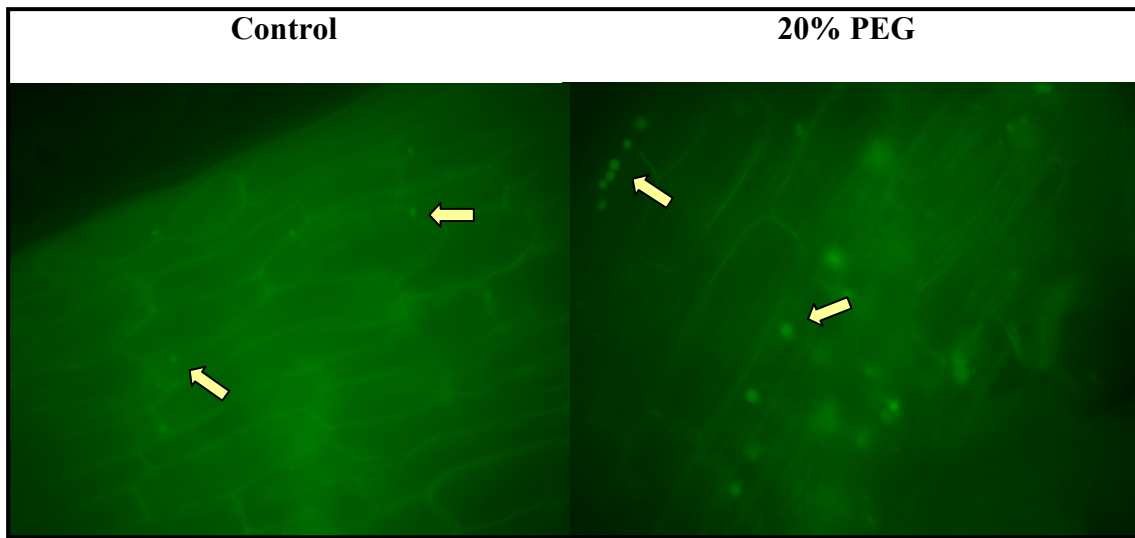
Fig. 4.10 MDC staining of *B. distachyon* roots. 4-weeks old *B. distachyon* plants were transferred to control and 20% PEG applied MS medium and grown for 3-4 days, followed by staining with MDC.
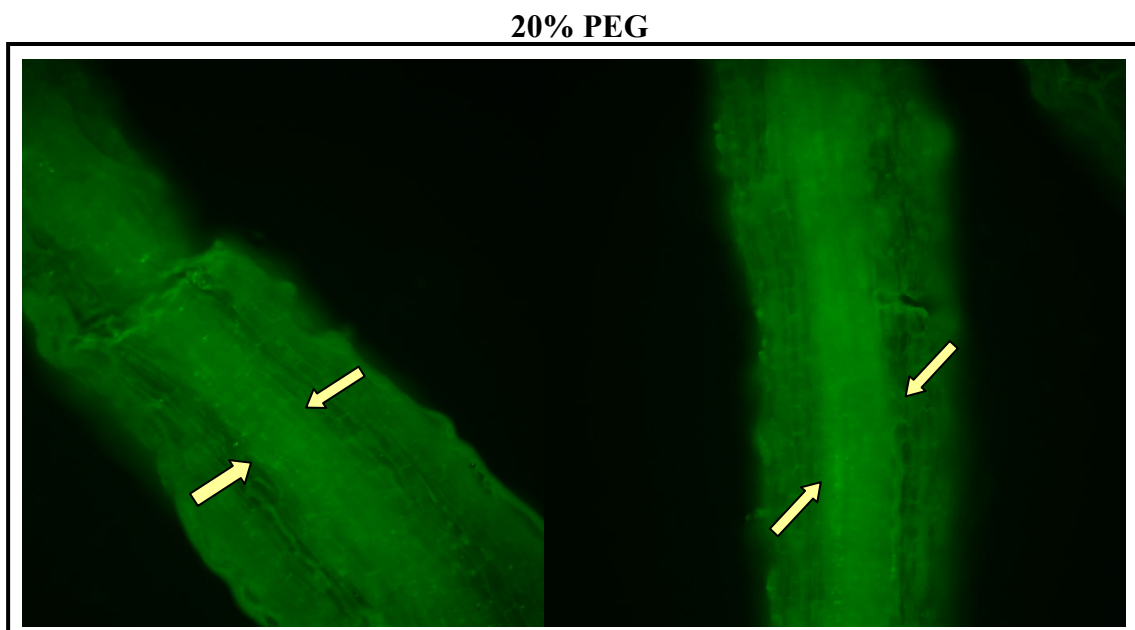
Fig. 4.11 MDC staining of roots of *B. distachyon* plants treated with 20% PEG. Arrows indicate MDC-stained autophagosomes localized next to vascular tissue.
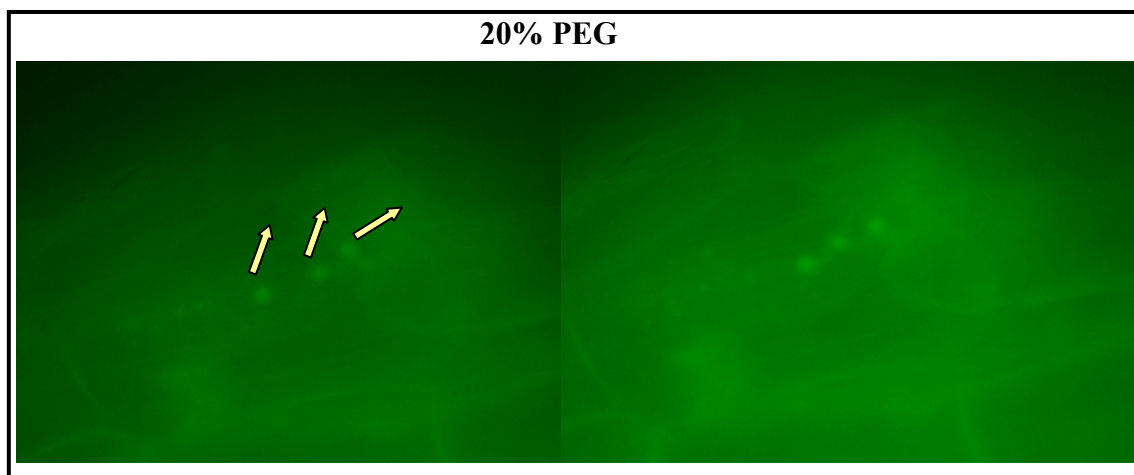
20% PEG

Fig. 4.12   Motile autophagosomes observed in the roots of *B. distachyon* treated with 20% PEG. Arrows indicate the direction of motility.

4.5 Functional analysis of BdATG8

4.5.1 Yeast complementation with *BdAtg8*

To test if the BdATG8 protein is a functional homolog of yeast ATG8, yeast *atg8Δ* mutant was complemented with *BdAtg8*. Functional complementation of yeast *atg8Δ* mutant with *BdAtg8* was performed by introducing pYES2 vector containing *BdAtg8* gene into yeast *atg8Δ* mutant strain. To check whether pYES2 vector containing *BdAtg8* could be successfully transformed to yeast *atg8Δ* mutant, colony PCR was carried out. The expected size of the PCR product was ~375 bp (Fig 4.13).
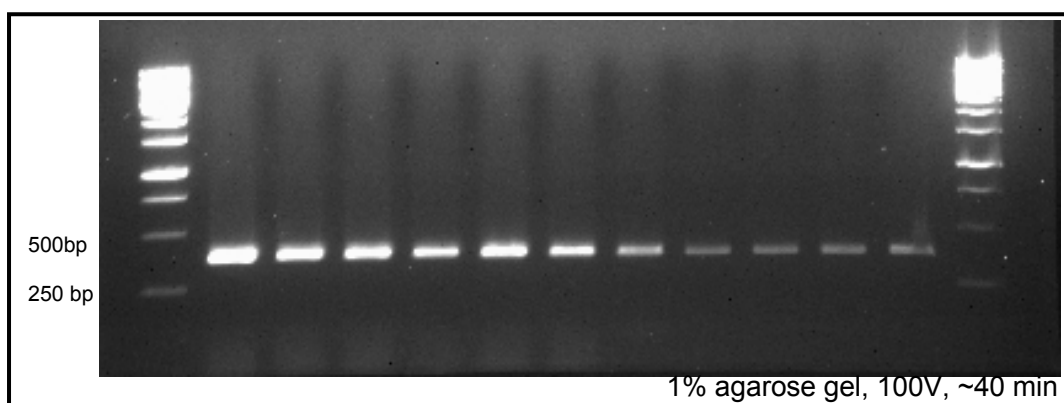
Fig. 4.13   Colony PCR of yeast transformants with pYES2 vector containing *BdAtg8*

