The role of *Will Die Slowly 1 (WDS1)* gene in the development and defense response of *Arabidopsis thaliana*

A thesis Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science in Biology.

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ABBREVIATIONS

WDS: will die slowly

PCD: programmed cell death

SA: salicylic acid

FB1: fumonisin B 1

SAG: senescence associated gene

ORF: open reading frame

MAPK: Mitogen-Activated Protein Kinase

WDR26: WD40 repeat protein 26

JA: Jasmonic acid

ET: ethylene

HR: hypersensitive response

ROS: reactive oxygen species

ABA: abscisic acid

GA: gibberellin

LD: long day

SD: short day

CO: CONSTANS

ABSTRACT

Programmed cell death (PCD) is a form of cellular suicide and involves a set of biochemical programs during the development of organisms and in their responses to pathogen attacks and other stress signals. We have isolated and characterized the *Will Die Slowly 1* (*WDS1*) gene of *Arabidopsis thaliana*, which encodes a 63kD protein similar to the *Will Die Slowly* (*WDS*) protein of *Drosophila melanogaster*. Real-time PCR reveals that the expression of the gene modulated at different developmental stages, reaching a peak when 50% flowers have open. Analyses on the phenotypes of three T-DNA knockout *wds1* mutants indicate that *WDS1* is not essential for development of *Arabidopsis*. However, deletion of *WDS1* accelerates the induction of *SAG12* expression triggered by FB1, suggesting a regulatory role of *WDS1* in stress-induced senescence. *WDS1* expression is up-regulated by salicylic acid (SA) and overexpression of *WDS1* in *Arabidopsis* enhances the tolerance to osmotic stress. Our results support the hypothesis of *WDS1* as a regulator for cell death progression triggered by both developmental and stress signals in *Arabidopsis*.

Chapter I

General Introduction

Programmed cell death (PCD), a concept first came from plants, is a broad term that refers to a process by which cells promote their own death through the activation of self-destruction systems (Quirino *et al.*, 2000). It is recognized as a ubiquitous process of cellular suicide that lays dormant in all living cells from prokaryotes to eukaryotes (Beers and McDowell, 2001; Koonin and Aravind, 2002; Lewis, 2000; Madeo *et al.*, 2004; Watanabe and Lam, 2006). Three strands of research came together to shape today's research agenda about plant PCD: terminal differentiation, senescence, and disease resistance (Jones, 2001). Cell death fulfills several essential functions in plant development: a) senescence removes cells by recycling much of its carbon, nitrogen, and phosphorous; b) cell death is important in sculpting tissues such as the formation of lysigenous aerenchyma, flower primordial during floral abortion, and aleurone layers during germination; c) cells invaded by pathogens may be self eliminated as part of a hypersensitive response against the pathogen; d) cell death also occurs during terminal differentiation and the classic example is the formation of vessel members and tracheids, collectively termed tracheary elements (TE); and e) cell death is programmed when the metabolism of cells is perturbed either by coping with abiotic stresses imposed upon it or by bioengineering (Jones, 2001). Many researches have indicated unequivocally that plant PCD defines an active process of death genetically dissectable and cytoplasmically driven (Gan and Amasino, 1995; Grbic and Bleeker, 1995; Thomas and Stoddart, 1980; Weymann *et al.*, 1995; Jones, 2001). Moreover, it is now clear that plant cells integrate death and survival signals to make decisions when to die (Jones, 2001). Although PCD has been widely accepted as an intrinsic process that is intimately linked to life-sustaining reactions of the organism, the components of the signal transduction pathways that lead to the activation of PCD are only beginning to be identified.

Leaf senescence, a developmentally programmed degeneration process that constitutes the final step of leaf development, has been for years recognized as a form of PCD (Quirino *et al.*, 2000). During senescence, leaf cells undergo dramatic changes in cellular metabolism and the sequential degeneration of cellular structures in an orderly manner (Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2003; Quirino *et al.*, 2000). The loss of chloroplast integrity occurs first, followed by the breakdown of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA, releasing nutrients such as nitrogen, phosphorus and metals which can be recycled by the rest of the plant (Buchanan-Wollaston *et al.*, 2003). It is likely that many genes are involved in regulation of these orderly, sequential changes in cellular physiology, biochemistry and gene expression (Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2003; Quirino *et al.*, 2000). Characterization of the processes that regulate leaf senescence will help manipulate leaf senescence through breeding or genetic engineering and might also help to improve crop yields by keeping leaves photo-synthetically active for longer.

