**Molecular genetic analysis of *Will Die Slowly 2 (WDS2)*
gene in *Arabidopsis thaliana***

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LIST OF ABBREVIATIONS

Et : Ethylene

FB1: Fumonisin B1

HR: Hypersensitive Response

JA: Jasmonic Acid

LD: Long Day

PR: Pathogenesis-related

PCD: Programmed Cell Death

ROS: Reactive Oxygen Species

SA: Salicylic Acid

SEFs: Senescence-enhanced factors

SAGs: Senescence Associated Genes

SAR: Systemic Acquired Resistance

SD: Short Day

TEs: Tracheary Elements

TUNEL: Terminal Deoxynucleotidyl transferase d-uridine triphosphate Nick end

Labeling

WD40-RPs: WD40 repeat proteins

WDS: Will Die Slowly

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ABSTRACT

Programmed cell death is a complex process that involves the selective elimination of unwanted cells and is controlled by multiple developmental and environmental signals. Senescence, as a visible form of cell death, is an active process that involves the increased expression of multiple genes and represents a highly regulated developmental stage. In this project, I characterized mutants of the *Will Die Slowly (WDS) 1* and *2* genes. The mutants *wds2* and *wds1 wds2* displayed accelerated leaf senescence, increased chlorophyll leaching and increased sensitivity to osmotic stress, indicating that expression of *WDS1* and *WDS2* genes during senescence has a protective role to retain viability during this fundamental developmental process. *WDS1* and *WDS2* gene expression in wild-type *Arabidopsis* leaves infiltrated with 0.5 mM salicylic acid was increased 48 h after induction. By using the pathogen-derived cell death elicitor FB1, we found that the *WDS2* is a novel gene important for plant cell death signaling related to defense responses. *WDS2* is predicted to be located in the nucleus and is thus likely to act as a regulator of nuclear gene expression. Our work has indicated the complexity of signalling pathways that involve *WDS* genes. Further study will help delineate the pathways and functions of *WDS* genes in senescence and in plant responses to stresses.

Chapter I

Introduction

Programmed cell death (PCD) has been well studied in animals, where it is called apoptosis (Krishnamurthy *et al.*, 2000), while the mechanisms and pathways that regulate PCD in plants remain poorly understood (Lim *et al.*, 2005). Plant PCD or animal apoptosis involve the same morphological and biochemical features, which are defined as physiological processes that involve the selective elimination of unwanted cells. It plays an important role in cell and tissue homeostasis, cellular differentiation, tissue sculpting, and disease resistance (Lam *et al.*, 1999; Lim *et al.*, 2005; Nood'en, 1988). This process takes place in plants for example, during developmental processes, such as gametogenesis, endosperm development, trachery element differentiation, and leaf senescence.

1.1 PCD in different categories of plant cells

In plants, the cell death mechanism has been studied in four categories: I) cells that die after performing a specific function, II) cells that die throughout terminal differentiation into specialized cell types, III) cells that are subjected to biotic and abiotic stresses, and IV) cell that are already unable to function from the beginning (Krishnamurthy *et al.*, 2000). The following is a description of the analyses done on these four categories of cell death.

In the first category, cells that undergo cell death after performing their function have a similar mechanism of PCD to that of animal apoptosis. It was found that they share common characteristics, such as cytoplasmic condensation and shrinkage, general cell shrinkage, presence of effector proteases, reactive oxygen

species (ROS) production, increase in cytosolic calcium, phosphorylation / dephosphorylation changes, chromatin condensation, and activation of endonucleases causing DNA fragmentation (Fukuda, 1997). The latter characteristic was measured by electrophoretic ladder formation and terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labeling (TUNEL) detection where the size of the DNA fragments obtained was around 180 bp (Fukuda, 1997).

The second category includes tracheary elements (TEs), sclerenchyma fibres, sclerids and cork cells, which all undergo significant morphological changes during cell death. These cells die so that the cytological events associated with cell death are oriented towards making them persist and function until the end of the life of the plant. They exhibit cell enlargement and elongation during PCD. They do not show cell shrinkage, which is common in all other cells that undergo PCD. The nuclei of the cells are metabolically very active and undergo endoduplication and polyploid changes throughout the differentiation process. They are found to be transcriptionally extremely active and are responsible for the production of several chemicals that constitute a significant fraction of the persisting specialized secondary cell wall. These cells have proteases, thought to function in degrading cytoplasmic proteins, which are then recycled for use in organizing the specialized secondary wall (Fukuda, 1997). These cells also contain endonucleases causing DNA fragmentation.

The third category includes cells that are subjected to different abiotic and biotic stresses resulting in a hypersensitive response (HR). This category is the most studied category in plants. Studies have indicated that among a population of cells subjected to environmental stresses, there may be cells that are undergoing PCD,

while others are depending on the strength of other stress factors and proximity of the cell to the stress agent. This is shown by the extent of cytological variations during stress-induced cell death in plants. Other stress-related PCD work have indicated that the overall changes observed may be due to PCD, which appear as necrotic in plant tissue (Krishnamurthy *et al.*, 2000).

Finally, for the fourth category, a failure occurs in the initial stage of plant development due to injury or any other reasons leading to cell damage. There is not enough research that has been carried out to determine the mechanisms of cell death and physiological roles. Krishnamurthy *et al.*(2000) indicated that the nonfunctional stamens in male flowers and nonfunctional carpel primordial cell in female flowers and/or nonfunctional cells formed during embryoid initiation in culture cells are examples of this type of cell death (Krishnamurthy *et al.*, 2000). There is not much evidence to support this category and so definite conclusions cannot be made.

1.2 PCD and leaf senescence

Leaf senescence is the end of life span of leaf development, which is a complex process that is controlled by multiple developmental and environmental signals and is marked by induced expression of a large number of different genes (Guo *et al.*, 2004). It is clearly marked by leaf colour change, which is associated with active physiological and biochemical processes such as chlorophyll breakdown, RNA and protein degradation, and translocation of nitrogen, carbon nutrients, and minerals from the senescing leaves to other parts of the plants. In other words, leaf senescence is the descent of age-dependence in plant development at the cellular, tissue, organ, or organism level, leading to death or the final stage of leaf

development (Nood'en, 1988). Therefore, even though the initiation of leaf senescence is a lethal process, it can be seen as beneficial for the plant. It significantly contributes to the fitness of entire plants promoting most favorable production of offspring and better survival of plants in their given temporal and spatial functions. Consequently, it is considered an evolutionarily selected developmental process and an important stage in the plant life cycle (Buchanan-Wollaston *et al.*, 2003; Lim and Nam, 2005). However, leaf senescence in agricultural aspects plays an essential role in limiting crop yield in plants by limiting the growth phase. Moreover, it may cause post harvest decaying such as leaf yellowing and nutrient loss in vegetable crops. Therefore, the study of this process not only improves our understanding of the basic biological mechanisms, but also provides us with means to improve crop yield and quality.

Onset of leaf senescence is essentially controlled by developmental age. Nevertheless, it is influenced by a variety of internal and environmental signals. The environmental factors that manipulate leaf senescence include abiotic and biotic factors. The abiotic factors are drought, nutrient limitation, temperature extreme, and oxidative stress by UV-B irradiation or ozone, for example. The biotic factors are pathogen infection and shading by other plants (Lim and Nam, 2003). Plant growth and development can be significantly altered under these critical environmental conditions. Leaf senescence involves cell death that is influenced by age under the control of endogenous and environmental factors. Programmed cell death (PCD) is a self-destructing cellular system activated by internal or external factors and mediated throughout an active genetic program (Quirino *et al.*, 2000). Since cell death in leaf senescence is influenced by many active genetic programs, the cell death occurring

in leaf senescence is a type of PCD (Cao *et al.*, 2003). A leaf is composed of many cell types. The mechanism of cell death in leaf senescence initiates from mesophyll cells and then proceeds to other cell types. Furthermore, it appears that cell death does not occur consistently, but rather begins with a restricted area of early dying cells and after that spreads through the entire leaf (Cao *et al.*, 2003). In most plants, PCD is also involved in the development of tracheary elements, germination-related degeneration of aleuron layer cells, and pathogen-induced hypersensitive response (HR) (Lara *et al.*, 2004; Van Doorn, 2005). Some features of PCD in leaf senescence are distinctive from other types of PCD. First, leaf senescence involves an organ level cell death that includes the whole leaf, while other types of PCD require rather localized cell death or take place in limited tissues and cell types. Second, the rate of cell death during leaf senescence is slower than that in other types of PCDs. Third, the molecular and biological function of PCD in leaf senescence is characterized by remobilization of nutrients from the leaf to other part of organs (Van Doorn and Woltering, 2004). Leaf tissue is the most important photosynthetic organ in plants. Therefore, the optimal use of nutrients accumulated through photosynthesis and nutrient metabolism is important for the strength of the plant and for crop yield. The slow degeneration of cells throughout leaf senescence may ensure effective remobilization of nutrients (Van Doorn and Woltering, 2004).

1.3 PCD and plant disease resistance

Plants have evolved multiple mechanisms to defend themselves against attacks of bacteria, viruses, fungi, oomycetes, nematodes and insects. Disease resistance in higher plants sometimes occurs in a 'gene-for-gene' mode, in which a plant expressing a specific resistance (*R*) gene recognizes a pathogen strain

expressing a corresponding effector (avirulence) gene (*avr*) and stimulates defense responses (Kirankumar *et al.*, 2002). This type of interaction has been proposed to involve four different steps: (1) delivery of a pathogen-produced elicitor molecule to the plant cell; (2) identification of this signal molecule by the plant cell; (3) signal transduction, which involves many pathways; and (4) the activation of defence responses (Ynag-Qiang and Gregory, 1998). To date, *R* genes have been identified that encode resistance to many pathogen types with very various lifestyles, outside or inside the plant cell (Bent, 1996).

Higher plants have multiple defense responses (Lamb *et al.*, 1994). One of them is called the hypersensitive response (HR), which results in plant cell death at the site of pathogen attack that can limit pathogen ingress (Hammond-kosack & Jones, 1996). In the past fifteen years, there has been considerable progress in the study of its role in plant defense (Jones, 2001; Lam *et al.*, 1999). Two major types of cell death can result when plants are inoculated with a pathogen: the rapid hypersensitive response (HR), which is typically associated with gene-for-gene resistance, and a slower form of death associated with disease. Hypersensitive cell death occurs at the site of attempted attack by an avirulent pathogen, and the hypersensitive response leads to the formation of necrosis that is clearly delimited from surrounding healthy tissues. HR cell death is an active process in which the accumulation of O_2^- and H_2O_2 leads to an elevation in cytosolic Ca^{2+} and triggers a protein kinase-mediated cell death process (Levine *et al.*, 1996). Certain mutations in maize and Arabidopsis cause the spontaneous appearance of lesions resembling HR lesions (Lamb and Dixon, 1997), suggesting that HR cell death is under genetic control. Also, localized inoculation of Arabidopsis with an avirulent strain of

Pseudomonas syringae leads to hypersensitive response and also triggers low-frequency cell death in uninoculated leaves (Danon *et al.*, 2000). This cell death in uninoculated leaves begins at approximately the same time as cell death in primary HR. Cell morphology studies and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) experiments suggest that cell death in uninoculated leaves is also PCD (Pennel and Lamb, 1997). The possibility that PCD occurs in uninoculated leaves which may contribute to systemic disease resistance, would represent a novel function for PCD.