To induce starvation, and hence autophagy in yeast, yeast transformants were grown in the presence of nitrogen deficiency. Galactose was selected as the carbohydrate source, since the pYES2 vector has GAL1, galactose inducible promoter. pRS316 vector with yeast *Atg8* gene was transformed to atg8Δ yeast mutant strain, as a positive control. URA was the auxotrophic marker in this experiment. Transformants were found to grow better than *atg8Δ* mutant under starvation conditions. Their overall growth performance was similar to yeast transformed with yeast *Atg8*, indicating that *BdAtg8* functionally complements the yeast *Atg8* gene (Fig. 4.14).
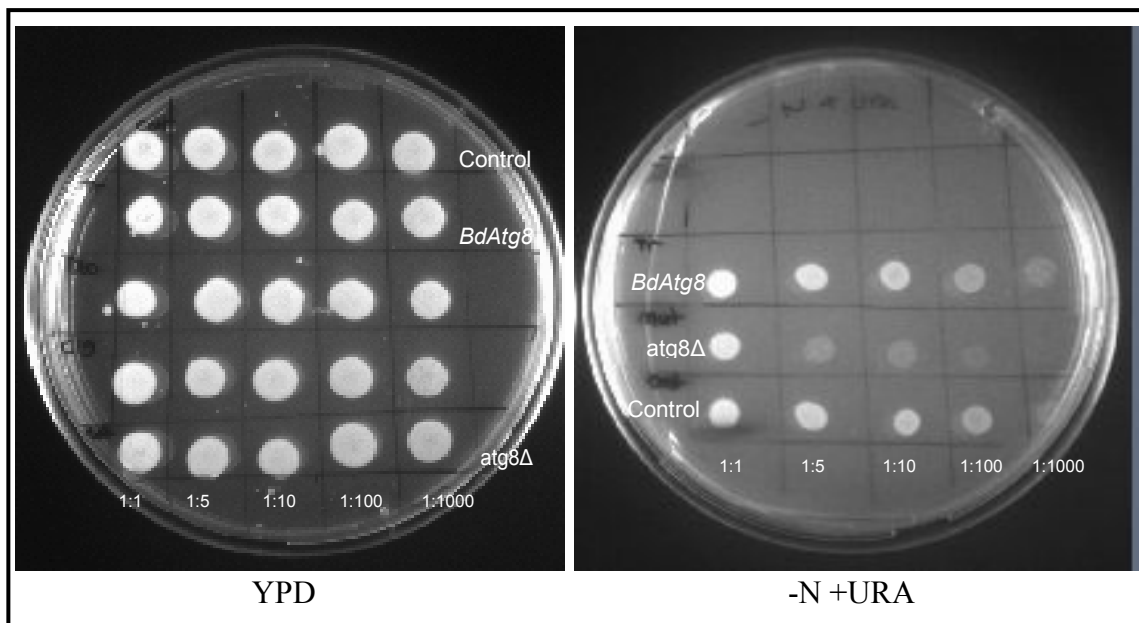
Fig. 4.14 Functional complementation of yeast *atg8Δ* mutant with *BdAtg8* gene. Transformants and yeast *atg8Δ* mutant were grown on standard rich growth medium (YPD) plates for control of equal loading and viability.

Transformants and yeast *atg8Δ* mutant were grown on YPD and YPD with 20% PEG plates for detecting the effect of BdATG8 on growth of yeast in the presence of osmotic stress. However, YPD treated with 20% PEG could not mimic osmotic stress conditions, since growth performance of transformants and yeast *atg8Δ* mutant were similar on both YPD and YPD treated with 20% PEG plates (Fig. 4.15).
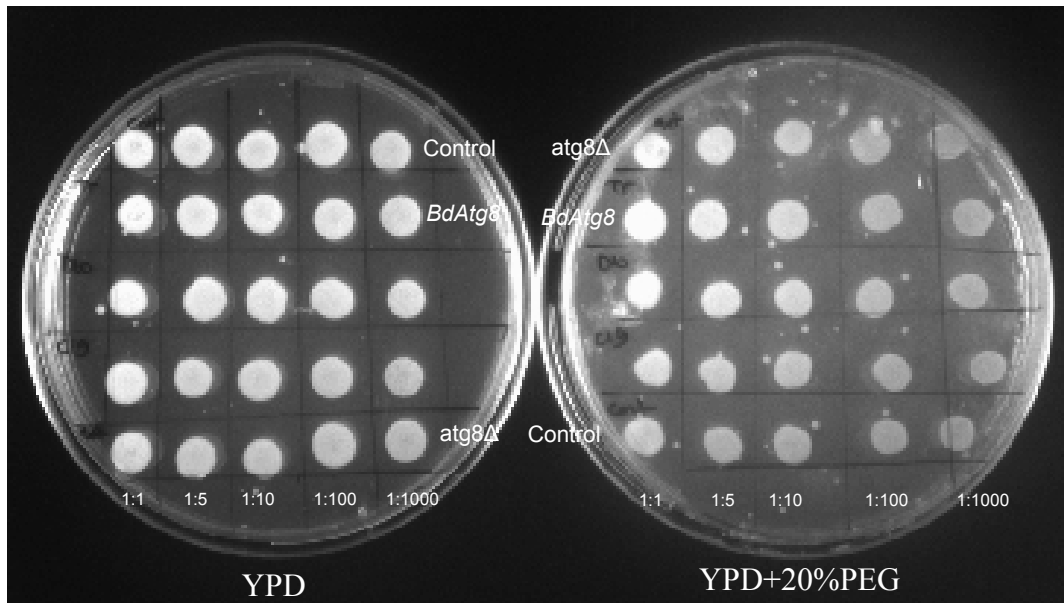
Fig. 4.15 Growth of transformants and yeast *atg8Δ* mutant under standard rich conditions (YPD) and YPD + 20% PEG.