Like many other genetically programmed developmental processes, leaf senescence, particularly its initiation, is subject to regulation by many environmental and autonomous (internal) factors (Lim *et al.*, 2003; Quirino *et al.*, 2000). The environmental cues include stresses such as drought and pathogen infection, whereas the autonomous factors include age, reproductive development, and phytohormone levels (Gan and Amasino, 1997). Among the environmental cues, limited water and nutrient availability (especially nitrogen) are major factors that adversely affect plant life in many ecosystems. Plants have evolved mechanisms by which leaf senescence can be induced by these stresses to reallocate nutrients to reproductive organs and to eliminate water consumption by older, less productive leaves (Gan and Amasino, 1997). In the absence of external stimuli that accelerate the senescence program, leaf age has a major influence on the initiation of senescence (Gan and Amasino, 1997). In one general model of what triggers the senescence program, leaf senescence is initiated when the photosynthetic rate drops below a certain threshold. That threshold may be at or near the compensation point at which the leaf no longer contributes fixed carbon to the rest of the plant (Gan and Amasino, 1997).

In response to various environmental stresses, plants have developed different physiological and biochemical mechanisms to adapt to or tolerate stress conditions (Baker *et al.*, 1988; Bartels & Salamini, 2001; Bray, 1997; Cushman & Bohnert, 2000; Ingram & Bartels, 1996). A large set of genes is transcriptionally activated, encoding proteins that accumulate in vegetative tissues exposed to biotic and abiotic stresses (Buchanan-Wollaston *et al.*, 2003; Weaver *et al.*, 1998). Many genes that are involved in responding to environmental changes have also been identified as being induced during leaf senescence (Buchanan-Wollaston *et al.*, 2003; Weaver *et al.*, 1998; He *et al.*, 2001). Thomas and Smart (1993) monitored the mRNA expression profiles of 402 potential transcription factors at different developmental stages and under various biotic and a biotic stresses. They found that among the 43 transcription factor genes that were induced during senescence, 28 were also induced by stress treatments, suggesting that there is extensive overlap in the responses to leaf senescence and stress. However, the extent and significance of the overlap between the leaf-senescence and defense programs requires further study.

Increasing efforts have been made to identify possible senescence-regulating and/or defense-related genes in plants. Two different approaches are commonly used in genetic studies. Genes with a role in senescence or defense can be identified by the isolation and characterization of mutants that are defective in some aspect of the senescence and/or defense pathway (Buchanan-Wollaston *et al.*, 2003). A mutant that showed an early senescence phenotype, *hys1* (*hypersenescence1*) was identified by Yoshida *et al.* (2002). This mutant showed early loss of chlorophyll and expression of senescence enhanced genes and was found to be allelic with *cpr5* (*constitutive expresser of pathogenesis related genes 5*). The *HYS1* gene is expressed at all stages of leaf development and may encode a protein that represses the initiation of senescence.

On the other hand, many research groups have used differential expression as a way to identify possible senescence-regulating genes. The rapid advances in genomics resources, especially for the model plant species *Arabidopsis*, provide scientists with many tools for the identification and functional analysis of the genes and pathways involved in senescence and defense (Buchanan-Wollaston *et al.*, 2003). Techniques include differential screening (Buchanan-Wollaston, 1994; Drake

et al., 1996; Lohman *et al.*, 1994; Park *et al.*, 1998; Smart *et al.*, 1995), subtractive hybridization (Buchanan-Wollaston and Ainsworth, 1997), differential display (Fujiki *et al.*, 2001; Hajouj *et al.*, 2000; Kleber-Janke and Krupinska, 1997; Yoshida *et al.*, 2001) and suppressive subtractive hybridization (Hinderhofer and Zentgraf, 2001). The availability of cDNA microarrays and Affymetrix GeneChips has considerably increased the speed by which differentially expressed genes can be identified in *Arabidopsis*. Zhu and Wang (2000) have used Affymetrix *Arabidopsis* GeneChips, which carries probes for around 8000 *Arabidopsis* gene, to analyse the gene expression levels at three stages of leaf development. Over 1400 genes were identified that show relative changes in expression during leaf development.