1.4 Genetic approaches in the analysis of PCD

Two main genetic approaches have been taken to study of PCD in senescence and disease resistance. First, genes can be determined by the isolation and characterization of mutants (Buchanan-Wollaston *et al.*, 2003). Second, determination of differential gene expression to identify senescence-regulated genes and molecular cell death markers. The rapid progress in genomics and bioinformatics resources and the use of the model plant species *Arabidopsis thaliana* has greatly facilitated these approaches. *Arabidopsis* is considered one of the best model plants for the molecular genetic study of leaf senescence and disease resistance due to many reasons: first, *Arabidopsis thaliana* has a short life cycle; second, its leaves undergo clear developmental stages and demonstrate a reproducible senescence program; third, the vast *Arabidopsis* genomic resources accelerate the functional analysis of senescence-associated genes (SAGs) (Bleecker and Patterson, 1997; Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2003).

Microarray technology and the availability of publically available genomic databases has also significantly accelerated the discovery and functional analysis of

novel genes. Zhu and Wang (2000) designed their microarray experiment to analyze the gene expression levels at three different stages of leaf development by using Affymetrix Arabidopsis GeneChips, which carried probes for around 8000 Arabidopsis gene. They found approximately 1400 genes that showed relative changes in expression patterns during leaf development, senescence and defense responses.

1.5 Salicylic acid has a role in regulating gene expression during leaf senescence

As described earlier, leaf senescence is a complicated process that is controlled by multiple internal and external factors and is marked by induced expression of many genes. Many studies have shown that the salicylic acid-signalling pathway has a role in the control of gene expression during leaf senescence. Salicylic acid (SA) is also known as a major plant growth regulator that is involved in defense against pathogens (Appendix B).

However, a recent study in programmed cell death identified a role of SA in the initiation of leaf senescence. In *Arabidopsis thaliana*, the endogenous SA concentration was found to be four times higher in senescencing leaves compared to younger leaves (Morris *et al.*, 2000; Ryals *et al.*, 1996; Ward *et al.*, 1991). Senescence at this stage appears to involve the up-regulation of many senescence-associated genes (SAGs). Gene expression of SAGs such as *PR1a* (*LSC94*), *chitinase*, and *SAG12* are altered in Arabidopsis plants defective in the salicylic acid signalling or biosynthetic pathway (*pad4* mutants, and *NahG* transgenic plants) (Morris *et al.*, 2000). Morris *et al.* (2000) also showed that the expression of the *PR1a* (*LSC94*) gene is strongly induced in green leaves treated with SA.

Nevertheless, the *SAG12* gene showed no induction, which indicated that expression of *SAG12* is not solely dependent on SA. In addition, Arabidopsis leaves from *pad4* mutants have reduced cell death compared to the wild type (Morris *et al.*, 2000). In this mutant, changes in gene expression patterns were accompanied by reduced necrosis indicating that there might be a genetic control switch regulating the transition from senescence to cell death. It is possible that senescing leaves are sustained long enough for efficient reclamation of nutrients. After that, individual cells are damaged by PCD. Also, the recessive *pad4* mutation suggests that *NPR1* and *PAD4* are directly involved in SA signaling. *Pad4* mutant was also isolated and characterized by screening for increased susceptibility to virulent strains (Jirage *et al.*, 1999). After pathogen infection, *pad4* mutant has reduced PR-1 expression and salicylic acid accumulation. Therefore, since the *pad4* mutant has reduced defense responses when treated with pathogens that elicit a strong hypersensitive response (HR), *PAD4* is considered to increase weak salicylic acid signals (Jirage *et al.*, 1999).

To date, few studies have addressed the regulatory network governing leaf senescence and coordinating with plant defense signaling pathways in Arabidopsis (Bekir, *et al.*, 2007; Li *et al.*, 2004). Morris *et al.* (2000) present a critical model of signalling networks leading to alterations in gene expression during leaf senescence. Morris and colleagues proposed that when the onset of senescence is induced, a number of different signalling factors (senescence-enhanced factors, SEFs) are involved in controlling the genes that carry out the senescence process. SA is a signalling factor essential for the expression of certain senescence-enhanced genes, some of which (*LSC94* and *LSC222*) are probably independent of any other factors.

However, other genes are independent of SA and at the same time are expressed in tissues that are not undergoing senescence (e.g. *LSC54*, *LSC7* and *LSC30*) (Morris *et al.*, 2000). The reason for enhanced-expression of these genes during senescence requires further study.

Jose *et al.* (2008) stated that an internal factor such as Acetylsalicylic acid (ASA) behaves as an agent regulating PCD. ASA is derived from the plant hormone salicylic acid that activates the expression of a variety of pathogenesis-related (PR) genes and induces resistance to a variety of pathogens. To illustrate, an important progress in understanding of SA-mediated signaling pathways was the isolation and characterization of *Arabidopsis npr1* mutant (Kinkema *et al.*, 2000; Yu, *et al.*, 2001). This mutant failed to express and induce pathogenesis-related gene expression in response to salicylic acid. This suggests that NPR1 is directly involved in SA signaling.

1.6 Fumonisin B1 (FB1)-induced cell death in *Arabidopsis* requires SA dependent signaling pathway

FB1 is a sphingoid-like compound secreted from different species of fungi and often associated with plant fungal diseases. The fumonisins have been shown to be pathogenicity or virulence factors in *Fusarium moniliforme* (Asai *et al.*, 2000). Gilchrist *et al.* (1998) found that FB1 enhances PCD in wild-type protoplasts. Nevertheless, FB1 only slightly affects the viability of protoplasts isolated from the *Arabidopsis NahG* mutant, in which SA is degraded, and from the *Arabidopsis pad4* mutant, in which SA signal amplification progress is impaired. Thus, treating *Arabidopsis* plants with FB1 will initiate degradation of the nuclear DNA membrane, preceding the loss of membrane integrity. Although little is known about its target

and the nature of its activity in plant tissues, FB1-induced cell death is dependent on active transcription and translation factors and reversible protein phosphorylation (Asai *et al.*, 2000; Chivasa *et al.*, 2005). It has been utilized as the basis of model systems to investigate cell death in pathogen-free-conditions, which is advantageous as it avoids both the confounding effects of pathogen growth in experimental media and tissues and the necessity of maintaining and propagating pathogenic organisms (Chivasa *et al.*, 2005). In addition, FB1-induced cell death in *Arabidopsis* requires SA-, jasmonic acid (JA) -, and ethylene (Et)-mediated signaling pathways.

1.7 Mannitol accumulation in response to abiotic and biotic stresses

Plants have a significant ability to deal with highly variable environmental stresses, including cold, drought, and hyperosmotic stress. Today, these stresses represent the major cause of crop loss worldwide such as reducing average yields for most major crop plants by more than 50%. On the contrary, the estimated yield loss caused by pathogens is found to be around 10% to 20%. Significant progress has been study to understand and manipulate abiotic stress responses (Galaud *et al.*, 1997; Kiedrowski *et al.*, 1992; Williamson *et al.*, 1995; Kerps *et al.*, 2002; Loescher *et al.*, 1992).

Mannitol, a six carbon sugar alcohol, has previously studied by plant scientists. They found that species that metabolize mannitol have several advantages over those that completely translocate sugars. One advantage is used as hyperosmotic stress indicator. Another advantage is probably play an important role in plant responses to pathogen attack. Therefore, mannitol metabolism may play roles in plant responses to both biotic and abiotic stresses. The molecular and

genetic basis of plant tolerance or sensitivity to hyperosmotic stress is poorly understood. (Chen *et al.*, 2005; Kerps *et al.*, 2002; Kiedrowski *et al.*, 1992 ;Williamson *et al.*, 1995; Loescher *et al.*, 1992).

1.8 Plant MAPK cascades play a role in leaf senescence

Today, biologists consider signal transduction as one of the most widely studied fields. Extracellular signals from the surface of a cell are translated into an intracellular response that involves complex signaling pathways. These signaling actions eventually control such cellular responses as proliferation, differentiation, secretion, and PCD. To illustrate, signal transduction cascades are activated by the binding of extracellular ligands (e.g. biotic and/or abiotic factors) to cell-surface receptors. These receptors transducer the extracellular stimulus to the intracellular fluid, where the signal activates other signaling factors such as the mitogen-activated protein kinase (MAPK) signaling pathways (Hopkins *et al.*, 2007; Murray, 1998; Xiong *et al.*, 2005). This pathway plays a significant role in signal transduction in animal and plant cells (Champion *et al.*, 2004). Plants are equipped with this defense system which consists of MAPKs, MAPK kinases (MAPKKs) and MAPK kinase kinases (MAPKKKs). There are twenty MAPKs, 10 MAPKKs, 60 MAPKKKs, 15 kinases of the Raf family in Arabidopsis (MAPKK Group, 2002). Doczi *et al.*, (2007) found that MPK1, MPK2, MPK7, and MPK14 were identified as downstream substrates of MKK3, as revealed by yeast two-hybrid. However, a complete identification of MAPK cascade in Arabidopsis has been a challenging problem (Champion *et al.*, 2004; Doczi *et al.*, 2007; MAPKK Group, 2002; Zhou *et al.*, 2009).

The pathway of MAPK cascades is associated with different physiological, developmental, and hormonal responses. In addition, molecular and biochemical studies have indicated that MAPKs cascades are associated with many factors such as pathogen infection, wounding, temperature, drought, high salinity, and reactive oxygen species (Champion *et al.*, 2004; MAPKK Group, 2002; Tena *et al.*, 2001; Zhou *et al.*, 2009). The MAPKK is stimulated by an upstream MAPKKK and, in turn, triggers the MAPK. Then, the stimulatory MAPK activates various other protein kinases, catalyses the phosphorylation of cytoskeletal components, or regulate the function of transcription factors (Hirt, 1997). The MAPK pathways also integrate signal convergence and divergence, transmit positive and negative regulatory mechanisms, and interact with scaffold proteins. Most of the substrates for stress-stimulated MAPKs in animal and yeast cells are transcription factors (Davis, 2000). Therefore, the activation of MAPK cascades leads to the phosphorylation of transcription factors, which stimulate gene expression. Similar roles in plants are supported by experiment in Liu *et al.*, (2000). They identified a MAPK that moves into the nucleus upon activation in parsley cells treated with Pep25 elicitor.

There is not much evidence showing that MAPK cascades play any roles in controlling leaf senescence. However, Zhou *et al.*, (2009) verified the interaction of a new signaling pathway in Arabidopsis that entails both *MKK9* and *MPK6* in leaf senescence at the biochemical, physiological, cell and molecular genetic levels (Zhou *et al.*, 2009). These genes have kinase activities, and the loss-of-function and gain-of-function analyses have revealed that MKK9-MPK6 cascade positively regulates leaf senescence in Arabidopsis (Zhou *et al.*, 2009).

1.9 Will Die Slowly (WDS) family members

Hollmann *et al.*, (2002) described the characterization of the essential *Drosophila melanogaster will die slowly (wds)* gene. They found that the predicted amino acid sequence contains seven WD40 repeats (Hollmann *et al.*, 2002). These structural and mechanistic aspects of WD40 repeats are described below.