4.5.2 Follow up of maturation of yeast Aminopeptidase I (API)

To further investigate complementation of yeast *atg8Δ* mutant with *BdAtg8*, maturation of API was examined. API is an enzyme synthesized in the cytosol as a precursor enzyme, prAPI (61kD). Maturation of this enzyme inside the vacuole into mAPI (50kD) depends on the presence of an active autophagy pathway in yeast. To test whether BdATG8 is a functional homolog of yeast ATG8, the CDS of *BdAtg8* was cloned into pYES2 vector, which were transformed into a yeast *atg8Δ* mutant strain, and selected with uracil auxotrophic marker. Transformants were grown under nitrogen deficiency to induce autophagy. Next, total protein extracts of transformants and *atg8Δ* mutant were analyzed by western immunoblotting with polyclonal anti-API antibody. Maturation of prAPI, which depends on the presence of an active autophagy pathway in yeast, was found to be somewhat reduced in *BdAtg8* transformants in comparison to the transformants with yeast *Atg8*. However, *BdAtg8* gene could still functionally complement the yeast *Atg8* gene. mAPI could not be detected in yeast *atg8Δ* mutant, as expected (Fig. 4.16).
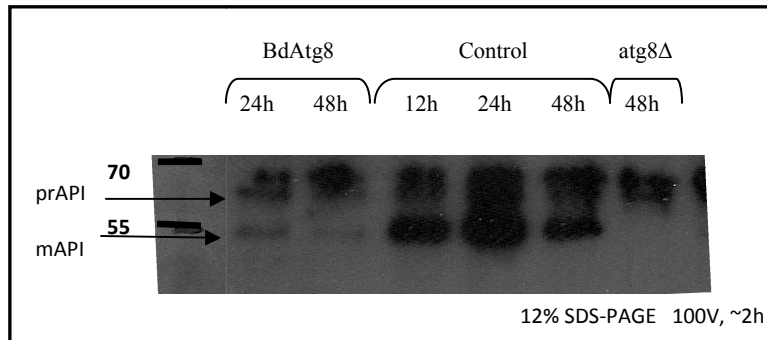
Fig. 4.16 Functional complementation of yeast *atg8Δ* mutant with BdATG8, monitoring API maturation. Maturation of API was detected only in yeast *atg8Δ* mutant transformed with yeast *Atg8* and *BdAtg8*.

4.5.3 Detection of BdATG8 recombinant protein expressed in fusion to GAL4 activation domain and HA epitope tag by immunoblot analysis with anti-HA antibodies

To further confirm whether BdATG8 protein was expressed and also functional in yeast, CDS of *BdAtg8* gene was subcloned into pACT2 vector (Clontech) and the recombinant vector was transformed into yeast *atg8Δ* mutant via LiAc/SS-DNA/PEG TRAFO protocol (Gietz and Woods, 2002). Yeast cell lysates were analyzed by western blotting with monoclonal anti-HA antibody. pACT2-HA-BdAtg8- recombinat vector that we constructed produced fusion protein containing GAL4 activation domain (AD) (768-881), an HA epitope tag and BdATG8 protein. When GAL4 AD- HA-BdATG8 fusion protein was expressed in yeast, a band ~27 kDa (size of BdATG8: ~13 kDa + size of GAL4 AD/HA tag: ~14 kDa) and was recognized by immunoblotting with monoclonal anti-HA antibody (Fig. 4.17). This result validated that BdATG8 protein was expressed in yeast. This fusion protein will be used as a prey for further investigation of the interaction partners of this protein in yeast two-hybrid study.
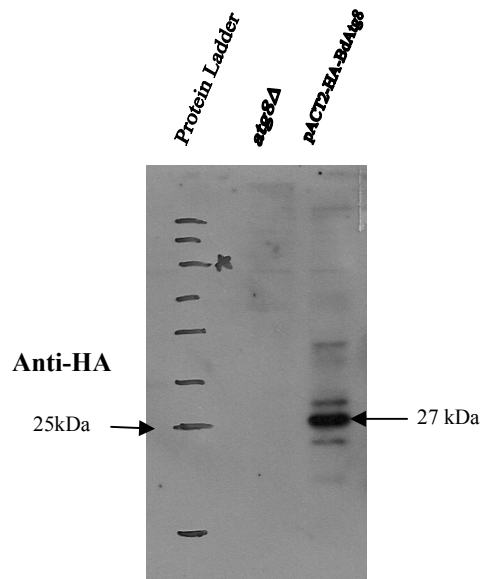
Fig. 4.17 Expression of BdATG8 in yeast *atg8Δ* mutant (Lane 1) and yeast *atg8Δ* mutant tranformed with recombinant expression vector pACT2-HA-BdAtg8 (Lane 2). Yeast cell lysates were analyzed by western blotting with monoclonal anti-HA antibody.

5 DISCUSSION

A number of study in plants has demonstrated the importance of autophagy in responses of plants to abiotic stress conditions, including nutrient deficiency (Hanaoka et al. 2002; Surpin et al. 2003; Yoshimoto et al. 2004; Xiong et al. 2005; Fujiki et al. 2007; Qin et al. 2007), oxidative stress (Xiong et al., 2007a, 2007b) and, most recently, the salt and osmotic stress (Liu et al., 2009). In this study, we identified an autophagy related gene, *Atg8* in *B. distachyon*, and demonstrated a role for autophagy in response of *Brachypodium* to drought/osmotic stress. First, the mRNA level of *BdAtg8* gene was found to be higher in roots of *Brachypodium* in the presence of osmotic stress, which revealed that roots are more active in autophagy under osmotic stress conditions. Similarly, expression level of *BdAtg8* gene was higher in younger leaves of Bd21 seedlings treated with osmotic stress in comparison to *BdAtg8* expression in the same-aged leaves of control seedlings (Fig. 4.9). Second, the number of MDC-stained autophagosomes increased with PEG treatment of the roots of *B. distachyon* plants, which showed the induction of autophagy in those plants during osmotic stress conditions (Fig. 4.10). These results suggest that autophagy is involved in responses of plants to osmotic stress. It has been demonstrated that salt and drought stress can induce autophagy in *Arabidopsis* plants (Liu et al., 2009). Our data support the expectations that autophagy is involved in survival of plants under environmental stress conditions, such as osmotic stress.

Sequence alignment of *BdAtg8* gene with genomic DNA sequence of *Brachypodium* revealed that CDS of *BdAtg8* gene consists of 5 exons and 4 introns (The Brachypodium Genome Initiative, 2010). Sequence comparison with orthologous genes in bread wheat, rice, *Arabidopsis* and yeast demonstrated that BdATG8 has 97% homology with the *T. aestivum* ATG8, based on aminoacid sequences. BdATG8 showed high homology to rice, *Arabidopsis*, and yeast ATG8s (90,8% *O. Sativa*, 82%

A. thaliana, 71,7% *S. cerevisiae)*. BdATG8 also contains highly conserved C-terminal Gly residue. All these data indicated that *BdAtg8* gene is highly conserved in *Brachypodium*. Presence of highly conserved Gly residue at the C-terminal of BdATG8 suggests that this protein is posttranslationally modified as in yeast and other plants such as *Arabidopsis* (Hanaoka et al., 2002) and rice (Wei et al., 2006).

Sequence alignment of *BdAtg8* to *Brachypodium* genome sequence revealed that *BdAtg8* is located on chromosome 1 of Bd21 genome and its gene label is Bradi1g26400 (The International Brachypodium Initiative, 2010). It has been annotated as a "beta-tubulin binding, microtubule binding protein". Previously, it has been shown that *Arabidopsis* ATG8 might be involved in linking the autophagy pathway to the microtubule network and relocation of autophagosomes to the vacuole (Ketelaar et al., 2004). Under the light of these facts, it is an interesting finding that BdATG8 protein has a microtubule binding character. Further studies are required to reveal the role that microtubule binding character plays in plant autophagy process.

MDC staining of a number of autophagosomes in roots of *Brachypodium* plants growing under normal conditions has revealed that there is constitutive autophagy in *Brachypodium*. Previously, it has been demonstrated that autophagy is constitutively present in *Arabidopsis* plants under normal growth conditions (Slavikova et al., 2005; Inoue et al., 2006). Our results contributed to the view that autophagy is a process exploited by plants to sustain their survival under normal growth conditions.

Autophagosomes were found to be mainly located in vicinity of the root vascular system (Fig 4.13). Similarly, a recent study has demonstrated that *Atg10b* gene in rice is also strongly expressed in the phloem tissue of old leaves (Shin et al., 2009). Mobilization of sugars, aminoacids, hormones, mRNAs, and proteins inside the phloem is essential for cell survival in plants. This re-mobilization of organic molecules through sieve tube elements becomes more crucial for sustainment of plant survival under stress conditions, including osmotic/drought stress. It has also been reported that disturbance in autophagy pathway might result in induction of PCD in plants (Phillips et al., 2008). Under the light of these data, active autophagy around the vascular tissue might be playing a role in delay of PCD, and hence could sustain the effective mobilization of degraded organic molecules inside the plant body.