Having an extensive collection of genes that show altered expression patterns during senescence and defense provides a very useful tool for the functional analysis of potential regulatory genes. Knock-out mutants and transgenic overexpression lines can be obtained for potential transcription factor or other regulatory genes and gene expression patterns in the mutant compared to the wild type (Buchanan-Wollaston *et al.*, 2003). The use of libraries of *Arabidopsis* insertion mutants allows a functional analysis of individual genes to be carried out more rapidly (Buchanan-Wollaston *et al.*, 2003). In essential genes, insertions will be lethal and will therefore not be available. There may be genes that have a role in senescence but are also essential in early stages of development. A functional analysis of these will require a more specific approach such as the use of antisense or RNAi (Buchanan-Wollaston *et al.*, 2003). Another potential problem with the insertion mutant approach for functional analysis is the possible functional homologues of particular genes (Buchanan-Wollaston *et al.*, 2003). The sequencing of the *Arabidopsis* genome revealed large areas of duplication that may contain genes that carry out the same function (Blanc *et al.*, 2000; Buchanan-Wollaston *et al.*, 2003).

Our present work has identified a putative *WDS* gene family in *Arabidopsis*. *WDS1* shows a sequence similarity to the *WDS* of *Drosophila melanogaster*. Mutation of *WDS1* in *Drosophila* led to the lethal phase throughout the larval stages (Shannon *et al.*, 1972; Hollmann *et al.*, 2002). In the thesis, the following work will be presented: (1) Bioinformatic analysis of *WDS* gene family in *Arabidopsis*; (2) Phenotypic and functional analysis of *WDS1* in knockout lines; (3) Phenotypic and functional analysis

of *WDS1* in overexpression lines. Data from these analyses support a role of *WDS1* as a regulator in the salicylic acid (SA) pathway, and it functions in both age-dependent and FB1-triggered senescence, as well as in plant responses to hyperosmotic stress. At the end of the thesis, we will discuss the possible future directions in the study of this novel gene family.

Chapter II

Bioinformatic Analyses of the *WDS* Gene Family in *Arabidopsis*

2.1 Introduction

The WD40 repeat is composed of ~40 amino acid residues with a conserved Gly-His (GH) dipeptide 11-24 residues from its N terminus and a Trp-Asp (WD) dipeptide at its C terminus, to form a propeller-like structure with several blades where each blade is composed of a four-stranded anti-parallel β -sheet (Garcia *et al.*, 1996). Each WD40 sequence repeat forms the first three strands of one blade and the last strand in the next blade; the last C-terminal WD40 repeat completes the blade structure of the first WD40 repeat to create the closed ring propeller-structure and residues on the top and bottom surfaces of the propeller are proposed to coordinate interactions with other proteins and/or small ligands (Figure 2-1, Garcia *et al.*, 1996) A complex may be able to accommodate sequential and/or simultaneous interactions involving several sets of proteins.

Despite the conservation of structures, WD-repeat proteins have diverse functions, including signal transduction, RNA synthesis/processing, chromatin assembly, vesicular traffic and fusion, cytoskeletal assembly, cell cycle control and apoptosis (Li and Roberts, 2001). However, defining the function of a WD-repeat protein is the current challenge (Temple *et al.*, 1999). WD-repeat proteins seem to have a regulatory function in general, but no member of this class of proteins has yet been shown to play a structural role or have an enzymatic activity (Li and Roberts, 2001). The stable closure of the circular beta-propeller structure is proposed to play a key role in the motion of surface residues during protein-protein interaction as well as the prevention of conformational change in its backbone geometry, which leads to the less flexibility required for catalytic activity (Li and Roberts, 2001). The best-characterized WD-repeat protein is the G-beta subunit of heterotrimeric G proteins and due to its extensive characterization, there has been an tendency to label any protein that contain WD repeats 'transducin-like' or 'G-beta-like', implying that there is some functional similarity (Li and Roberts, 2001). We