WD40-RPs (repeat proteins) play fundamental roles in such distinct mechanisms as signal transduction, nuclear export, cytoskeletal assembly, vesicular traffic and fusion, and RNA synthesis/processing (Letunic *et al.*, 2008). In addition, WD40-RPs are intimately involved in chromatin modification and transcriptional mechanisms (Dan-Qiao *et al.*, 2005; Letunic *et al.*, 2008; Myoung *et al.*, 2006; Neer *et al.*, 1994). Moreover, WD40-RPs play a key role in different cellular and organismal processes such as cell division and cytokinesis, apoptosis, light signaling and vision, cell motility, flowering, floral development, and meristem organization (Neer *et al.*, 1994).

Furthermore, WD40-RPs typically have four to ten repeat units, each composed of approximately 44-66 amino acids which form core and variable regions. Each repeat unit ends with the dipeptides Gly-His (GH) at the N termini and Trp-Asp (WD) at C termini (Smith *et al.*, 1999). The structure of the WD40-repeat proteins has been determined for a mammalian G β subunit of heterotrimeric GTPases (Letunic *et al.*, 2008; Hollmann *et al.*, 2002). The repeated Trp-Asp units form a series of four-stranded, and antiparallel β -sheets, which fold into a higher-order structure termed a β -propeller. Chothia *et al.* (1997) stated that the structure of WD40-RPs can be visualized as a short, open cylinder where the strands form the walls and at least four repeats together are required to form a β -propeller.

Where studied, WD40-RPs domains within proteins work as sites for interaction with other proteins, which allows for three general functional roles. First of all, the interaction of WD40-repeat proteins within one protein can provide binding sites for more than one other protein, as well as promote transient interaction amongst these other proteins. Secondly, WD40-RPs have a potential role as an integral component of protein complexes. Finally, WD40-RPs act as a modular interaction domain of larger proteins (Letunic *et al.*, 2008; Tullio-Pelet *et al.*, 2000; Van der-Voorn and Ploegh, 1992).

Mutation of *WDS* gene in *Drosophila* led to lethality in the larval stages (Shannon *et al.*, 1972). The role for *WDS* in *Drosophila* development has not been further investigated. The lethality of mutant alleles in gynandromorphs and germ cell clones indicates that the gene is important in most of the cells and the presence of WD40 repeats is indicative of protein-protein interactions (Hollmann *et al.*, 2002; Komachi *et al.*, 1997; Tie *et al.*, 1998). Five predicated proteins in *Arabidopsis thaliana* shows significant amino acid sequence similarity to *WDS* of *Drosophila melanogaster* (Xiong, 2007). The *Drosophila* homolog has been implicated in cell death and this provided the initial rationale for testing the role of *Arabidopsis WDS2*.

Previously, Xiong (2007) identified and described the five-member gene family, *Will Die Slowly (WDS)*, of *Arabidopsis* (Figure 1.1). The *WDS* proteins contain seven WD40 repeats (also called Trp-Asp repeat) (Figure 1.1). There are around 269 different proteins in *Arabidopsis* that have WD40 repeats, representing distinct cellular and biochemical pathways (van Nocker and Ludwig, 2003).

Figure 1.1: Phylogenetic tree (a) and domain structure (b) of WDS family members. Protein sequences of WDS1-5 were aligned using ClustalW version 1.83. The phylogenetic tree was generated from this sequence alignment indicating that WDS2 is mostly related to WDS1. Sequence analysis in SMART showed that the protein of WDS1, WDS2, and WDS3 contain three conserved motifs: the 33-residue LisH motif, CTLH domain and seven WD40 repeat (Letunic *et al.*, 2008; Smith *et al.*, 2000).

1.10 Hypothesis and Objectives

In this project, we characterized the *WDS2* gene and analyzed its potential functions in cell death and disease resistance. The *WDS2* gene was initially investigated using the genomic tools available in the Arabidopsis Information Resource (TAIR). Furthermore, I studied the nature of members of the *WDS* gene family and determined the *WDS1* and *WDS2* gene expression profiles during leaf senescence and PCD. As mentioned earlier, Xiong (2007) studied *WDS1* and found that a knockout of *WDS1* accelerates the induction of *SAG12* expression triggered by FB1, indicating a regulatory role of *WDS1* in stress-induced senescence. In addition, *WDS1* expression was found to up-regulated by SA as well as the overexpression of *WDS1* in Arabidopsis enhances the tolerance to osmotic stress. These results supported her hypothesis that the *WDS1* gene is involved in cell death progression triggered by both developmental and stress signals in Arabidopsis. Therefore, in this project, characterizing the related *WDS2* gene including studying the phenotype of the *wds2* mutant, and a double mutant of *wds1 wds2*. One of the most important objectives in this study was to investigate how *WDS2* is coordinately regulated during this process. For example, is *WDS2* involved in the FB1 and SA pathway?

My hypothesis is that *WDS2* has an important role in leaf senescence and plant development by regulating PCD. My goals were:

- (1) phenotypic and functional analysis of *WDS2* in knockout lines;
- (2) create a double mutant between *wds1* and *wds2* for phenotypic and functional analysis;

- (3) construct tagged (GFP) fusions of *WDS2* for subcellular localization by confocal microscopic analysis;
- (4) study the function of *WDS2* in SA pathways, responses to hyperosmotic stress, and treatment with the fungal elicitor Fumonisin B1.

Chapter II

Materials and Methods

2.1 Plant materials and growth conditions

Arabidopsis T-DNA insertion lines of *WDS2* in Columbia-0 (Col-0) ecotype background were obtained from the Arabidopsis Biological Resource Centre (ABRC) (Ohio State University). Plants were grown either in a long day condition (16 hours of light, 8 hours of dark) or a short day condition (8 hours of light, 16 hours of dark) at 22 °C in growth chambers (ENCONAIR Technologies Inc, Winnipeg, Monitoba). Seeds were surface sterilized for 2 min in 70% ethanol and then soaked for 8 min in sterilization solution (25% Bleach v/v and 0.01% Triton X-100 (v/v). Seeds were then rinsed 10 times with autoclaved water and then suspended in 0.1% agarose solution. For the growth and development experiments, sterilized seeds were sowed directly onto autoclaved soil (BM1, Montreal, Quebec, Canada). For phenotypic and hyperosmotic stress analyses, seeds were germinated on petri plates containing 1 x Murshige and Skoog (MS) basal medium (Sigma, St Louis, MO, USA), 3.0% sucrose (w/v) and 0.8% agar (w/v) (EMD Chemicals Inc. Darmstadt, Germany). The pH of the media was adjusted to 5.8. The plates were kept in the dark at 4°C for 4 days. The plates were then moved to the growth chamber for 7-10 days. The seedlings were transferred to autoclaved soil at the 2-rosette leaf stage.

2.2 Genotyping loss-of-function T-DNA lines

Two different SALK T-DNA insertion lines (SALK_095495 and SALK_100917) of *wds2* mutant were characterized (Alonso *et al.*, 2003). *Arabidopsis thaliana* ecotype Columbia-0 (Col0) is used as the wild type control for these lines. Extract-N-AmpTM plant PCR kit (Sigma Aldrich, Oakville, ON, Canada) was used for DNA extraction. Leaf tissues (0.5-0.7 cm long) were collected in 1.5 mL Eppendorf tubes and frozen in liquid nitrogen. A 100 µL aliquot of extraction solution was added to the collection tube. The tube was then incubated at 95 °C for 10 min. After that, 100 µL of dilution solution was added and the sample was then stored at 4°C. The following reagents were then added to 0.5 mL PCR microcentrifuge tube: 1 µL PCR reagent, 10 µL Extract-N-AMP PCR reaction, 3 µL of 10 µM forward primer, 3 µL of 10 µM reverse primer and 3 µL leaf disk extract. The homozygous mutant was identified by using PCR with primers that flank the T-DNA insertion under the following conditions: 94 °C for 3 min; 30s at 94°C; 30s at 59°C; 30s at 72°C for 34 cycles; and then 10 min at 72°C. The primer sequences were: 5'-CAAACTCAATTCAGGCAAGC-3' (LP) and 5'-CTGATGAGAAGGCTGTCAAGG-3' (RP) for SALK_095495, and 5'-CAAACTCAATTCAGGCAAGC-3' (LP) and 5'-CCATTAAGCTGTTTCTGCAGC-3' (RP) for SALK_100917. The presence of the T-DNA insertion was confirmed using gene specific primers and a primer designed to the left border (either LBa1 or LBb1) of the T-DNA insertion. The primer sequence of LBa1 was 5'-TGGTTCACGTAGTGGGCCATCG-3' and LBb1 primer was 5'-GCGTGGACCGCTTGCTGCAACT-3'.

A complete loss-of-function of *wds1* and *wds2* were obtained as described above. A loss-of-function mutant of *wds1 wds2* double mutant was generated by crossing the *wds1* and *wds2* mutants and genotypic identification in the F2 generation.

2.3 Confirmation of loss-of-function using reverse transcriptase PCR (RT-PCR) to monitor transcript levels

Total RNA was extracted from the loss-of-function SALK T-DNA lines after growth for ~10 days on solid media (as described earlier in section 2.1). TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction according to manufacturer's protocol. Leaf tissue (100 mg) was homogenized in 1 mL of TRIzol. The homogenized sample was incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. An aliquot of 200 μ L of chloroform was then added to the sample, mixed vigorously by hand for 15 seconds, and incubated at RT for 2-3 min. The sample was centrifuged for 15 min at 12,000 x g at 4°C. Following centrifugation, the mixture separated into a lower, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase was about 60% of the volume of TRIzol reagent used for homogenization. The aqueous phase was transferred into a fresh tube and 500 μ L of isopropyl alcohol was added. The sample was incubated for 10 min at RT and centrifuged for 10 min at 12,000 x g at 4°C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 1 mL of 75%

ethanol (diluted with DEPC water). The sample was mixed by vortexing and centrifuged 5 min at 7,500 x g at 4°C. At the end of the procedure, the RNA pellet was air-dried for 5 min. The pellet was dissolved in 50 µL of RNase-free water and stored at -80°C.

After TRIzol extraction, Deoxyribonuclease I kit (amplification grade, Invitrogen, Carlsbad, CA, USA) was used to eliminate genomic DNA contamination in the sample. The following was added to an RNase-free, 0.5 mL microcentrifuge tube on ice: 1-5 µg RNA sample, 1 µL 10X DNase I reaction buffer (100mM Tris-HCL (pH 7.5), 25mM MgCl₂, 5 mM CaCl₂), 1 µL of 1U/ µL DNase I, and DEPC-treated water to 10 µL. The sample was then incubated for 15 min at RT. DNase I was inactivated by addition of 1 µL of 25 mM EDTA solution to the reaction mixture. The sample was heated 10 min at 65°C.