We further investigated whether *BdAtg8* gene is a functional homolog of yeast *Atg8* gene. Yeast *atg8Δ* mutant was complemented with *BdAtg8* gene, therefore it is likely that *Brachypodium Atg8* gene performs a similar function to that of its yeast ortholog in *Brachypodium* autophagy pathway.

Maturation of API inside the vacuole only takes place when there is an active autophagy pathway (Ketelaar et al., 2004). In order to find further evidence for functionality of *BdAtg8* gene in yeast, maturation of API protein has been assayed. We demonstrated that yeast Atg8 gene fully complemented yeast *atg8Δ* mutant under starvation conditions, since mature API was detected using western blotting with anti-API antibody. *BdAtg8* gene also complemented yeast *atg8Δ* mutant, however, in this case, to a lesser extent, which implied that *BdAtg8* is functional in yeast autophagy pathway. Based on these data, it is highly likely that *BdAtg8* gene is involved in autophagy in *Brachypodium* via a molecular mechanism quite similar to that of yeast.

GFP-Atg8 is offered as a useful marker to monitor autophagy in plants (Yoshimoto et al., 2004). Exploitation of this technique seems to be useful for visualization of autophagic bodies in live plant cells. GFP-labelled ATG8s could also help following up of autophagosome formation and, additionally, may enable purification of these structures for further analysis of their cargo (Thompson et al., 2005).

We also expressed BdATG8 as a fusion protein in yeast and detected its expression by western immunoblotting with anti-HA antibody. This result also supported the assumption that BdATG8 could function in yeast. Additionally, BdATG8 protein expressed in fusion to GAL4 AD could be used as a prey protein for further investigation of the interacting partners of this protein in yeast two-hybrid study. These studies will allow researchers to identify proteins playing roles in *Brachypodium* autophagy pathway and, hence reveal the details of autophagy mechanism in plant systems.

6 CONCLUSION

Autophagy is a bulk degradation process for breakdown of cytoplasmic components in either lytic vacuoles or lysosomes. Although, autophagy has been found to be constitutively active in plants, it is also induced by a variety of abiotic stress conditions, including nutrient deficiency, oxidative stress, salinity, and osmotic stress. However, there is not any study investigating the role of autophagy in molecular responses of economically important cereals to osmotic stress. As a model representative of temperate cereals, like wheat, barley, and rye, *Brachypodium*, opens up a new venue for understanding involvement of autophagy process in responses to osmotic/drought stress factors.

This study, to our knowledge, is the first to identify an autophagy related gene, *Atg8*, from *Brachypodium*. Our data also suggest a role for autophagy in the responses of *Brachypodium* plants to osmotic/drought stress. Expression profile analyses conducted in various tissues and MDC staining results demonstrate that autophagy is active in plants exposed to osmotic stress conditions. MDC staining data also indicate that autophagy is a constitutively active process in *Brachypodium*. Functional studies carried out in yeast *atg8Δ* mutant show that *BdAtg8* gene complements yeast *atg8Δ* mutant, which is a strong evidence for functionality of BdATG8 protein in yeast and *Brachypodium*. Presence of a highly conserved Gly residue at the C-terminal of BdATG8 protein suggests that BdATG8 is posttranslationally processed and active in *Brachypodium* autophagy with a molecular function as in yeast.

Future investigation of BdATG8 protein might pave the way to better understanding of autophagy in *Brachypodium* at both molecular and physiological levels. Identification of novel autophagy related genes in *Brachypodium* should also provide insight into the mechanism of autophagy in plants. Research in *Brachypodium*

autophagy might contribute essential data to ongoing molecular studies in cereals in the pursuit of understanding molecular responses to osmotic/drought stress.

7 REFERENCES

1. Xiong, Y., Contento, A.L., Nguyen, P.Q., Bassham, D.C. 2007a. Degradation of oxidized proteins by autophagy during oxidative stress in *Arabidopsis*. Plant Physiol. 143, 291-299.

2. Xiong, Y., Contento, A.L., Bassham, D.C. 2007b. Disruption of autophagy results in constitutive oxidative stress in *Arabidopsis*. Autophagy. 3, 257-258.

3. Liu, Y., Xiong, Y., Bassham, D.C. 2009. Autophagy is required for tolerance of drought and salt sress in plants. Autophagy, 5, 954-963.

4. Liu, Y., Schiff, M., Czymmek, K., Talloczy, Z., Levine, B., Dinesh-Kumar, S.P. 2005. Autophagy regulates programmed cell death during the plant innate immune response. Cell 121, 567-577.

5. Ozdemir, B.S., Hernandez, P., Filiz, E., Budak, H. 2008a. *Brachypodium* genomics. Int. J. Plant Genomics. Article ID 536104

6. Ozdemir, B.S. 2009. Application of biotechnological tools to model plant, *Brachypodium distachyon*. PhD. Thesis. Sabancı University.

7. Garvin, D.F. 2010. An SSR-based genetic linkage map of the model grass Brachypodium distachyon. Genome. 53, 1-13.

8. "The International Brachypodium Initiative" 2010. Genome Sequencing and Analysis of The Model Grass *Brachypodium distachyon*. Nature 11;463 (7282):763-768.

9. Draper, J., Mur, L.A.J., Jenkins, G., Ghosh-Biswas, G., Bablak, P., Hasterok, R., Routledge, A.P.M. 2001. *Brachypodium distachyon*. A new model system for functional genomics in grasses. Plant Physiol. 127, 1539-1555.

10. Filiz, E., Ozdemir, B.S., Budak, F., Vogel, J.P., Tuna, M., Budak, H. 2009. Molecular, morphological, and cytological analysis of diverse *Brachypodium distachyon* inbred lines. Genome. 52, 876-890.

11. Vogel, J. P., Tuna, M., Budak, H., Huo, N., Gu, Y. Q., Steinwand, M. A. 2009. Development of SSR markers and analysis of diversity in Turkish populations of *Brachypodium distachyon*. BMC Plant Biol. 9, 88.

12. Filiz, E., Ozdemir, B.S., Tuna, M., Budak, H. 2008. Diploid *Brachypodium distachyon* of Turkey: Molecular and morphological analysis; T. Yamada and G. Spangenberg 9eds. Molecular Breeding of Forage and Turf. Springer Sci. 7, 83-89.

13. Vogel, J.P. et al. 2006. EST sequencing and phylogenetic analysis of the model grass *Brachypodium distachyon*. Theor. Appl. Genet. 113, 186-195.

14. Christiansen, P., Andersen C.H., Didion, T., Folling M., Nielsen, K.K. 2005. A rapid and efficient transformation protocol for the grass *Brachypodium distachyon*. Plant Cell Rep. 23, 751-758.

15. The *Arabidopsis* Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature. 408, 796-815.

16. Keller B. and Feıillet, C. 2000. Colinearity and gene density in grass genomes. Trends Plant Sci. 5, 246-251.

17. Bennetzen, J.L., San Miguel, P., Chen, M., Tikhonov, A., Francki, M., Avramova, Z. 1998. Grass Genomes. Proc. Natl. Acad. Sci. USA. 95, 1975-1978.

18. Devos, K.M., Beales, J., Nagamura, Y., Sasaki, T. 1999. *Arabidopsis*-rice: will colinearity allow gene prediction across the eudicot-monocot divide? Genome Res. 9, 825-829.

19. Bennett, M.D., Bhandol, P., Leitch, I.J. 2000. Nuclear DNA amounts in angiosperms and their modern uses: 807 new estimates. Ann. Bot. 86, 859-909.