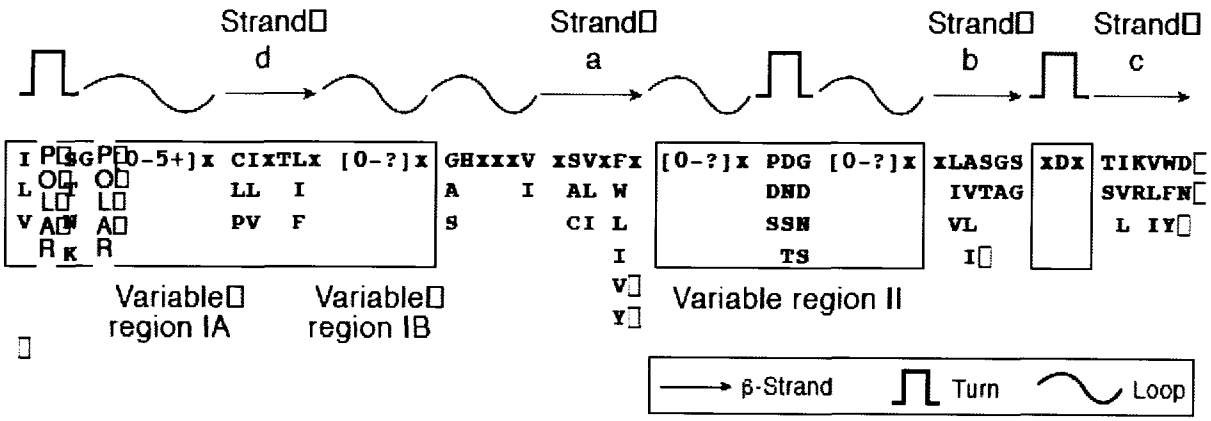
have identified and characterized a novel WD-repeat protein, *Will Die Slowly1 (WDS1),* in *Arabidopsis thaliana*. This protein contains seven WD40 repeats and is similar to the WDS protein of *Drosophila melanogaster*, whose transcript is able to rescue two different lethal *wds* alleles (Hollmann *et al.*, 2002). Structural analysis implies this new WD40 repeat protein is more similar to the beta-transducin structure than G-beta-like proteins.

Figure 2-1: Schematic illustration of the structural elements within a single WD repeat.

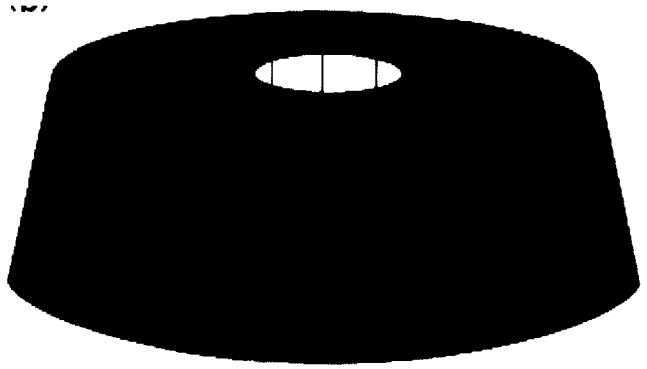
a) Each repeat sequence comprises of four-strands, d, a, b and c. In a WD-repeat propeller, the repeat structure does not correspond to the four strands of a single blade of the propeller but, rather, to the first three strands of one blade and the fourth strand of the next. The alternative amino acids for each position are listed in approximate order of their frequency of occurrence. In the case of strand d, the listed alternative amino acids are only evident in about a third of the repeats. The arrows above indicate the position of these strands, with 'a' being the strand closest to the central pore and 'd' at the external surface of the folded protein. In the G protein beta subunit, each of the seven repeats folds into a small antiparallel beta-sheet, which are arranged around a central pseudosymmetry axis into a beta propeller.

b) Schematic representation of the positions of the elements shown in a) in the three-dimensional structure of the fold. The predicted bottom or larger of the flat surface regions is shown in red; the top flat surface is shown in lighter blue. The circumference, which is composed primarily of the d β-strand residues, is shown in green. Polar, polar residue.

a)



b)



2.2 Results

2.2.1 Discovery of *WDS1* gene in *Arabidopsis*

The *WDS1* gene was discovered when we studied the down-regulation of MAPK pathways in *Arabidopsis*. We were looking for negative regulators of this pathway; and based on the evolutionary biology, our search began from literature on mammalian and yeast MAPK pathways, studies of which started much earlier than that done with plants. Nineteen genes were found in the search and their predicted protein sequences were blasted against the *Arabidopsis* database (www.*Arabidopsis*.org) for the homologues. When one of the nineteen genes *WDR26* (Zhu *et al.*, 2004) was used as a query, the gene At5g43920 was retrieved with the commend 'similar to will die slowly protein (*Drosophila melannogaster*)'.

2.2.2 Molecular characterization of the *WDS1* gene At5g43920

The *WDS1* is a gene in the *Arabidopsis* genome and encodes a cDNA of 2515 bp long (accession number NP_199205). Comparison of the cDNA and genomic sequences revealed that the *WDS1* gene is composed of five exons and four introns. The transcripted RNA of *WDS1* consists of an ORF of 1572 bp from 729 to 2962, a 728 bp 5'-untranslated region and a 215 bp 3'-untraslated region. The predicted protein is made up of 523 amino acids with an estimated molecular mass of 63 kD (Figure 2-2). A canonical poly (A) addition signal (AATAAA, Ashwin *et al.*, 2004) is not found at the expected distance from the 3' end. There is, however, such a signal 850 nt downstream from the end of 3' UTR.