Next, the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis according to the manufacturer's protocol. A master mixture of 4 µL of 5x cDNA synthesis buffer (250 mM Tris acetate (pH8.4), 375 mM potassium acetate, 40 mM magnesium acetate, stabilizer, 20 µg/mL BSA), 1 µL of 0.1 M DDT, 1 µL of RNaseOUT (40U/µL), 1.5 µL of DEPC-treated water and 0.5 µL of cloned AMV RT (15U/µL) was prepared and kept on ice. Also, in a 0.5 mL tube, the following components were mixed: 1 µL of 50 µM Oligo(dt)₂₀, 1-5 µg RNA sample, 2 µL of 10 mM dNTP mix and DEPC-treated water to 12 µL. The mixture was incubated for 5 min at 65°C. Eight µL of the master mixture was added to the above 0.5 mL reaction tube. Then, the reaction tube was transferred to a thermal cycle and heated for 48 min at 48°C, and then for 5 min at 85 °C. RNA and DNA

quantity was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The primers used for RT-PCR were, 5'-ATCGCCAGTGGAAGTGAGGATTC-3' and 5'-AGTTCAGTGGTTTGTTCGGTTTGC-3' for *WDS1*; 5'-CCAAGGGGATGACGAACTA-3' and 5'-GGAAACCGATTCTGTGCAAT-3' for *WDS2*; 5'-AGACGCTGGGATGTTGATTC-3' and 5'-CATGGCCTGGTAGCTCAACT-3' for *WDS2*; 5'-CGGTTAGCGTTGGAATTGAAGGAG-3' and 5'-TCCGTTAGTAGATTGCGCGTATCC-3' for *SAG12*; 5'-CCTCATGCCATCCTCCGTCTTG-3' and 5'-TTCCATCTCCTGCTCGTAGTCAAC-3' for *ACTIN2* (*At3g18780*); and 5'-TTCTTCCCTCGAAAGCTCAA-3' and 5'-AAGGCCCACCAGAGTGTATG-3' for *PR-1* (*At2g14610*). PCR was carried out under the following conditions: 94 °C for 3 min; 30s at 94°C, 30s at 59°C, and 30s at 72°C for 25 cycles; and then 10 min at 72°C. All the primers were designed to amplify products in the range of 100-300 bp.

2.4 Root length analyses

Sterilized seeds of Col-0, *wds1*, *wds2* and *wds1 wds2* were sowed in vertical MS-agar-plate and kept in the growth chamber with long day condition. After 9 days, the root length (cm) of the above seedlings plants was measured.

2.5 Treatment with Salicylic acid (SA) and chlorophyll content analysis

Leaves from three-to-four-week-old plants (Col-0, *wds1*, *wds2*, and *wds1 wds2*) were collected, cut to squares of about 0.6-0.8 mm² and then were treated with 0.5 mM and 2.0 mM SA solution. These leaf discs were infiltrated by a vacuum pump for 20 min and then were placed upward on the filter paper in a Petri dish so that they were just covered by the SA solution. Water was used as a control. Samples after 0h, 24h, 48h, and 72h incubation were collected for various analyses.

Fresh tissues (50 mg) from Col-0, *wds1*, *wds2*, and *wds1 wds2* were collected after treatment. 1 ml of 95% EtOH was added to tissues and incubated at 65°C for 2 hours. The OD649 and OD665 were measured. The micromolar concentration of total chlorophyll per gram of fresh weight of tissue was calculated using the following equation:

Total micromoles [chlorophyll a + Chlorophyll b] = 6.1 OD665+ 20.04 OD649.

2.6 Treatment with Fumonisin B1 (FB1)

Approximately seven fully expanded leaves were harvested from 3-week-old plants. Detached leaves were infiltrated with three different concentrations of FB1 (1 µM, 2 µM and 5 µM) as an elicitor to trigger cell death. After infiltration, leaves were incubated in FB1 solution in the growth chamber at 22°C under 16-h light/8-h dark condition. Samples after 0h, 24h, 48h, 72h, 96h, and 120h of incubation were collected for staining assays (section 2.7).

2.7 Trypan blue and aniline blue staining

Cells of *Arabidopsis* rosette leaves undergoing cell death were photographed using an Axioplan 2 microscope (Carl Zeiss). Methods described by Tang *et al.* (1999) and Stone *et al.* (2000) were used with slight modifications. Falcon tubes containing the leaves were boiled at 95°C for 4 min and kept at room temperature for 20 min instead of 30 min for the detection of autofluorescent materials.

Arabidopsis rosette leaves were immersed in 10 mL of ethanol-lactophenol (2 volumes of ethanol and 1 volume of phenol-glycerol-lactic acid-water (1:1:1:1)) that contained 0.05% trypan blue. The leaves were placed in 15 ml Falcon tubes and covered with ethanol-lactophenol-trypan blue. The samples were incubated at 95°C for 4 min and then kept at room temperature for 20 min. The staining solution was removed and 1.5 mL chloral hydrate destaining solution (2.5 g/mL of nano pure water) was added to each tube. The leaves were cleared for 2 days by replacing the destaining solution twice. After destaining, leaves were suspended in 50% glycerol and examined under Axioplan 2 microscope (Cark Zeiss) with white light.

Arabidopsis rosette leaves were immersed and vacuum-infiltrated in 10 mL of ethanol-lactophenol (2:1 v : v) and then incubated at 60°C for 30 min. Leaves were rinsed in 50% ethanol and stained overnight with aniline blue (0.01% aniline blue powder in 150 mM K_2HPO_4 , pH 9.5). Leaves were equilibrated in 50% glycerol and aniline blue staining was visualized using a UV epifluorescence (Axioplan 2 microscope, Carl Zeiss) with a DAPI filter (Scan range: 320-520 nm).

2.8 DNA construct for fusion of WDS2 with green fluorescent protein, transformation in to *Agrobacterium tumefaciens*, and transient delivery into plant cells

The cDNA of *WDS2* was obtained from ABRC (Ohio State University). The primers used for amplification were At5g08560-F*Sall*: 5'-AGAGTCGACATGGGAGTTGTGGAGGATACTG-3' and At5g08560-R*Bam*HI: 5'-AGAGGATCCTCAATTCCCATTGCATCGGT-3'. Two different flanking restriction enzyme sites, *Sall* and *Bam*HI, were introduced. Both the *WDS2* and *pVKH18::GFPN* vector were digested using the corresponding restriction enzymes to generate 'sticky' ends. The cDNA fragments were ligated using T4 DNA ligase (Invitrogen, Burlington, Canada). The ligation mixtures were used to transform competent DH5 α *E. coli* to generate recombinant plasmid *pVKH18::GFP-WDS2*. A total of 10 μ l of DNA per reaction was sent for sequencing (National Research Council of Canada, Saskatoon, SK). Then, the *pVKH18::GFP-WDS2* construct was transferred to *Agrobacterium tumefaciens* strain GV3101 using a modified freeze-thaw method. *Agrobacterium tumefaciens* strain GV3101 carrying the binary plasmid construct was streaked on LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 7 g/L agar) with the added antibiotics (kanamycin (50 μ g/mL), gentamycin (25 μ g/mL) and rifampicin (50 μ g/mL)) and incubated at 28°C, and for 3 days. A single *Agrobacterium* colony was inoculated into 5 mL of sterilized LB medium (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) containing the same antibiotics and grown at 28°C, and at 225 rpm for 3 days.

2.9 Subcellular localization

Cell cultures of *Agrobacterium* were grown for 3 days. Cells were collected in 1.5 mL Eppendorf tubes by centrifugation for 10 min at 1000 *g* at RT. Then, the supernatant was discarded and the pellets were resuspended in 1 mL of infiltration medium (500 mM MES, 20 mM Na₃PO₄·12H₂O, 1 M acetosyringone and 1 M D (+)-glucose). The cells were pelleted again and then resuspended in 1 mL infiltration medium. The second wash with 1 mL infiltration medium was repeated once to remove any trace of antibiotics that may kill the leaves after infiltration. Following this, 0.3 mL of infiltration medium was injected by syringe into leaves of 5-week-old tobacco leaves. Subcellular localization was examined using an LSM 510 META confocal microscope (Carl Zeiss).

2.10 Osmotic stress treatment

Sterilized seeds of *Arabidopsis thaliana* Col-0 were sown in Magenta boxes containing MSagarmedia and plants were grown in a long day chamber. Sixteen-day-old seedlings of Col-0 were sprayed with 0.6 M D-mannitol or water as a mock control. The boxes were transferred back to the growth chamber. Leaves were collected 0, 6, 12, 24 h after treatment. The primers used for RT-PCR were: 5'-CCAAGGGGATGACGAACTA-3' and 5'-GGAAACCGATTCTGTGCAAT-3' for *WDS2*; 5'-CCTCATGCCATCCTCCGTCTTG-3' and 5'-TTCCATCTCCTGCTCGTAGTCAAC-3' for *ACTIN2*; and 5'-TGTCACACCGATGTTTAC-3' and 5'-GGTCGAATCTTTTAGAGTTAA-3' for *ADH*. Conditions of PCR were as follows: 94 °C for 3 min, 30s at 94°C, 30s at 59°C, and

30s at 72°C for 28 cycles; and then 10 min at 72°C. For *ADH*, the annealing temperature was modified to 55°C for 1 min and extension temperature of 72°C for 1 min were used.

To test the possible role of WDS2 in hyperosmotic stress response, wild type, *wds1*, *wds2* and *wds1 wds2* mutants were grown on MS plates (control) and MS plates containing 300 mM D-mannitol (hyperosmotic stress). 10 seeds were sowed in each plate and kept in long day condition for 25 days. Microscopic images of the average seedling size were taken at 11d, 18d and 25d using a Discovery V12 SteREO microscope (Carl Zeiss).

Chapter III

Results

3.1 Isolation and molecular characterization of *WDS2*

Two different SALK T-DNA insertion lines in the Col-0 wild-type background, both with a T-DNA insertion in the first exon of the gene At5g08560 (*WDS2*) (Table 3.1) (Figure 3.1), were obtained from Arabidopsis Biological Resource Center (ABRC). T-DNA insertion and homozygous lines were confirmed by using PCR-based markers that can distinguish between the Col-0 wild-type plants, T-DNA hemizygotes, and T-DNA homozygotes. Based on the reported insertion location and flanking genomic sequences, left primers (LP) and right primers (RP) were designed to identify the homozygous mutants. The T-DNA insertions were confirmed by using the left border sequence of the T-DNA (LBa1) and right primer (RP) of each SALK line (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Of the two SALK lines tested (SALK_095495 and SALK_100917), T-DNA insertion homozygous line was found only in SALK_100917 in the first exon (Figure 3.1) (Figure 3.2).

The T-DNA homozygous mutant of *wds1* (SALK_106274) (Xiong, 2007) was confirmed and used as a female to cross with the *wds2* mutant (SALK_100917), which was used as a male (pollen donor) (Figure 3.2). The seeds of the first generation (F1) were collected and allowed to self-pollinate in order to identify homozygote mutants, predicted to occur in a $\frac{1}{4}$ *wds1 wds2*. The seeds of the

T-DNA homozygous line of the double crossing was collected and verified in the next generation (Figure 3.2).

SALK lines of *wds2* were screened for the absence of mRNA transcript by using semi-quantitative reverse transcription PCR (RT-PCR). Total mRNA was isolated from 16-day old seedling (*wds2* and Col-0 wild-type). The control gene *ACTIN* was equally expressed in the mutant and Col-0 wild-type plants. The transcript of *WDS2* was absent in the SALK_100917 after 30 cycles of PCR (Figure 3.3).

Table 3.1: Members of the Arabidopsis *WDS* gene family and SALK T-DNA insertion lines.