20. Dickson, D. and Cyranoski, D. 2001. Commercial sector scores success with whole rice genome. Nature. 409, 501.

21. Gale, M.D. and Devos, K.M. 1998. Comparative genetics in the grasses. Proc. Natl. Acad. Sci. USA. 95, 1971-1974.

22. Kellogg, E.A. 2001. Evolutionar history of grasses. Plant Physiol. 125, 1198-1205.

23. Shi, Y. 1991. Molecular studies of the evolutionary relationships of *Brachypodium* (Poaceae). PhD thesis, University of Leicester, Leicester, UK.

24. Shi, Y., Draper, J., Stace, C. 1993. Ribosomal DNA variation and its phylogenetic implications in the genus *Brachypodium* (Poaceae). Plant Systematics Evol. 188, 125–138

25. Catalan, P., Shi, Y., Armstrong, L., Draper, J., Stace, C.A. 1995. Molecular phylogeny of the grass genus *Brachypodium* P-Beauv based on RFLP and RAPD analysis. Bot. J. Linnean Soc. 117, 263–280.

26. Catalan, P., Kellogg, E.A., Olmstead, R.G. 1997. Phylogeny of Poaceae subfamily Pooideae based on chloroplast *ndhF* gene sequences. Mol. Phylogen. Evol. 8, 150–166.

27. Catalan, P., Olmstead, R.G. 2000. Phylogenetic reconstruction of the genus *Brachypodium* P-Beauv. (Poaceae) from combined sequences of chloroplast *ndhF* gene and nuclear ITS. Plant System. Evol. 220, 1–19.

28. Aragon-Alcaide, L. Miller, T., Schwarzaher, T., Reader, S., Moore, G. 1996. A cereal centromeric sequence. Chromosoma. 10, 261-268.

29. Bevan M.W. Establishing a BAC-based physical map of *Brachypodium distachyon* as an aid to physical mapping in bread wheat", in *Plant&Animal Genomes XIV Conference*, San Diego, Calif, USA, January 2006.

30. Luo, M., Ma, Y., Huo, N. Construction Of physical map for *Brachypodium distachyon* in *Plant & Animal Genomes XV Conference*, San Diego, Calif, USA, January 2007

31. Kalendar R. and Schulman, A.H. Retrotransposons and their use as molecular markers in *Brachypodium* in *Plant&Animal Genomes XIV Conference*, San Diego, Calif, USA, January 2006.

32. Bartel, B. and Bartel, D.P. 2003. MicroRNAs: at the root of plant development. Plant Physiol. 132, 709–717.

33. Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 116, 281–297.

34. Jones-Rhoades, M.W., Bartel, D.P., Bartel. B. 2006. MicroRNAs and their regulatory roles in plants. Annu. Rev. Plant. Biol. **57**, 19-53.

35. Wei, B., Cai, T., Zhang, R., Li, A., Huo, N., Li, S., Gu, Y.Q., Vogel, J., Jia, J., Qi, Y., Mao, L. 2009. Novel microRNAs uncovered by deep sequencing of small RNA transcriptomes in bread wheat (*Triticum aestivum* L.) and *Brachypodium distachyon* (L.) Beauv. Funct. Integr. Genomics. 9, 499-511.

36. Unver, T. and Budak, H. 2009. Conserved miRNAs and their targets in model grass species Brachypodium distachyon. Planta. 230, 659-669.

37. Zhang, J., Xu, Y., Huan, Q., Chong, K. 2009. Deep sequencing of *Brachypodium* small RNAs at the global genome level identifies microRNAs involved in cold stress response. BMC Genomics. 10, 449.

38. Bevan, M., McKenzie, N., Trick, M. et al. Developing a genetic map of *Brachypodium distachyon* Bd21 in *Plant & Animal Genomes XV Conference*, San Diego, Calif, USA, January 2007.

39. Bossolini, E., Wicker, T., Knobel, P.A., Keller, B. 2007. Comparison of orthologous loci from small grass genomes *Brachypodium* and rice: implications for wheat genomics and grass genome annotation. Plant J. 49, 704–717.

40. Paterson, A.H., Bowers, J.E., Chapman, B.A. 2004. Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. PNAS. 101, 9903–9908.

41. Charles, M., Choisne, N., Samain, S., Boudet, N., Chalhoub, B. *Brachypodium* species as intermediate models for wheat genomics in *Plant & Animal Genomes XIV Conference*, San Diego, Calif, USA, January 2006.

42. Foote, T.N., Griffiths, S., Allouis, S., Moore, G. 2004. Construction and analysis of a BAC library in the grass *Brachypodium sylvaticum*: its use as a tool to bridge the gap between rice and wheat in elucidating gene content. Funct. Integr. Genomics. 4, 26–33.

43. Bray, E.A., Bailey-Serres, J., Weretilnyk, E. 2000. Responses to abiotic stresses. **In:** Biochem. Mol. Biol. Plants. Gruissem, W., Buchnnan, B., Jones, R. eds. American Society of Plant Physiologists, Rockville, MD, 1158-1249.

44. Chaves, M.M., Maroco, J.P., Pereira, J.S. 2003. Understanding plant responses to drought: from genes to the whole plant. Funct. Plant Biol. 30, 239–264.

45. Bartels, D. and Sunkar, R. 2005. Drought and salt tolerance in plants. Crit. Rev. Plant Sci. 24, 23–58.

46. Valliyodan, B. and Nguyen, H.T. 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr. Opin. Plant Biol. 9, 189–195.

47. Barnabas, B., Jager, K., Feher, A. 2008. The effect of drought and heat stress on reproductive processes in cereals. Plant, Cell Environ. 31, 11–38.

48. Sairam, R.K. and Saxena, D.C. 2000. Oxidative stress and antioxidants in wheat genotypes: possible mechanism of water stress tolerance. J. Agron. Crop Sci. 184, 55–61.

49. Shinozaki, K. and Yamaguchi-Shinozaki, K. 2007. Gene networks involved in drought stress response and tolerance. J. Exp. Bot. 58, 221–227.

50. Ergen, Zahide Neslihan and Budak, Hikmet, "Sequencing over 13 000 expressed sequence tags from six subtractive cDNA libraries of wild and modern wheats following slow drought stress", Plant Cell and Environment, Vol.32, No.3, March 2009, 220-236

51. Ergen, Zahide Neslihan and Thimmapuram, Jyothi and Bohnert, Hans J. and Budak, Hikmet, "Transcriptome pathways unique to dehydration tolerant relatives of modern wheat", Functional and Integrative Genomics, Vol.9, No.3, August 2009, 377-396

52. Kaur, N. and Gupta, A.K. 2005. Signal transduction pathways under abiotic stresses in plants. Curr. Sci. 88, 1771–1780.

53. Mishra, N.S., Tuteja, R., Tuteja N. 2006. Signaling through map kinase networks in plants. Arch. Biochem. Biophys. 452, 55–68.

54. Nakashima, K. and Yamaguchi-Shinozaki, K. 2005. Molecular studies on stress-responsive gene expression in *Arabidopsis* and improvement of stress tolerance in crop plants by regulon biotechnology. Jpn. Agr. Res. Q. 39, 221–229.

55. Zhu, J.K. 2001. Cell signaling under salt, water and cold stresses. Curr. Opin. Plant Biol. 4,401-406.

56. Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., Kobayashi, H. 1999. A recessive *Arabidopsis* mutant that grows photoautotrophically under salt stress shows enhanced active oxygen detoxification. Plant Cell. 11, 1195-206.

57. Kerepesi, I. and Galiba, G. 2000. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Sci*. 40,482-487.

58. Murillo-Amadaor, B., Lopez-Aguilar, R., Kaya, C., Larrinaga-Mayoral, J., Flores-Hernandez. A. 2002. Comparative effects of NaCl and polyethylene glycol on germination, emergence and seedling growth of cowpea. J. Agron. Crop Sci. 188, 235–247.

59. Abebe, T., Guenzi, A.C., Martin, B., Cushman, J.C. 2003. Tolerance of mannitol accumulating transgenic wheat to water stress and salinity. Plant Physiol. 131, 1748-1755.