2.2.3 Conserved domains of predicted WDS1 proteins

Sequence analysis in SMART showed that the WDS1 proteins contain three conserved motifs: LisH domain from amino acid 16 to 48, CTLH domain from amino acid 49 to 107 and seven WD40 repeats (Figure 2-3). The LisH motifs are suggested to contribute to the regulation of microtubule dynamics, either by mediating dimerization or else by binding cytoplasmic dynein heavy chain or microtubules directly (Smith *et al.*, 2000). CTLH, predicted to be alpha-helical, is C- terminal to LisH motif. The LisH motifs occur to be associated with homologues of G beta- propeller subunits, which suggests that they are analogues of G gamma subunits and might associate with the periphery of beta-propeller

Figure 2-2: Nucleotide sequence and deduced protein sequence of the *Arabidopsis Will Die Slowly* 1 (*WDS1*) gene. The annealing sites for the primers of real-time PCR are shown in bold. *WDS1* encodes a polypeptide of 523 amino acids. The initiation ATG and termination TGA codons are shaded in yellow. The 5'UTR and 3'UTR sequences are shaded in gray. Amino acids are identified by their one-letter code. The amino acids of the seven WD40 repeats are underlined. Both nucleotides and amino acids are numbered at the right side of each line respectively. The putative polyadenylation signal sequence AATAAA is boxed.

```
AGGGATTAGCGATAGGACTTAACGATATAAACACATATGTATATGGTTTTCGACCTCTCT    60
TCTCCAAAACCATCACAGAGAGAGTGGCCGAAATAAATTGAAATCCGGTGAGAAGATTTT   120
GGATTCACTCCTTCCTTCTTTCTTCTTCTTTATCATCATTTGAGCTGGTTTTGGTATCTC   180
TCCTGACGAATTTGAATCTCTCAGCCATTTTCTCTTCTCGCATCTGTGTTTTCGTGCTTG   240
GAGCTGTACACGTTTCTTTAATTTGAATAGTGATTTTGCGTTCGTTAATTAGCCTTCTCG   300
TACGAGTGGGCTATTTTGGGAATGATTTAGGTGGTTTTAAGTTACTGGGTAGTAGTCACT   360
GCTGAGCGATTTCTGTTTCTCCTGTTGAATGATTGATTCAGAAATTTGAAATTCACATCG   420
AAACCAAATCTTAAATCATATTGGTAGCTTTTTTTTTTGTTTTGTGATTAGATACTGTTTT   480
TTTATAGTATTAAATTCGTTTTGGTGTGAGTGATTTAGTGTTTGGGAAATTATTTAATTT   540
AGTGGTTATTTTCTGATTTAGTGATGATTTGAGAATTTTGTGTTGTTTATATTCTTGGTA   600
GCGGGAAATTTGTACTTTCCTTTGTGGACCTTTCAACTATTGAATGTGGAAGATTTGCTG   660