Gene Locus	Description of Encoded Protein	Physical Map Position kbp	Full Sequence Length (bp)			T-DNA SALK
			CDS	Genomic DNA	cDNA	
<i>At5g43920</i> (<i>WDS1</i>)	Transducin family protein / WD-40 repeat family protein	17.69	1572	3177	2515	SALK_106274.37.55.x (exon) SALK_022240.33.40.x (300-UTR5)
<i>At5g08560</i> (<i>WDS2</i>)	Transducin family protein / WD-40 repeat family protein	2.77	1770	3799	2299	SALK_095495.45.95.x (exon) SALK_100917.52.75.x (exon)
<i>At1g73720</i> (<i>WDS3</i>)	Transducin family protein / WD-40 repeat family protein	27.73	1536	4930	1802	SALK_123852.55.00.x (exon) SALK_051163.50.20.x (intron)
<i>At5g27570</i> (<i>WDS4</i>)	Transducin family protein / WD-40 repeat family protein	9.73	1236	1236	1236	SALK_087779.52.45.x (exon) SALK_040079.55.50.x (exon)
<i>At5g64730</i> (<i>WDS5</i>)	Transducin family protein / WD-40 repeat family protein	25.89	900	2156	1180	SALK_126634.23.15.x (exon) SALK_075672.53.25.x (300-UTR5)

Figure 3.1: Schematic map of the genomic regions corresponding to *WDS2* gene with mutation sites indicated.

The location of the T-DNA insert and genomic regions was mapped using SIGnAL “T-DNA Express” Arabidopsis Gene Mapping Tool (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and the Arabidopsis Information Resource (TAIR; <http://www.arabidopsis.org/>). Blue boxes stand for 5' and 3' untranslated regions (UTRs); red boxes represent exons and lines between red boxes represent introns. The positions of the T-DNA insertion of both SALK_095495 and SALK_100917 are mapped. The start codon ATG and stop codon TGA are indicated by an arrow.

Figure 3.2: PCR amplification for identification of homozygous mutant lines of *wds1*, *wds2* and *wds1 wds2* mutants.

Leaves from wild type, single mutant (SALK_106274 for *wds1* and SALK_100917 for *wds2*) and double mutant lines were collected for DNA extraction. PCR amplification with the T-DNA (LBa1) and gene-specific (LP and RP) primers was performed on the corresponding genomic DNA to identify the homozygous line and T-DNA insertion. a) No band was observed in the *wds1* mutant indicating that *wds1* is homozygous when LP and RP primers were used. However, a band was observed in *wds1* mutant indicating the T-DNA insertion line was located in *wds1* mutant when LBa1 T-DNA primer was used. b) No band was show in the *wds2* mutant indicating that *wds2* is homozygous when LP and RP primers were used. However, a band was observed in *wds2* mutant indicating the presence of the T-DNA insertion when LBa1 primer was used. c) The confirmation of the homozygous line and T-DNA insertion in *wds1 wds2* double mutants.

a) SALK_106374 (*wds1*)

b) SALK_100917 (*wds2*)

c) *wds1 wds2* mutant

Figure 3.3: Semi-quantitative RT-PCR analysis of mRNA accumulation of *WDS2* in Col-0 wild-type and *wds2* mutant.

The upper panel shows the *WDS2* transcript in Col-0 and *wds2* mutant line. The absent band on *wds2* mutant indicates the loss of function. The lower panel shows the expression of the constitutive control gene, *ACTIN2*.

3.2 Mutant phenotypes of *wds1*, *wds2* and *wds1 wds2*

Although the homozygous *wds1* mutant grew normally, *wds2* and *wds1 wds2* double mutants displayed a range of phenotypic abnormalities including early leaf senescence, early flowering, reduced rosette and cauline leaves number and increased silique length (Figure 3.4) (Table 3.2). Plants homozygous for each of the mutant alleles were grown under long day condition (16 h light/ 8 h dark) and short day condition (8 h light/ 16 h dark) and compared with Col-0 wild-type plants (Figure 3.4). Both *wds2* and *wds1 wds2* mutants displayed rapid senescence observed after 32-day-old and 51-day-old in long day and short day condition, respectively. The leaf yellowing proceeded from the top margins toward the bottom of the leaves. First, rapid senescence was observed in cotyledons. Over time, senescence became more evident and spreaded randomly in the other part of rosette leaves. Accelerated leaf senescence and the differences in level of necrosis were also clear in both *wds2* and *wds1 wds2* double mutants. Whereas cotyledons and rosette leaves of *wds1* mutant and wild-type plants were still green, those of *wds2* and the double mutant were yellow and already dry (Figure 3.4-a and-b). Also, we observed a decrease in chlorophyll content in *wds2* and *wds1 wds2* double mutants of 26% and 31%, respectively, while Col-0 wild-type and *wds1* mutant chlorophyll leached of 6% (Figure 3.4-c). These results suggest that *WDS2* plays a role in the regulation of senescence, but not *WDS1*. There was no significant phenotypic difference in root elongation between each mutant and Col-0 wild-type plants (Table 3.2). *wds2* and *wds1 wds2* mutants flowered slightly earlier (28 and 25 days, respectively) than did

the *wds1* and Col-0 wild-type (35 and 36 days, respectively) and with fewer leaves and longer siliques (Table 3.2).

Figure 3.4: Phenotypic analysis of *wds1*, *wds2* and *wds1 wds2* mutants in long-day and short-day condition.

Col-0 wild type, *wds1*, *wds2*, and *wds1 wds2* mutants were germinated on MS plates for 11 days in a growth chamber under long day condition (16 h of light, 8 hours of dark) and short day condition (8 hours of light, 16 hours of dark). Small plants were then transferred to pots and grown in the same growth chamber under the same condition. The plants were photographed from day 32 to day 45 in long day condition and at 51 day-old in short day condition. Phenotypes were also recorded on detached leaves. Bar, 2cm. The chlorophyll content of the plants were also measured in long day condition. Results are based on three replicate experiments where n= 216 for each.

a) phenotype in long day (LD) condition.

b) phenotype in short day (SD) condition.

c) the change of chlorophyll content of the indicated detached leaves at 32, 35, 40 and 45 at the long day condition were recorded.

a) LD

b) SD

c) The change of chlorophyll content in LD

Table 3.2: Flowering time, leaf numbers (35-day-old), silique size (mm)(49-day-old) and root length (cm)(9-day-old) of *wds1* and *wds2* mutants.

Plants	Rosette leaf number ^{a,c}	Cauline leaf number ^{a,c}	Silique length ^{a,c} (mm)	Root length ^{b,c} (cm)	Flowering time ^{a,c} ± 1 day
Col-0	13.90 ± 0.30	3.24 ± 0.43	13.86 ± 0.36	3.25 ± 0.63	35-day-old ± 1
<i>wds1</i>	13.61 ± 0.58	3.14 ± 0.36	13.67 ± 0.58	3.13 ± 0.64	36-day-old ± 1
<i>wds2</i>	11.19 ± 1.24	2.76 ± 0.43	17.76 ± 1.26	3.03 ± 0.88	28-day-old ± 1
<i>wds1 wds2</i>	10.80 ± 1.07	2.28 ± 0.56	16.71 ± 1.23	3.12 ± 0.77	25-day-old ± 1

^a : Data based on three repeats of experiments

^b : Data based on four repeats of experiments

^c : Standard deviation

3.3 Response of *WDS2* to hyperosmotic stress

The effect of 300mM mannitol during seedling development of *wds1*, *wds2*, *wds1 wds2*, and Col-0 wild type plants was examined to determine the role of *WDS* genes in response to osmotic stress. Sterilized seeds of all the indicated genotypes of plants were germinated on MS plates or MS plates containing 300mM mannitol for 25 days in a growth chamber under long day conditions (16 h light/ 8 h dark). After that, seedlings were photographed at 11, 18 and 25 days using a Carl Zeiss Discovery V12 SteREO microscope. As shown in Figure 3.5, there was similar growth inhibition on all mutants and control seedlings grown with 300 mM mannitol at 11 and 18-day-old. However, at 25-day-old, seedling death was much more significant in *wds2* and double mutants. The *wds1* mutant plants showed no

significant difference in size comparing to the other mutants. The mutants and control plant were shrunk due to osmotic stress at each time points. The results show that the *WDS2* may play an important role in response to osmotic stress.

Figure 3.5: The effect of hyperosmotic stress on wild type, *wds1*, *wds2*, and *wds1 wds2* in tissue culture.

Col-0 wild-type, *wds1*, *wds2*, and *wds1 wds2* plants were grown in standard MS medium or MS containing 300 mM mannitol for 25 days in a growth chamber under long day conditions (16 h light/ 8 h dark). Then, microscopic photographs were taken using a Carl Zeiss Discovery V12 SteREO microscope at 11, 18 and 25-day-old. Results are based on three replicate experiments where n= 50 for each.

3.4 Response of *WDS2* gene to abiotic stress treatment

To investigate the expression pattern of *WDS1* and *WDS2* genes in response to Osmotic stress, RT-PCR was conducted using total RNA isolated from 16-day-old *Arabidopsis* wild type seedlings that were subjected to 0.6 M D-mannitol treatment. After D-mannitol treatment, the expression of *WDS2* was induced after 12 h and 24 h. However, there was no change in the gene expression of *WDS1*. This result suggests that the expression of *WDS2* gene can be modulated by abiotic stresses and may be involved in the plant response to hyperosmotic stresses. In addition, the expression of the *ADH* gene is affected by other environmental conditions, such as osmotic stresses, in several plant species as expected (Dolferus *et al.*, 1994; Xie and Wu, 1989). Therefore, we used it as a marker gene in this work and observed increase in *ADH* expression 24 h after treatment (Figure 3.6).

Figure 3.6: RT-PCR analysis of *WDS2* expression in wild-type seedlings with 0.6 M D-mannitol treatment.

Total RNAs were isolated from 16-day-old of wild type seedlings as outlined in Materials and Methods. The *ADH* was used as marker gene and *ACTIN2* was used as constitutive expression control. Results are based on four replicate experiments.

	Water	0 h	6 h	12 h	24 h
WDS1					
WDS2					
ADH					
ACTIN2					

3.5 Up-regulation of *WDS1* and *WDS2* transcripts by SA

To investigate the expression pattern of *WDS1* and *WDS2* genes in response to salicylic acid, RT-PCR was conducted using total RNA isolated from leaves of three-to-four-week-old *Arabidopsis* wild type that were subjected to 0.5 mM SA treatment. After SA treatment, the expression of *WDS1* was increased after 48 hours, and the expression of *WDS2* was induced after 24 hours (Figure 3.7). This result suggests that the expression of *WDS1* and *WDS2* genes downstream of SA pathway. In addition, the expression of the *PR1* gene (*AT2G14610*) is affected by SA as expected (Wang *et al.*, 2007). Therefore, we used it as a marker gene in this work.

Figure 3.7: RT-PCR analysis of *WDS1* and *WDS2* expression in wild-type Arabidopsis leaves with 0.5mM SA treatment.

Total RNAs were isolated from three-to-four-week-old Arabidopsis wild type as outlined in Materials and Methods. *PR1* was used as marker gene and *ACTIN2* was used as constitutive expression control. Results are based on three replicate experiments.