60. Liu, H.P., Dong, B.H., Zhang, Y.Y., Liu, Z.P., Liu Y.L. 2004. Relationship between osmotic stress and the levels of free, conjugated and bound polyamines in leaves of wheat seedlings. Plant Sci. 166, 1261-1267.

61. Turkan, I., Bor, M., Ozdemir F., Koca, H. 2005. Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci. 168, 223-231.

62. Klionsky D.J. and Ohsumi,Y. 1999. Vacuolar import of proteins and organelles from the cytoplasm. Annu. Rev. Cell Dev. Biol*., 15, 1–32.

63. Klionsky, D.J. 2005 The molecular machinery of autophagy: unanswered questions, J. Cell Sci. 118, 7–18.

64. Bassham, D.C. 2009. Function and regulation of macroautophagy in plants. Biochim. Biophys. Acta, Mol. Cell. Res. 9, 1397-1403.

65. Mitou, G., Budak, H., Gozuacik, D. 2009. Techniques to study autophagy in plants. Int. J. Plant Genomics. Vol.2009, 1-14.

66. Aubert, S., Gout, E., Bligny, R., MartyMazars, D., Barrieu, F., Alabouvette, J., Marty, F., Douce, R. 1996. Ultrastructural and biochemical characterization of autophagy in higher plant cells subjected to carbon deprivation: control by the supply of mitochondria with respiratory substrates. J. Cell Biol. 133, 1251-1263.

67. Bassham, D.C., Laporte, M., Marty, F., Moriyasu, Y., Ohsumi, Y., Olsen, L.J., Yoshimoto, K. 2006. Autophagy in development and stress responses of plants. Autophagy. 2, 2–11.

68. Rose, T.L., Bonneau, L., Der, C., Marty-Mazars, D., Marty, F. 2006. Starvation-induced expression of autophagy-related genes in *Arabidopsis*. Biol. Cell. 98, 53–67.

69. Shin, J-H., Yoshimoto, K., Ohsumi, Y., Jeon, J-S., An, G. 2009. *OsATG10b*, an autophagosome component, is needed for cell survival against oxidative stresses in rice. Mol. Cells. 27, 67-74.

70. Yano, K., Hattori, M., Moriyasu, Y. 2007. A novel type of autophagy occurs together with vacuole genesis in miniprotoplasts prepared from tobacco culture cells. Autophagy. 3, 215-221.

71. Thompson, A.R. and Vierstra, R.D. 2005. Autophagic recycling: lessons from yeast help define the process in plants. Curr. Opin. Plant Biol. 8, 165–173.

72. Seay, M., Patel, S., Dinesh-Kumar, S.P. 2006. Autophagy and plant innate immunity. Cell. Microbiol. 8, 899–906.

73. Yoshimoto, K., Jikumaru, Y., Kamiya, Y., Kusano, M., Consonni, C., Panstruga, R., Ohsumi, Y., Shirasu, K. 2009. Autophagy negatively regulates cell death by controlling NPR1-depending salcylic acid signaling during senescence and the innate immune response in *Arabidopsis*. The Plant Cell. 21, 2914-2927.

74. Chen, M.H., Liu, L.F., Chen, Y.R., Wu, H.K., Yu, S.M. 1994. Expression of alpha-amylases, carbohydrate metabolism, and autophagy in cultured rice cells is coordinately regulated by sugar nutrient. Plant J. 6, 625–636.

75. Moriyasu, Y. and Hillmer, S. 2000. Autophagy and vacuole formation. In: Robinson DG, Rogers JC (eds) Vacuolar compartments. Sheffield Academic Press, Sheffield, pp 71–89.

76. Brouquisse, R., Gaudillère, J.P., Raymond, P. 1998. Induction of a carbon-starvation-related proteolysis in whole maize plants submitted to light/dark cycles and to extended darkness. Plant Physiol. 117, 1281–1291.

77. Mortimore, G.E., Lardeux, B.R., Adams, C.E. 1988. Regulation of microautophagy and basal protein turnover in rat liver. Effects of short-term starvation. J. Biol. Chem. 263, 2506–2512.

78. Dunn Jr., W.A., Cregg, J.M., Kiel, J.A. et al. 2005. Pexophagy: the selective autophagy of peroxisomes. Autophagy. 1, 75–83.

79. Levanony, H., Rubin, R., Altschuler, Y., Galili, G. 1992. Evidence for a novel route of wheat storage proteins to vacuoles. J. Cell Biol. 119, 1117-1128.

80. Shy, G., Ehler, L., Herman, E., Galili, G. 2001. Expression patterns of genes encoding endomembrane proteins support a reduced function of the golgi in wheat endosperm during the onset of storage protein deposition. J. Exp. Bot. 52, 2387-2388.

81. Toyooka, K., Okamoto, T., Minamikawa, T. 2001. Cotyledon cells of *Vigna mungo* seedlings use at least two distinct autophagic machineries for degradation of starch granules and cellular components. J. Cell Biol. 154, 973-982.

82. Bassham, D.C. 2007. Plant autophagy- more than a starvation response. Curr. Opin. Plant Biol. 10, 587-593.

83. Inoue, Y., Suzuki, T., Hattori, M., Yoshimoto, K., Ohsumi, Y., Moriyasu, Y. 2006. *AtATG* genes, homologs of yeast autophagy genes, are involved in constitutive autophagy in *Arabidopsis* root tip cells. Plant Cell Physiol. 47, 1641-1652.

84. Harding, T.M., Morano, K.A., Scott, S.V., Klionsky, D.J. 1995. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. J. Cell Biol. 131, 591–602.

85. Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M.,Veenhuis, M., Wolf, D.H. 1994. Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. FEBS Lett. 349, 275–280.

86. Tsukada, M., Ohsumi, Y. 1993. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett. 333, 169–174.

87. Wang, C.W. and Klionsky, D.J. 2003. The molecular mechanism of autophagy. Mol. Med. 9, 65–76.

88. Noda, T., Suzuki, K., Ohsumi, Y. 2002. Yeast autophagosomes: de novo formation of a membrane structure. Trends Cell Biol. 12, 231–235.

89. Klionsky, D.J., Cregg, J.M., Dunn, W.A., Jr., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., Ohsumi, Y. 2003. A unified nomenclature for yeast autophagy-related genes. Dev. Cell. 5, 539–545.

90. Doelling, J.H., Walker, J.M., Friedman, E.M., Thompson, A.R., Vierstra, R.D. 2002. The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in *Arabidopsis thaliana*. J. Biol. Chem. 277, 33105-33114.

91. Hanaoka, H., Noda, T., Shirano, Y., Kato, T., Hayashi, H., Shibata, D., Tabata, S., Ohsumi, Y. 2002. Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an *Arabidopsis* autophagy gene. Plant Physiol. 129, 1181–1193.

92. Surpin, M., Zheng, H., Morita, M.T., Saito, C., Avila, E., Blakeslee, J.J., Bandyopadhyay, A., Kovaleva, V., Carter, D., Murphy, A., et. al. 2003. The VTI family of SNARE proteins is necessary for plant viability and mediates different protein transport pathways. Plant Cell. 15, 2885–2899.

93. Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T., Ohsumi, Y. 2004. Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell 16, 2967–2983.

94. Thompson, A.R., Doelling, J.H., Suttangkakul, A., Vierstra, R.D. 2005. Autophagic nutrient recycling in *Arabidopsis* directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol. 138, 2097-110.

95. Noda T. and Ohsumi. Y. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. 273, 3963–3966.

96. Matsuura, A., Tsukada, M., Wada, Y., Ohsumi, Y. 1997. Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. Gene. 192, 245–250.

97. Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., Ohsumi, Y. 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J. Biol. Chem. 150, 1507–1513.

98. Abeliovich, H., Zhang, C., Dunn Jr., W. A., Shokat, K. M., Klionsky,.D. J. 2003. Chemical genetic analysis of Apg1 reveals a non-kinase role in the induction of autophagy Mol. Biol. Cell. 14, 477–490.