AGAATCTTAGAAAGTCATCATCATCCATACGTAAGAGAACTTTTTTTTAGCTTGAGAGTG   720
ATTTTCAGATGGAGAATGGGTTATGGGAGGTTTTAGGTTCAAAAGGGTTGTTGAAGAAAC   780
          M  E  N  G  L  W  E  V  L  G  S  K  G  L  L  K  K      17
ATGAATTCATTAGGATTTTGGTTCAATGCTTATACTCGTTAGGATTCAAGAACTCTGCTT   840
H  E  F  I  R  I  L  V  Q  C  L  Y  S  L  G  F  K  N  S  A      37
CTTGTCTGGAATTCGAGTCAAAGATCTTGTATAAAACAGCTGATTCTGAGTTTCTTGAAA   900
S  C  L  E  F  E  S  K  I  L  Y  K  T  A  D  S  E  F  L  E      57
AGCAAGTTTTGAGTGGGAACTGGGATAGTTGCGTACAGGTTCTAGACAGAATTTTTGATA   960
K  Q  V  L  S  G  N  W  D  S  C  V  Q  V  L  D  R  I  F  D      77
ATTCCATGGATGATACGAGGAACACGGCTTTATATCTAGTGTTCAAGCAATGTTTGTTGG  1020
N  S  M  D  D  T  R  N  T  A  L  Y  L  V  F  K  Q  C  L  L      97
AGTATTTGAAACGTGGGGATGTTTCTTTGGCCTTGAATGTGTTACGGAAGCAAGCTCCGT  1080
E  Y  L  K  R  G  D  V  S  L  A  L  N  V  L  R  K  Q  A  P     117
TGTTACGGATGGGAAAAGAGAAGATTCATAGGCTTGCTTGTGATATTGTTTATTCGAAAG  1140
L  L  R  M  G  K  E  K  I  H  R  L  A  C  D  I  V  Y  S  K     137
AGATGGAATCCGGTGAAGTAGACAACTGTTTAGTTCTAGATTTGAGGAGAAAGTTGTTGG  1200
E  M  E  S  G  E  V  D  N  C  L  V  L  D  L  R  R  K  L  L     157
TTGAACTGGAGAAGTTGATTCCCTTGCCAATTGTTATTCCTGAGAGAAGGTTGGAACATT  1260
V  E  L  E  K  L  I  P  L  P  I  V  I  P  E  R  R  L  E  H     177
TGGTTGAGACTGCTGTGATGGACCAGATTGATACGTGTATGTATCATAACTCATGTGATG  1320
L  V  E  T  A  V  M  D  Q  I  D  T  C  M  Y  H  N  S  C  D     197
CAGTATCGCTTTACAAGGATCACTGTTGCGGTAGAGACCAAATTCCTTCAGAGACAGTCC  1380
A  V  S  L  Y  K  D  H  C  C  G  R  D  Q  I  P  S  E  T  V     217
AGATTTTGGTGGCACACAAAAATGAAGTGTGGTTTGTGCAATTCTCTAATAGTGGCAAAT  1440
Q  I  L  V  A  H  K  N  E  V  W  F  V  Q  F  S  N  S  G  K     237
ATCTGGCCACTGCATCAAGCGATTGTACAGCTATAATATGGAAGGTACTGGATGACAACA  1500
Y  L  A  T  A  S  S  D  C  T  A  I  I  W  K  V  L  D  D  N     257
AAGTCGAACTGAAGCACACACTTGAGAGCCACCAAAATCCAGTTTCTTTTGTCTCGTGGA  1560
K  V  E  L  K  H  T  L  E  S  H  Q  N  P  V  S  F  V  S  W     277
GTCCTGACGATACTAAACTGCTTACATGCGGAAACGCTGAGGTTCTTAAGCTATGGGATG  1620
S  P  D  D  T  K  L  L  T  C  G  N  A  E  V  L  K  L  W  D     297
TTGACACAGGTGTGTTGAGACACACATTTGGAAACAACAATACTGGATTCACTGTCAGCT  1680
```