	Water	0 h	24 h	48 h	72 h
WDS1	[REDACTED]				
WDS2	[REDACTED]				
PR1	[REDACTED]				
ACTIN2	[REDACTED]				

3.6 Leaf senescence induced by senescence-accelerating hormones such as salicylic acid (SA) is accelerated in *wds2*

Our gene expression data in section 3.5 revealed that WDS1 and WDS2 are induced by SA. Thus, we examined the response of the mutants as well as the Col-0 wild-type plants to the senescence-accelerating hormone SA (Figure 3.8). When leaves detached from four-week-old Col-0, *wds1*, *wds2*, and *wds1 wds2* mutant plants were treated with two different concentration of SA (0.5 mM and 2.0mM), we observed a decrease in chlorophyll content in all mutant lines. However, at 0.5 mM and 2.0mM SA, after 96 hours of incubation, the chlorophyll contents in *wds2* and double mutants were less than 12%, in contrast to Col-0 wild type and *wds1* which retained 18% and 23% of its chlorophyll (Figure 3.8-b and c). The chlorophyll level at 0 h of all plants was subtracted from the level at 96 h incubation time in order to determine how much chlorophyll was leached in each mutant line and Col-0 wild type plants. The differences were recorded and plotted in Figure 3.8-d. This indicated that more chlorophyll was leached in *wds2* and double mutants than in Col-0 wild-type and *wds1* mutant when treated with SA (Figure 3.8-d).

Figure 3.8: The change of chlorophyll content in response to SA treatment.

The change of chlorophyll content in detached leaves was recorded at 0, 24, 48, 72 and 96 hours after treatment with 0.5 mM, 2.0 mM SA or water (as control). Results are based on three replicate experiments.

- a) Water treatment in Col-0 wild-type and *wds1*, Col-0 and *wds2*, and Col-0 and double mutants.
- b) 0.5 mM SA treatment in Col-0 and *wds1*, Col-0 and *wds2*, and Col-0 and double mutants.
- c) 2.0 mM SA treatment in Col-0 and *wds1*, Col-0 and *wds2*, and Col-0 and double mutants.
- d) Re-plotting to show the difference of chlorophyll leaching from 0h to 96h in all lines.

a) Water

b)

c)

d) Difference in chlorophyll leaching

3.7 Fumonisin B1 induces spontaneous cell death in *wds1*, *wds2* and *wds1 wds2* mutants

Cell death occurred on leaves detached from three-week-old Col-0 wild-type, *wds1*, *wds2* and *wds1 wds2* mutant plants when they were treated with different concentrations of FB1 (1 μ M, 2 μ M and 5 μ M) and incubated in long day conditions for up to 120h. Trypan blue is an important stain used to selectively color dead tissues or cells blue. Dead cells form dark spots when stained with trypan blue and they can be distinguished from the surrounding, living green cells by using a microscope with white light (Axioplan 2, Carl Zeiss). The cell death was constrained in a limited area and did not visibly accelerate the death of the whole leaf. Leaves were collected from each treatment at 0, 24, 48, 72, 96, and 120h. The dark speckles were scattered on the adaxial surface of leaves, but hardly visible to the naked eye, and did not have defined margins. Water treatment was used as a control for all plants (Figure 3.9-a). When all mutants and Col-0 wild-type plants were treated with 1 μ M FB1, the earliest cell death in the double mutant was observed at 72 h (Figure 3.9-b). Then, cell death occurred on *wds2* mutant and *wds1 wds2* at 120 h. There was no effect on *wds1* mutant and Col-0 wild-type (Figure 3.9-b). However, when we increased the concentration of FB1 to 2 μ M and 5 μ M, cell death was observed on all mutants. Cell death occurred earlier in the double mutant than on *wds2* mutant. Cell death was then observed on *wds1* and Col-0 wild-type plant, respectively (Figure 10 c, d, and e). The dark spots appeared in all plants when we increased the concentration of FB1 and incubated for longer time.

To determine whether the cell death in the dark spots indicated using the trypan blue assay is associated with callose deposition, we determined the presence of callose with aniline blue in Col-0, *wds1*, *wds2* and *wds1 wds2* leaves at 0h, 72h, 96h, and 120h. The accumulation of callose deposition around the site of infection is usually part of the complex cell wall-strengthening process that halts pathogen invasion (Stanghellini and Aragati, 1966). The stain of aniline blue is used to detect callose deposition and produces UV-stimulated fluorescence (Eschrich and Currier, 1964). The fluorescence produced by callose appears as a bright blue fluorescent ring along the cell wall and is distinct from the white autofluorescence under the epifluorescence microscope (Figure 3.10). Cell death was induced more in the double mutant, and *wds2* than on *wds1* mutant after 72 h.

Figure 3.9: Microscopic image of whole-mounted Col-0, *wds1*, *wds2*, *wds1 wds2* leaves.

All plants of the indicated genotypes were germinated on MS plates for 11 days in a growth chamber under long day conditions (16 h light/ 8 h dark). Small plants were then transferred to pots and grown in the same growth chamber under the same condition. Leaves detached from three-week-old plants were treated with 1 μ M, 2 μ M, 5 μ M FB1 and water. It was then stained with trypan blue and mounted in 70% Glycerol. A microscopic image was taken with white light by Axioplan 2 microscope (Carl Zeiss).

- a) Col-0, *wds1*, *wds2*, *wds1 wds2* leaves under the control (water) treatment. Scale bar, 10 μ m
- b) Col-0, *wds1*, *wds2*, *wds1 wds2* leaves when treated with 1 μ M FB1. Scale bar, 10 μ m
- c) Col-0, *wds1*, *wds2*, *wds1 wds2* leaves when treated with 2 μ M FB1. Scale bar, 10 μ m
- d) Col-0, *wds1*, *wds2*, *wds1 wds2* leaves when treated with 5 μ M FB1. Scale bar, 10 μ m
- e) Macroscopic image of 5 μ M FB1 and water treatments in *wds1*, *wds2*, double mutants and Col-0 wild-type leaves. Scale bar, 2 cm.

a) Water

b) 1 μ M FB1

c) 2 μ M FB1

d) 5 μ M FB1

e) Water and 5 μ M FB1

Figure 3.10: Microscopic image of callose deposition in Col-0, *wds1*, *wds2*, *wds1 wds2* leaves after 5 μ M FB1 treatment.

All plants were germinated on MS plates for 11 days in a growth chamber under long day conditions (16 h light/ 8 h dark). Small plants were then transferred to pots and grown in the same growth chamber under the same condition. Leaves detached from three-week-old plants were treated with water and 5 μ M FB1 and stained with aniline blue. The whole leaves were mounted in 70% glycerol. A microscopic image was taken using a microscope equipped with a UV light source and DAPI filter (scan range: 320-520 nm) (Axioplan 2, Carl Zeiss). Scale bar, 10 μ m.

3.8 Subcellular localization of *WDS2*

Heazlewood *et al.* (2007) are using various data sources together to build SUBA, a SUBcellular location database for Arabidopsis proteins. This provides subcellular prediction information for proteins based on amino acid sequence using 6743 non-redundant proteins and characterizes the proteins encoded in the transcripts responsible for 51% of Arabidopsis expressed sequence tags (Heazlewood *et al.*, 2007). Based on Heazlewood *et al.* (2007), I predicted that *WDS2* is probably located in the nucleus. To examine the subcellular localization of *WDS2*, a green fluorescent protein (GFP)-*WDS2* fusion protein was transiently expressed in 5-week-old tobacco leaves. The GFP-*WDS2* fusion appeared to be located in the nuclei of cells in the tobacco leaf (Figure 3.11). DAPI, blue fluorescent stain was used to label nuclei (Figure 3.11-d). Also, water infiltration was used as a control, only revealed autofluorescence in chloroplast (Figure 3.11-e and -f).

Figure 3.11: Nuclear localization of GFP-WDS2 in DAPI blue-treated transgenic tobacco plants.

(A) Diagram of *WDS2* constructs in the pVKH18::GFPN vector. GFP, green fluorescent protein and 35S promoter is located in pVKH18 vector. (B) Nuclear localization of GFP-WDS2. The full length *WDS2* gene was fused to the coding sequence of GFP located in pVKH18 and the binary construct introduced into tobacco plants by *Agrobacterium* mediated transient delivery. After 4 days of incubation, localization of *WDS2*-GFPN was examined by confocal microscopy. Nuclear speckle was detected in the *WDS2* under the following conditions: (a) fluorescence image of cell putatively expressing GFP-WDS2 (505-650 nm); (b) autofluorescence image of panel a (>650 nm); (c) bright field image of panel a; (d) DAPI staining of panel a; (e) control (water) injection showing autofluorescence from chloroplasts; and (f) bright field image of the control. Bar, 10 μ m.

A)

B)

Chapter IV

Discussion, Conclusion and Future Research

Discussion:

Programmed cell death is a physiological process that involves the selective elimination of unwanted cells. It plays an important role in cell and tissue homeostasis, cellular differentiation, tissue sculpting, and disease resistance. Senescence, as a visible form of cell death, is a complex process and represents a highly regulated developmental stage (Hammond-kosack & Jones, 1996; Kim *et al.*, 2008). Senescence results in chlorophyll breakdown, RNA and protein degradation, and translocation of nitrogen, carbon nutrients, and minerals from the senescing leaves to other parts of the plants, and eventually leads to cell death (Lim and Nam, 2005). The application of physiological and molecular biological methods to study leaf senescence has enabled the isolation and characterization of a large range of senescence associated genes (SAGs) that have increased gene expression in senescing leaves. Studies of these genes and identification of the functions of the encoded proteins have given further insights into the complex processes that take place during senescence. The combined action of several biotic and abiotic factors may be involved in the induction of senescence (Lim and Nam, 2005). The initiation of leaf senescence can be induced by many different internal and external factors. Environmental factors such as temperature, wounding, nutrient supply and pathogen attack may all result in the initiation of leaf senescence (Bleecker and Patterson, 1997). However, today, scientists struggle to answer many questions regarding the

process of leaf senescence and cell death, such as how and when leaf senescence is initiated, what is the threshold that triggers leaf senescence and cell death, and how the developmental age is recognized.

Today, bioinformatics allows scientists to study genome-wide gene expression patterns in a single experiment, relatively quickly and efficiently. Thus, with the aid of bioinformatics, we can start to analyze functions and functional correlations of multiple genes on a genome scale, such as in the case of genes that are specifically up-regulated during leaf senescence (e.g. *SAG12*, *LSC*, *OLD*, etc). Meanwhile, genomic analysis has also indicated that there are many unknown genes that are involved in leaf senescence and other types of cell death (Zhu and Wang, 2000). An important model for leaf senescence at the molecular level is *Arabidopsis thaliana* (Buchanan-Wollaston *et al.*, 2003; Lim and Kim, 2005). *Arabidopsis* is classified as a monocarpic plant, which has a short life cycle. Boyes *et al.* (2001) have defined the growth stages of *Arabidopsis*, which allows an accurate sampling of material for comparative analysis. The leaves of *Arabidopsis* plants undergo obvious developmental stages. It shows a well-defined and reproducible senescence program that makes genetic analysis of leaf senescence reasonable (Boyes *et al.*, 2001). A wide-range of genomic resources available for *Arabidopsis* plants allows efficient identification and functional analysis of senescence regulatory genes.