99. Menand, B., Desnos, T., Nussaume, L., Berger, F., Bouchez, D., Meyer, C., Robaglia, C. 2002. Expression and disruption of the *Arabidopsis* TOR (target of rapamycin) gene. Proc. Natl. Acad. Sci. U.S.A. 99, 6422–6427.

100. Crespo, J.L., Diaz-Troya, S., Florencio, F.J. 2005. Inhibition of target of rapamycin signaling by rapamycin in the unicellular green alga *Chlamydomonas reinhardtii*. Plant Physiol. 139, 1736–1749.

101. Diaz-Troya, S., Florencio, F.J., Crespo, J.L. 2008. Target of rapamycin and LST8 proteins associate with membranes from the endoplasmic reticulum in the unicellular green alga *Chlamydomonas reinhardtii*. Eukaryot. Cell. 7, 212–222.

102. Kihara, A., Noda, T., Ishihara, N., Ohsumi, Y. 2001. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. J. Cell Biol. 152, 519–530.

103. Xie Z. and Klionsky, D. J. 2007. Autophagosome formation: core machinery and adaptations. Nature Cell Biol. 9, 1102–1109.

104. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M.,Ohsumi, Y. (1998) A protein conjugation system essential for autophagy. Nature 395:395–398.

105. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., Ohsumi, Y. 2000. A ubiquitin-like system mediates protein lipidation. Nature. 408, 488–492.

106. Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., Ohsumi, Y. 2001. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J. 20, 5971–5981.

107. Kuma, A., Matsui, M., Mizushima, N. 2007. LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. Autophagy. 3, 323–328.

108. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., Ohsumi, Y. 2000. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J. Cell Biol. 151, 263–276.

109. Wei, S., Ma, H., Liu, C., Wu, J., Yang, J. 2006. Identification and characterization of two rice autophagy associated genes, *OsAtg8* and *OsAtg4*. Mol Biol. Rep. 33, 273-278.

110. Kirisako T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori,T., Noda, T., Ohsumi,Y. 1999. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J. Cell Biol. 147, 435–446.

111. Ketelaar, T., Voss, C., Dimmock, S. A., Thumm, M., Hussey, P. J. 2004. *Arabidopsis* homologues of the autophagy protein Atg8 are a novel family of microtubule binding proteins. FEBS Lett. 567, 302–306.

112. Fischer von Mollard, G. and Stevens, T.H. 1999. The *Saccharomyces cerevisiae* v-SNARE Vti1p is required for multiple membrane transport pathways to the vacuole. Mol. Biol. Cell 10, 1719-1732.

113. Sanmartin, M., Ordonez, A., Sohn, E.J., Robert, S., Sanchez-Serrano, J.J., Surpin, M.A., Raikhel, N.V., Rojo, E. 2007. Divergent functions of VTI12 and VTI11 in trafficking to storage and lytic vacuoles in *Arabidopsis*. Proc. Natl. Acad. Sci. 104, 3645-3650.

114. Darsow, T., Rieder, S. E., Emr, S. D. A. 1997.multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. J. Cell Biol. 138, 517–529.

115. Ungermann C. and Langosch, D. 2005. Functions of SNAREs in intracellular membrane fusion and lipid bilayer mixing. J. Cell Sci. 118, 3819–3828.

116. Kim, I., Rodriguez-Enriquez, S., Lemasters, J. J. 2007. Selective degradation of mitochondria by mitophagy. Arch. Biochem. Biophys. 462, 245–253.

117. Marty, F. 1999. Plant vacuoles. Plant Cell. 11, 587–600.

118. Rojo, E., Martin, R., Carter, C., Zouhar, J., Pan, S., Plotnikova, J., Jin, H., Paneque, M., Sanchez-Serrano, J.J., Baker, B., Ausubel, F.M., Raikhel, N.V. 2004. VPEgamma exhibits a caspase-like activity that contributes to defense against pathogens. Curr. Biol. 14, 1897–1906.

119. Eskelinen, E-L. 2006. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. Mol. Aspects Med. 27, 495-502.

120. Qin, G., Ma, Z., Zhang, L., Xing, S., Hou, X., Deng, J., Liu, J., Chen, Z., Qu, L.J., Gu, H. 2007. *Arabidopsis* AtBECLIN 1/AtAtg6/AtVps30 is essential for pollen germination and plant development. Cell Res. 17, 249-263.

121. Fujiki, Y., Yoshimoto, K., Ohsumi, Y. 2007. An *Arabidopsis* homolog of yeast ATG6/VPS30 is essential for pollen germination. Plant Physiol. 143, 1132-1139.

122. Ghiglione, H., Gonzalez, F., Serrago, R., Maldonado, S., Chilcott, C., Curá, J., Miralles, D., Zhu, T., Casal, J. 2008. Autophagy regulated by day length determines the number of fertile florets in wheat, Plant J. 55, 1010–1024.

123. Xiong, Y., Contento, A.L., Bassham, D.C. 2005. AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in *Arabidopsis thaliana*. Plant J. 42, 535–546.

124. Horner, H.T., Healy, R.A., Cervantes-Martinez, T., Palmer, R.G. 2003. Floral nectary fine structure and development in *Glycine max* L. (*Fabaceae*). Int. J. Plant Sci. 164, 675-690.

125. Gaffal, K.P., Friedrichs, G.J., El-Gammal, S. 2007. Ultrastructural evidence for a dual function of the phloem and programmed cell death in the floral nectary of *Digitalis purpurea*. Ann. Bot. (Lond) 99, 593-607.

126. Moriyasu, Y., Hattori, M., Jauh, G.Y., Rogers, J.C. 2003. Alpha tonoplast intrinsic protein is specifically associated with vacuole membrane involved in an autophagic process. Plant Cell Physiol. 44, 795-802.

127. Slavikova, S., Shy, G., Yao, Y.L., Giozman, R., Levanony, H., Pietrokovski, S., Elazar, Z., Galili, G. 2005 The autophagy-associated *Atg8* gene family operates both under favourable growth conditions and under starvation stresses in *Arabidopsis* plants. J. Exp. Bot. 56, 2839-2849.

128. Phillips, A.R., Suttangkakul, A., Vierstra, R.D. 2008. The ATG12-conjugating enzyme ATG10 is essential for autophagic vesicle formation in *Arabidopsis thaliana*. Genetics. 178, 1339–1353.

129. Ashford, T.P. and Porter, K.R. 1962. Cytoplasmic components in hepatic cell lysosomes. J. Cell Biol. 12, 198–202.

130. Liu, Y., Schiff, M., Czymmek, K., Talloczy, Z., Levine, B., Dinesh-Kumar, S.P. 2005. Autophagy regulates programmed cell death during the plant innate immune response. Cell 121, 567-577.

131. Contento, A.L., Xiong. Y., Bassham, D.C. 2005. Visualization of autophagy in *Arabidopsis* using the fluorescent dye monodansylcadaverine and a GFP-AtATG8e fusion protein. Plant J. 42, 598-608.

132. Lepperdinger, G., Strobl, B. and Kreil, G. 1998. *HYAL2*, a human gene expressed in many cells, encodes a lysosomal hyaluronidase with a novel type of specificity. J. Biol. Chem. 273, 22466–22470.

133. Tarasova, N.I., Stauber, R.H., Choi, J.K., Hudson, E.A., Czerwinski, G., Miller, J.L., Pavlakis, G.N., Michejda, C.J., Wank, S.A. 1997. Visualization of G protein-coupled receptor trafficking with the aid of the green fluorescent protein. J. Biol. Chem. 272, 14817–14824.

134. Biederbick, A., Kern, H. F., Elsasser, H. P. 1995. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur. J. Cell Biol. 66, 3–14.