V D T G V L R H T F G N N N T G F T V S 317

CTTGCGCATGGTTCCCTGACTCAACTCGGCTTGTCTGTGGCAGTTCTGACCCAGAAAGAG 1740

S C A W F P D S T R L V C G S S D P E R 337

GGATTGTAATGTGGGACACTGATGGAAACGAGATCAAAGCCTGGAGAGGAACGAGAATTC 1800

G I V M W D T D G N E I K A W R G T R I 357

CAAAGGTGGTGGATTTGGCTGTGACGCCGGATGGCGAGAGTATGATTACAGTGTTTTCGG 1860

P K V V D L A V T P D G E S M I T V F S 377

ATAAAGAGATCCGGATCTTGAATTTGGAGACAAAAGTCGAACGTGTTATCTCTGAGGAAC 1920

D K E I R I L N L E T K V E R V I S E E 397

AGCCAATTACTTCCCTTTCAATTTCTGGTGATGGTAAGTTTTTTATAGTCAACTTGAGCT 1980

Q P I T S L S I S G D G K F F I V N L S 417

GCCAAGAGATACATCTTTGGGATCTTGCTGGAGAGTGGAAACAACCATTGAAGTTCTCGG 2040

C Q E I H L W D L A G E W K Q P L K F S 437

GTCACAGGCAGAGCAAATACGTGATACGGTCATGTTTTGGTGGGTTGGATAGTTCGTTC**A** 2100

G H R Q S K Y V I R S C F G G L D S S F 457

TCGCCAGTGGAAGTGAGGATTCACAGGTTTACATATGGAATCTAAAGAACACAAAGCCGC 2160

I A S G S E D S Q V Y I W N L K N T K P 477

TTGAAGTGTTATCAGGTCATTCGATGACCGTAAACTGTGTAAGCTGGAACCCGAAAAATC 2220

L E V L S G H S M T V N C V S W N P K N 497

CTCGGATGCTAGCCTCTGCTAGTGATGACCAAACCATCCGTATTTGGGGACCGG**GCAAAC** 2280

P R M L A S A S D D Q T I R I W G P G K 517

CGAACAAACCACTGAACTGATTAGATAACCAGAAACCGTAGTAGCTTTAAACCAGGACCT 2340

P **N** K P L N 523

AG**TT**CTAGTAGTTCCACATTTGTGTATAGTTCATTTCTGGTTTTTTCTTTTTTGGTGGTC 2400

CT**TA**TTGGCTTTGCAGAACAATCT**CTT**GCTTTTTGAGTTTTGGTACACTGGTTTTGGAGA 2460

ACT**TG**ATTTGAGATGAGCCATGAATT**G**AAACCAAACCGGTTTAGGCGGTAAACTGaacca 2520

agctgtgttgaggagttaggtggcaattgtttggtgttcagagcaaaacttagctaacta 2580

cttgtttcgttggtcatttgctaccatgtgggttccccgttattttaaacagaaaattta 2640

attataatataaacttcggaaacgcccctaaacttatgatatttaagaatctgccaatat 2700

tgttacttttttccttagtacagagacattatttttctaacaaacctgtttagaccaa 2760

aacttctgaaataattggcaatgcgtagattacaccaaaaaaaaagtttgaaagaaaatg 2820

tgttgcaatgattctaatatcttgaaaaggtatatgagtttcaattatagaactcaagaa 2880

gattaataagtaattgcgagttcgaacatttgtgggtttcaattatataattaacaaaaa 2940

aatctactaagtgcatacataagaagtatttcacctaatacgattaagaatatacatgat 3000

ttcagttttagtatctactatataaatatgaataccaacgtaatttaatcatatatttaa 3060

actacatatacatctatttgaaacatggaatttacatctttctatatttaaaaatatttg 3120

aaatgaagtttcaaaattttcatatgtaaagtatacgttatacaatctaaactagtattc 3180

aaaagatgataacatctacacaaaacaatcacatgaataggttaaatcaaaattaaaaat 3240

agaactgatataccctcttagatctcgaaatatttatcagtatgggccagaagggctata 3300

gttattcttagaaaaaaaatggaggtaatgtcgaaattgacgaaattaataggcgtattt 3360

ttttaaa<u>ataaa</u>ggttgtttaagataatcatgaaaaattagcagccactgagta 3415

domain.

Seven WD40 repeats were recognized in the region of amino acids 212 to 514 (Figure 2-2) in the alignment of the WDS1 protein against the WD consensus sequence. The WD 40 repeat is also found in all the matches when databases were searched for sequences related to the WDS1 protein (Figure 2-7). It is characteristic of a number of regulatory proteins, and may require post-translational modification for activation (Neer *et al.*, 1994; Temple *et al.*, 1999; Joyce *et al.*, 2000). All of the repeats in *WDS1* amino acid sequence are complete and four of them contain the typically conserved core sequence (Neer *et al.*, 1994). This suggested that WDS1 is a WD-protein according to former rules (Neer *et al.*, 1994). Each of the seven WD40 repeats is composed of ~40 (range from 38 to 45) amino acid residues. Despite a few variations in three repeats, the amino acids in positions 7-10 (mostly VSSV) as well as 15-17 (mostly PDP) are quite conserved (Figure 2-4). Besides, six out of seven repeats contain a Trp-Asp (WD) dipeptide at its C terminus (Figure 2-4). Therefore, it is likely that the WDS1 protein is able to form a complex that can be recruited to accommodate interactions with other protein (Neer *et al.*, 1994).

2.2.4 Analyses of *WDS* gene family

To find out whether there are other genes that share the same potential function as *Arabidopsis* WDS1, we compared the predicted amino acid sequences against the *Arabidopsis* database using BLAST. Our search revealed another four genes (At5g08560, At5g64730, At1g73720 and At5g27570) annotated the same as 'similar to will die slowly protein'. Further analyses of their sequence showed that peptides encoded by the four genes all contain 7 copies of WD40 repeats (Figure 2-5), which gave strong support that all the five genes belong to the same family that could be related to the cell death program. Of the four genes, only At5g08560 and At1g73720 encoded the two domains LisH and CTLH other than the WD40 repeats, which indicated that these two genes may share more similarity with *WDS1* gene in structure and function. This was confirmed by the phylogenetic analysis of the protein sequences of these five genes (Figure 2-6).