Previously, Xiong (2007) identified and described a bioinformatics analysis of a novel gene family, *Will Die Slowly* (*WDS*). Five *WDS* family members were found in *Arabidopsis thaliana* (*WDS1-WDS5*). The predicted proteins of the *WDS* genes

contain seven WD40 repeat motifs, which are conserved across many different species, such as *Oryza sativa*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Bos Taurus*, *Canis familiaris*, and *Gallus gallus*. These genes show sequence similarity (54%) and identities (35%) to the WDS of *Drosophila melanogaster* (Hollmann *et al*, 2002). The amino acid sequence of *Drosophila melanogaster* WDS shows that the gene codes for a protein with seven WD40 repeats and it belongs to the WD40-repeat family. The *Drosophila melanogaster* homolog has been implicated in cell death (Hollmann *et al*, 2002) and it provided a rationale for testing the roles of Arabidopsis WDS proteins.

By using quantitative PCR, Arabidopsis *WDS1* was found to be expressed in all developmental stages (young and old rosette leaves and flower). Xiong (2007) found that *wds1* knockout mutant showed a normal developmental phenotype. However, it exhibited an earlier onset of *SAG12* expression than wild-type upon FB1 treatment. Thus, this observation indicates that *WDS1* may have an important role in PCD. Moreover, Xiong (2007) found that the expression of *WDS1* in detached wild-type leaves is up-regulated by salicylic acid (SA). Furthermore, overexpression of *WDS1* resulted in an enhanced tolerance to hyperosmotic stress, which suggests an involvement of *WDS1* in plant responses to stresses (Xiong, 2007). Senescence associated genes that show induction of gene expression in response to developmental factors are likely to encode proteins that have a role to play in controlling or manifesting the developmental changes (Guo and Gan, 2006; Miao *et al.*, 2004; van der Graaff *et al.*, 2006). Therefore, identification of senescence gene regulators that are up-regulated or down-regulated during senescence is a starting

point for functional analysis followed by characterization of knockout mutations to identify the phenotypes in each gene during the senescence and cell death process. Studies taking molecular, genetic, and genomics strategies facilitate the understanding of the molecular mechanisms that are involved in controlling leaf senescence.

In this project, we characterized the Arabidopsis *WDS2* gene and assessed its functions in senescence and cell death. The *in vivo* characterization of the *WDS2* gene was first analyzed using the genomic tools available in the Arabidopsis information resource (<http://www.arabidopsis.org>), which has a large collection of T-DNA insertion lines. I also studied the molecular phenotypes of *wds1*, *wds2* and *wds1 wds2* mutants. It should be noted that leaf senescence occurs at the final stage of leaf development. Consequently, *WDS* family genes that function in leaf senescence might also be involved in other biological pathways. Thus, one of the most important challenges in this study was to investigate how *WDS2* is coordinately regulated during this process. For example, is *WDS2* involved in the FB1, SA and/or MAPK pathways? If it is involved in one of these pathways is *WDS2* involved upstream or downstream of these pathways? Our aim is to understand the molecular nature of the *Will Die Slowly2* (*WDS2*) gene and the mechanisms that control leaf senescence. The goal of this research was to understand the genetic role of the *WDS2* gene product by characterizing the function of *WDS2* in leaf senescence, salicylic acid signaling pathway and Fumonisin B1-induced cell death (used as a pathogen elicitor).

4.1 Structure of WDS2

We have characterized the Arabidopsis *WDS2* gene (*At5g08560*), whose 2.2 kb cDNA encodes a novel of 589-amino-acid protein containing seven WD40 repeats and has two other domains on the N terminus, LisH and CTLH (Figure 1.1) (Table 3.1). The Arabidopsis *WDS1-5* protein has been found to be similar to the WDS protein of *Drosophila melanogaster* (Hollmann *et al.*, 2002). Originally, the WD40 repeats were found in the β -subunit of heterotrimeric G-protein transducin, G β , and it is generally found in regulatory proteins, such as those involved in cell division, cell-fate determination, gene transcription, mRNA modification, transmembrane signaling, protein-protein interactions and vesicle fusion (Adler *et al.*, 1999; Neer *et al.*, 1994). Different studies show that crystal structures of two WD40-repeat proteins have a circularized seven-bladed beta-propeller structure, and each propeller blade consists of a four-stranded anti-parallel beta sheet (Voegtli *et al.*, 2003; Wall *et al.*, 1995). *WDS2* protein likely has such a β -propeller structure, but its interacting proteins and interaction mechanism have not been investigated yet.

In order to understand how *WDS2* is functionally organized, it is necessary to know where the regulatory protein is located in specific plant cells and under particular environmental conditions. Thus, a GFP in frame fusion with *WDS2* was used to determine its subcellular location. The GFP-*WDS2* fusion appeared to be in the nuclei of cells in tobacco leaves after transient delivery of a T-DNA harbouring the fusion and driven by the 35S promoter (Figure 3.11). 4',6'-diamidino-2-phenylindole (DAPI) blue staining was used to label nuclei and in this cell co-labelled with GFP

fluorescence (Figure 3.11-d). The result found in section 3.8 is not definitive and I cannot draw a final conclusion that WDS2 is located only in the nucleus. However, given that WDS2 might be nuclear localized, it may play regulatory roles in diverse cellular processes such as signal transduction, chromatin remodeling, chromosome condensation, cell cycle regulation, transcriptional induction/repression, vesicle trafficking, regulation of microtubule dynamics, and RNA processing (Neer *et al.*, 1994; Smith *et al.*, 1999; van Nocker and Ludwig, 2003; Vodemaier, 2001). The subcellular localization experiment of WDS2 should be repeated using stable transformants of *Arabidopsis* plant. Also, the fusion of GFP-WDS2 should be checked by Western blot using an anti-GFP antibody in order to make sure that the GFP-WDS2 fusion does not get cleaved.

4.2 WDS2 functions in the regulation of leaf senescence and plant development

The reproductive phase of plants depends on initiation of flowering time occurring under most favorable conditions. Plants have complicated mechanisms to sense biotic and abiotic factors, as well as their developmental and nutritional status, and to integrate this information to regulate their leaf senescence and many other internal processes. *Arabidopsis* plants initially grow in a vegetative phase producing rosette leaves, which are the repetitive production of leaves from lateral primordial initiated at the flanks of the apical meristem (Telfer *et al.*, 1997). Moreover, juvenile and adult phases have been distinguished during rosette leaf growth on the basis of leaf morphology, trichome distribution, and acquisition of meristem competence to

flower (Telfer *et al.*, 1997). Thus, when all vegetative phases have completed their cycle and the floral phase is induced, leaf production is inhibited, lateral primordia develop into flowers, and the main stem elongates to give rise to an inflorescence (Boyes *et al.*, 2001). Leaf senescence occurs in a coordinated manner at the whole leaf level, usually starting from the top margins toward the bottom of the leaves. Nevertheless, when environmental stress is involved locally on a leaf, the stressed leaf region undergoes earlier senescence than the other parts (Guo *et al.*, 2004; Lim and Nam, 2005; Zhou *et al.*, 2009).

Analysis of Arabidopsis mutants has allowed the identification of many genes involved in the regulation of leaf senescence. Physiological, genetic, and molecular analyses of leaf senescence mutants have shown that senescence is induced or delayed in several mutants, some of whose corresponding genes are dependent on hormones and/or environmental factors (Gracia-Heredia *et al.*, 2008; Nam, 1997; Yoon *et al.*, 2008). To investigate the role of *WDS1* and *WDS2* in leaf senescence, we tested loss-of-function mutants of *wds1* and *wds2*. T-DNA mutant lines of *wds1* (SALK_106374) and *wds2* (SALK_100917) were obtained. Also, we performed double mutant analysis between *wds1* and *wds2* to determine the potential functional interactions or redundancy between *WDS1* and *WDS2* genes. In addition, T-DNA insertion, homozygous lines and absence of mRNA of these mutants were confirmed (Figure 3.1, 3.2, and 3.3). We found that the growth and development of *wds1* mutant appeared normal, with no significant phenotype difference when compared to wild-type plant in leaf, flower or root development (Figure 3.4, Table 3.2). However, *wds2* and double mutants exhibited a range of phenotypic

abnormalities including early leaf senescence, early flowering, reduced rosette and cauline leaf numbers, and increased silique length (Figure 3.4, Table 3.2). The early leaf senescence phenotype of *wds2* and *wds1 wds2* mutants indicate that *wds2* mutant is a new locus involved in the regulation of senescencing and plant development. The pleiotropic phenotype shown by *wds2* mutant, which include early flowering time, reduce rosette and cauline leaf numbers and increased silique length, suggests the involvement of *WDS2* in other developmental processes in addition to the regulation of senescence processes. Also, there are many genes that control these developmental processes (e.g. *BOP1-1*, *WRKY70*, *OLD*, etc.) and these may work with *WDS2* to control leaf senescence. Furthermore, the results shown in Figure 3.4 were consistent with the visible yellowing on the leaf of the mutants, suggesting an overall acceleration of leaf senescence in the *wd2* and *wds1 wds2* double mutants. The reason that we did not observe early leaf senescence in *wds1* mutants is because the *WDS2* gene is functioning normally. Thus *WDS2* may have a more important role in the regulation of senescence. Also, the reason why leaf senescence showed earliest in the double mutants is because the two family members of *WDS* are knocked out. Therefore, there might be also functional redundancy within other *WDS* family members, but the *WDS2* might have the most important role in regulation and controlling leaf senescence.

The senescence process also occurs in a highly regulated mechanism and the cell components are dismantled in an ordered progression. Chlorophyll degradation is the first visible symptom of senescence (Buchanan-Wollaston and Ainsworth, 1997; Lee *et al.*, 2001). According to many studies, chlorophyll content is

used as a relevant metabolic indicator of the early and late stages of senescence (Buchanan-Wollaston and Ainsworth, 1997; Hortensteiner and Feller, 2002; Lee *et al.*, 2001; Lohman *et al.*, 1994). The loss of green color is a quantifiable measure of leaf senescence (Thomas, 1997). To examine the physiological changes during senescence, the chlorophyll content was measured in the leaves of the *wds1*, *wds2* and *wds1 wds2* mutants compared to wild-type as shown in Figure 3.4-c. In the whole rosette leaf (Figure 3.4-a), measurement of chlorophyll fluorescence during leaf development showed a significantly higher loss of chlorophyll content in the *wds2* (26%) and *wds1 wds2* mutants (31%) compared to the *wds1* mutant and wild type (6%) (Figure 3.4-c). This observation supports the phenotypic analysis and the observed acceleration of leaf senescence in the *wds2* and *wds1 wds2* double mutants.