135. Niemann, A., Takatsuki, A., Elsasser, H.P. 2000. The lysosomotropic agent monodansylcadaverine also acts as a solvent polarity probe. J. Histochem. Cytochem. 48, 251–258.

136. Patel, S. and Dinesh-Kumar, S.P. 2008. *Arabidopsis* ATG6 is required to limit the pathogen-associated cell death respose. Autophagy. 4, 20-27.

137. Moriyasu, Y. and Ohsumi, Y. 1996. Autophagy in tobacco suspension-cultured cells in response to sucrose starvation. Plant Physiol. 111, 1233-41.

138. Robinson, D.G., Albrecht, S., Moriyasu, Y. 2004. The V-ATPase inhibitors concanamycin A and bafilomycin A lead to Golgi swelling in tobacco BY-2 cells. Protoplasma. 224, 255–260.

139. Takatsuka, C., Inoue, Y., Matsuoka, K., Moriyasu, Y. 2004. 3-methyladenine inhibits autophagy in tobacco culture cells under sucrose starvation conditions. Plant Cell Physiol. 45, 265–274.

140. Inoue, Y. and Moriyasu, Y. 2006. Degradation of membrane phopholipids in plant cells cultured in sucrose-free medium. Autophagy. 2, 244-246.

141. Suzuki, N.N., Yoshimoto, K., Fujioka, Y., Ohsumi, Y., Inagaki, F. 2005. The crystal structure of plant ATG12 and its biological implication in autophagy. Autophagy 1:119-26.

142. Sambrook, J., Manniatis, T., Fritsch, E. F., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press. 3rd edition.

143. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

144. Muller, P.Y., Janovjak, H., Miserez, A.R., Dobbie, Z. 2002. Processing of gene expression data generated by quantitative real-time PCR. Biotechniques. 32, 1372-1379.

145. Kawamata, T., Kamada, Y., Suzuki, K., Kuboshima, N., Akimatsu, H., Ota, S., Ohsumi, M., and Ohsumi, Y. 2005. Characterization of a novel autophagy-specific gene, *ATG29*. Biochem. Biophys. Res. Commun. 338, 1884–1889.

146. Gietz, R.D. and Woods, R.A.. 2002. Transformation of yeast by the Liac/SS carrier DNA/PEG method. Methods in Enzymol. 350, 87-96.

147. Klionsky, D.J. and Ohsumi, Y. 2003. Vacuolar import of proteins and organelles from the cytoplasm. Annu. Rev. Cell. Dev. Biol. 15, 1-32.

Full CDS of *BdAtg8* gene

5'
ATGGCCAAGACTTCGTTCAAGCTCGAGCACCCCATGGAGAGGAGGCAGGCT
GAATCTGCTAGGATCCGAGAGAAGTACCCAGACAGAATTCCTGTGATCGTT
GAGAAGGCGGATAAGTCTGATGTTCCAGAGATTGACAAGAAGAAGTATCTT
GTACCAGCCGACCTAACTGTTGGTCAGTTTGTCTACGTGGTGCGGAAGAGG
ATTAAGCTGAGCCCAGAAAAGGCCATCTTTGTGTTTGTGAACAGCACCTTGC
CACCAACTGCATCTTTGATGTCTGCGATCTATGAAGAGAACAAGGATGAAG
ACGGCTTTCTTTACATGACTTACAGTGGCGAGAACACATTTGGCTTGTTGGC
CTAA
        3'

APPENDIX B

Map of pYES2
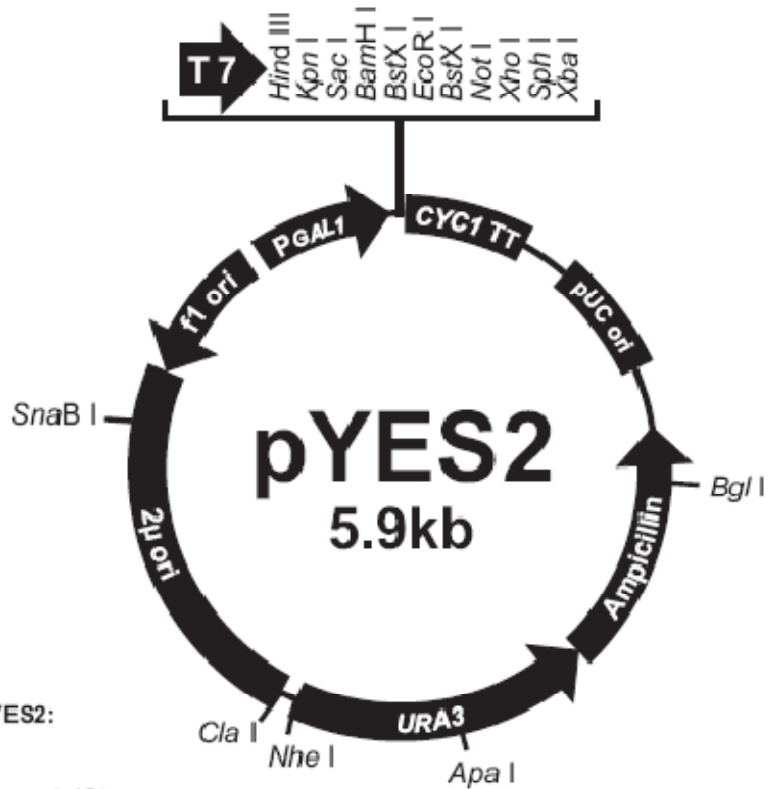
Comments for pYES2:
5856 nucleotides

GAL 1 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

Map of pACT2

pACT2 AD Vector Information

GenBank Accession No.: U29899.
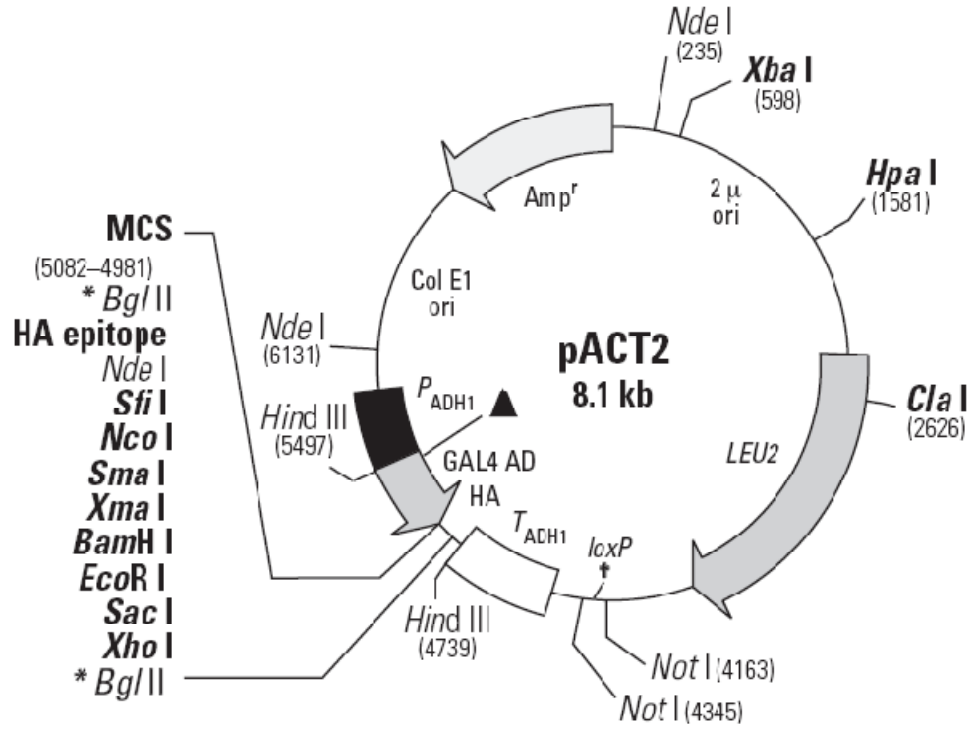
APPENDIX D

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

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