4.3 WDS2 and osmotic stress

As described earlier, leaf senescence is widely influenced by different factors and stress conditions. Thus, it is thought that genes regulating leaf senescence may also affect stress responses (e.g. hyperosmotic stress). Different studies of gene expression have shown that many of genes differentially expressed in senescing leaves are also influenced by various internal or external stresses, indicating that there is extensive signaling crosstalk between stress conditions and leaf senescence. To illustrate, along with the 43 transcription factor genes that are specifically induced during senescence, there are 28 transcription factors genes induced by various stresses in addition to senescence (Chen *et al.*, 2005; Hanfrey *et al.*, 1996; John *et al.*, 1997; Quirino *et al.*, 1999; Weaver *et al.*, 1998). Hyperosmotic

stress activates several protein kinases such as MAPK, which may mediate osmotic homeostasis and/or detoxification responses (Zhu, 2002). For example, some of the phospholipids-mediated signaling systems are triggered by osmotic stress, producing an array of secondary messenger molecules. Some of these molecules may have an important role upstream of the protein kinases activated by osmotic stress (Zhu, 2002). Figure 3.5 shows that phenotypic differences in *wds2* and *wds1* *wds2* compared to *wds1* and wild-type plant were influenced by osmotic stress conditions after 25 d. Figure 3.5 provides experimental evidence that *WDS2* is probably a molecular link that connects osmotic stress responses and leaf senescence. Also, as shown in Figure 3.6, there was no significant change in *WDS1* transcript levels. However, I observed that treatment of plants with D-mannitol enhanced *WDS2* transcription, suggesting that *WDS2* gene expression is modulated by hyperosmotic stresses. This result suggests that *WDS2* is not only a critical component in the regulation of leaf senescence, but also may play an important role in plant responses to hyperosmotic stress. Thus, our results are most consistent with the hypothesis that *WDS2* is involved in the regulation of leaf senescence and cell death triggered by other environmental stresses.

4.4 Up-regulation of *WDS2* by salicylic acid.

Senescence is known as a developmentally programmed process (Buchanan-Wollaston 1997; Smart, 1994). Nonetheless, it can be modulated by different plant hormones, such as ABA, ethylene and salicylic acid (Aharoni, 1989; Gan and Amasino, 1997; Nam, 1997). These hormones influence development and

environmental responses in plants. The hormonal pathways tend to control all the stages of leaf senescence, progression, and the terminal phases. For leaf senescence, intimate interactions of senescence associated genes are involved and may have an important role in hormonal pathways that controlled senescence and PCD (Gan and Amasino, 1997; Nam, 1997). Each hormone is involved in a variety of developmental processes working in a complex manner, which causes difficulties in finding the specific functions of the hormonal pathways in leaf senescence (Gan and Amasino, 1997; Nam, 1997). Thus, hormones likely control genes during senescence in order to coordinate the senescence process with various physiological processes (Nam, 1997). Genes that show induced expression upon SA treatment may encode proteins that have a function in the senescence and PCD processes (Guo *et al.*, 2004). As illustrated in Figure 3.7, the transcripts of *WDS2* increased after 48 hours. The up-regulation of gene transcription of *WDS2* following the treatment of 0.5 mM SA suggest that the *WDS2* gene rapidly responded to signaling molecules triggered by biotic and abiotic stresses in the SA-dependent signaling networks. Most of the genes that are expressed during senescence require a combination of pathways for appropriate expression (Guo *et al.*, 2004). Therefore, partial functional redundancy of cross-linking of signaling pathways between *WDS2* and the other *WDS* family members may play an important role in SA-dependent signaling pathway. Also, creating triple loss-of-function *wds* mutants would give more insight into the role of *WDS* and allow the measurement of a significant difference in the process of leaf senescence as well as assist in revealing the specific roles of each member and functional redundancy within this family.

Salicylic acid is the hormone involved in pathogen response and pathogen-mediated cell death (Morris *et al.*, 2000). Based on the gene expression patterns of *WDS1* and *WDS2* above and to determine whether *WDS2* is involved in the senescence process induced by SA, we examined cell death in wild-type, *wds1*, *wds2* and *wds1 wds2* mutants after treatment with 0.5 mM and 2.0 mM SA. I found that the chlorophyll content of wild-type and *wds1* leaves decreased rapidly to 18% and 23%, with 0.5 mM and 2.0 mM SA treatment respectively (Figure 3.8 a-d), whereas the chlorophyll content in *wds2* and *wds1 wds2* leaves were less than 12% at the same times (Figure 3.8 a-d). This result indicated that the change in the chlorophyll content was significantly different between wild-type and *wds2* and *wds1 wds2*, but not between *wds1* and wild type. This result is consistent with the phenotypic analysis of these mutants in long day and short day conditions, which shows the acceleration of leaf senescence and degradation of chlorophyll content in both *wds2* and *wds1 wds2* mutants. Thus, these observations after SA treatment provide further evidence to support the hypothesis that *WDS2* may function as a negative regulator in leaf senescence.

4.5 Interaction of senescence and defense responses

Genes that show increased expression in response to biotic or abiotic factors are likely to encode proteins that have a role to play in controlling or manifesting the developmental change (Gracia-Heredia *et al.*, 2008; Lim and Nam, 2005; Yoon *et al.*, 2008). As discussed earlier, identification of putative *WDS* genes that are upregulated during senescence was a starting point for functional analysis, using

knockout mutations, to study the function of each gene in the senescence process. We found that the *WDS2* gene is highly expressed during leaf senescence and is important for the plant to maintain viability during senescence process. Further investigation is needed to determine how the interaction between different senescence associated genes and signalling networks are linked together.

In addition, cell death associated with the hypersensitive response (HR) results in changes of cell structure and in the extracellular matrix, including callose deposition. Both senescence and HR are visible forms of programmed cell death even though they are not identical in their mechanisms and outcomes (Bostock and Stermer, 1989; Kim *et al.*, 2008; Kus *et al.*, 2002; Lim and Nam, 2005; Quirino *et al.*, 1999). We observed that *wds2* and *wds1 wds2* exhibited accelerated HR compared to wild-type and *wds1*. Our data indicate a significant role of *WDS2* in both senescence and defense signaling pathways. Therefore, activation of defense signaling pathways during the senescence process may provide a mechanism for resistance against pathogens. This mechanism might also protect susceptible organs against pathogen attack, which could impede the fundamental process of nutrient remobilization (Quirino *et al.*, 1999, 2000). Consequently, *WDS2* may be implicated in both developmentally regulating leaf senescence and defense signaling pathways.

Conclusion:

In conclusion, in this thesis, I described the analysis of *wds1* and *wds2* knockout mutants. *wds2* and *wds1 wds2* mutants show accelerated leaf senescence, increased chlorophyll leaching and sensitive to osmotic stress, indicating that expression of *WDS1* and *WDS2* genes during senescence has a protective role to retain viability during this fundamental developmental process. Expression of *WDS1* and *WDS2* in wild-type plant is up-regulated by SA and *WDS2* by hyperosmotic stress (D-mannitol). Also, by using the pathogen-derived cell death elicitor FB1, we found that the *WDS2* is a novel gene for plant cell death signaling related to defense responses. The putative *WDS2* protein contains seven WD40 repeats and might be located in the nucleus. Further studies on the loss-of-function *wds* mutants, isolation of *WDS* family members and elucidation of their roles in senescence and PCD will certainly help us understand the sophisticated regulatory mechanisms.

Future Direction

Although much progress has been made in identifying and characterizing *WDS1* and *WDS2*, significant work remains. First, quantitative real-time PCR should be applied to confirm the induction of *WDS2* in SA and osmotic stress in order to follow up with the rest of the experiments. To further investigate the developmental role of *WDS1* and *WDS2*, the transcript level of *WDS1* and *WDS2* should be also evaluated in response to age-induced senescence by collecting leaves at different developmental stages from *Arabidopsis* wild-type plants at 4-10 weeks and performing RT-PCR with gene specific primers. In this case, *SAG12* gene would be used as a marker gene for the induction of leaf senescence as described by Kim *et al.*, (2008).

In addition, phenotypic and expression level analysis on the rest of the *WDS* family will clarify the role of *WDS* family in plant development and leaf senescence. Looking at the expression pattern of all *WDS* family members might give insight into whether they function redundantly. To continue with our phenotypical analysis and investigation of the *WDS* family role, triple loss-of-function mutants should be obtained, as well as many possible losses of function members that can be obtained. This would allow for a more accurate phenotypical analysis that would distinguish the role of each *WDS* member in plant development and reveal whether a functional redundancy exist between those members.

At the mechanistic level, other challenges include the identification of leaf senescence and PCD initiation signals, and the recognition of developmental stages. Therefore, investigation of *WDS2* function by identifying interacting partners of *WDS*

members through yeast-two hybrid and the associated pathways will provide better understanding of the molecular mechanisms of longevity control in Arabidopsis and other systems. *WDS1* and *WDS2* were found to be induced by SA. *WDS1* and *WDS2* transcripts may also be enhanced by some additional factors present in senescing leaves. Thus, identifying the key signalling pathways involved in *WDS* gene regulation will give more insight into the role of *WDS* in leaf senescence and PCD. The work may also reveal more sophisticated coordination of all the *WDS* members in the fine regulation of plant development and in response to stresses.

Furthermore, site-directed mutagenesis of protein sequences is a powerful tool of modern recombinant DNA technology to determine the effects of amino acid substitutions on the functional characteristics of proteins. The ScanSite database reveal protein motifs that may be recognized by modular signaling domains, e.g. some motifs in a protein that are recognized and phosphorylated by Ser/Thr or Tyr-Kinases (Obenauer *et al.*, 2003). The amino acids S182, S363, I71, L166 and V525 of *WDS2* are predicted to be sites of phosphorylation by MAPKs using Scansite. These should be mutated for further studies. The mutant *WDS2* would be introduced into Arabidopsis to determine the function of *WDS2* protein in the MAPK pathway. This type of analysis will reveal a critical component in the protein in determining the specificity, fidelity, and efficiency of mitogen activated protein kinase.

The PCD pathways remain unclear even in the model plant Arabidopsis. Based on our results and those in Asai *et al.* (2000), Morris *et al.* (2000) and Zhang and Klessig (2001), a model for *WDS*-mediated pathways is proposed (Figure 4.1). However, the function all *WDS* family members still has to be determined.

Figure 4.1: A proposed model showing signalling pathways leading to *WDS* gene expression during PCD process.

Based on our results, bioinformatics analysis on MAPKs (data not shown) and proposed pathways in Asai *et al.* (2000), Morris *et al.* (2000) and Zhang and Klessig (2001), I hypothesized a model for *WDS*-mediated pathway. There might be two different pathways for *WDS* genes. The first pathway proposes the induction of *WDS* members in relation to each other on the MAPK cascade. Available evidence indicates that *WDS* genes are involved in the MAPK pathway based on the amino acids which are predicted to be sites of phosphorylation by MAPK using Scansite or published observations (Zhu *et al.*, 2004). The other pathway in which *WDS* genes might be involved in is the salicylic acid pathway. Since both *WDS1* and *WDS2* are induced by SA, therefore they might be located upstream of *PR1*, which is a marker for SA induction. After pathogen infection, *pad4* mutant show decreases in *PR-1* expression and salicylic acid accumulation (Asai *et al.*, 2000; Jirage *et al.*, 1999; Morris *et al.*, 2000). *PAD4* is found to be involved in leaf senescence and might play a role in the initiation of leaf senescence. *WDS* genes might and might not link both pathways to each other as well as activate other genes and signaling networks that might lead to PCD.

APPENDIX

Appendix A: Diagram of WDS2 Construct Map

Appendix B: (a) Proposed salicylic acid biosynthetic pathways in plants (Mary *et al.*, 2001). (b) Classical model for systemic acquired resistance (SAR) based on the presence of salicylic acid (Malamy *et al.*, 1990; Metraux *et al.*, 1990).

(a)

(b)

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