Figure 2-3: Conserved domains of the predicted proteins encoded by *WDS1* gene. The predicted WDS1 protein contains three domains: the LisH motif, the CTLH motif and seven WD40 repeats. The domains are not in scale to the sequences.

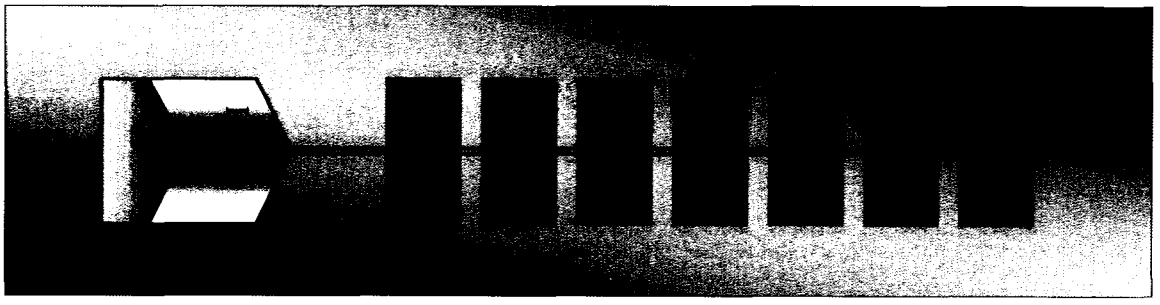


Figure 2-4: Comparison of the seven repeats in WDS1 with the WD-repeat consensus. Consensus amino acids are highlighted with yellow; deviations from the consensus in WD40 repeats are shown in green rectangles while the typical consensuses are marked with pink rectangles. WD40 repeats indicated with * contain all the typically conserved core amino acids.

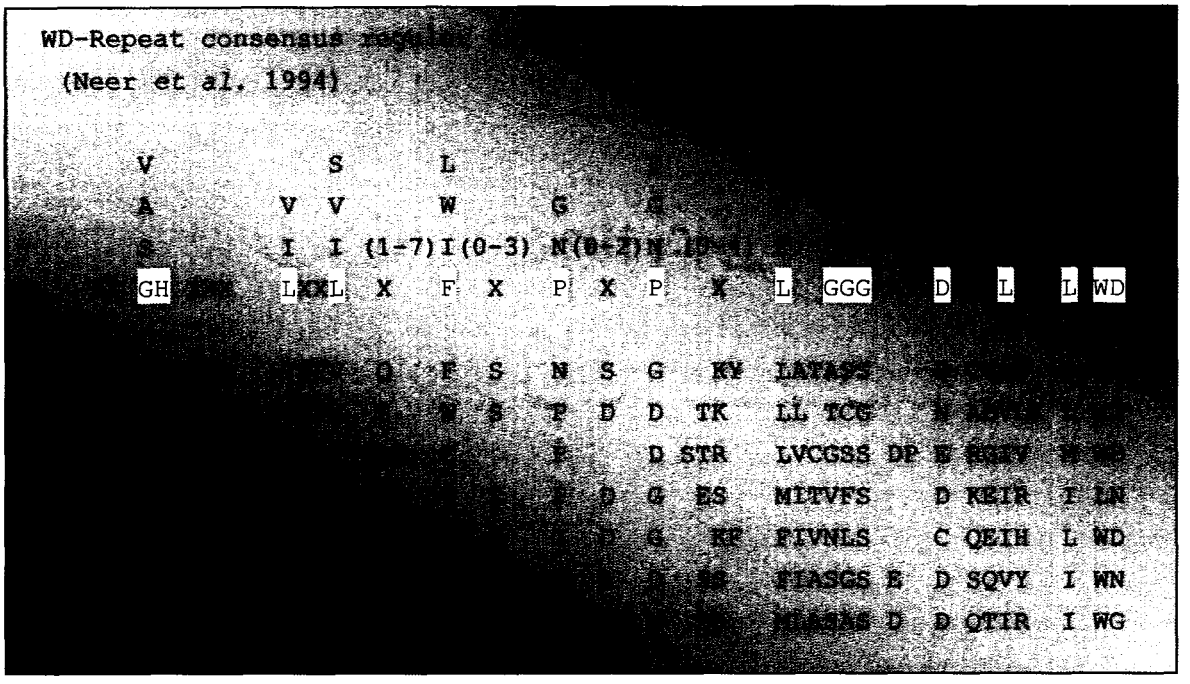
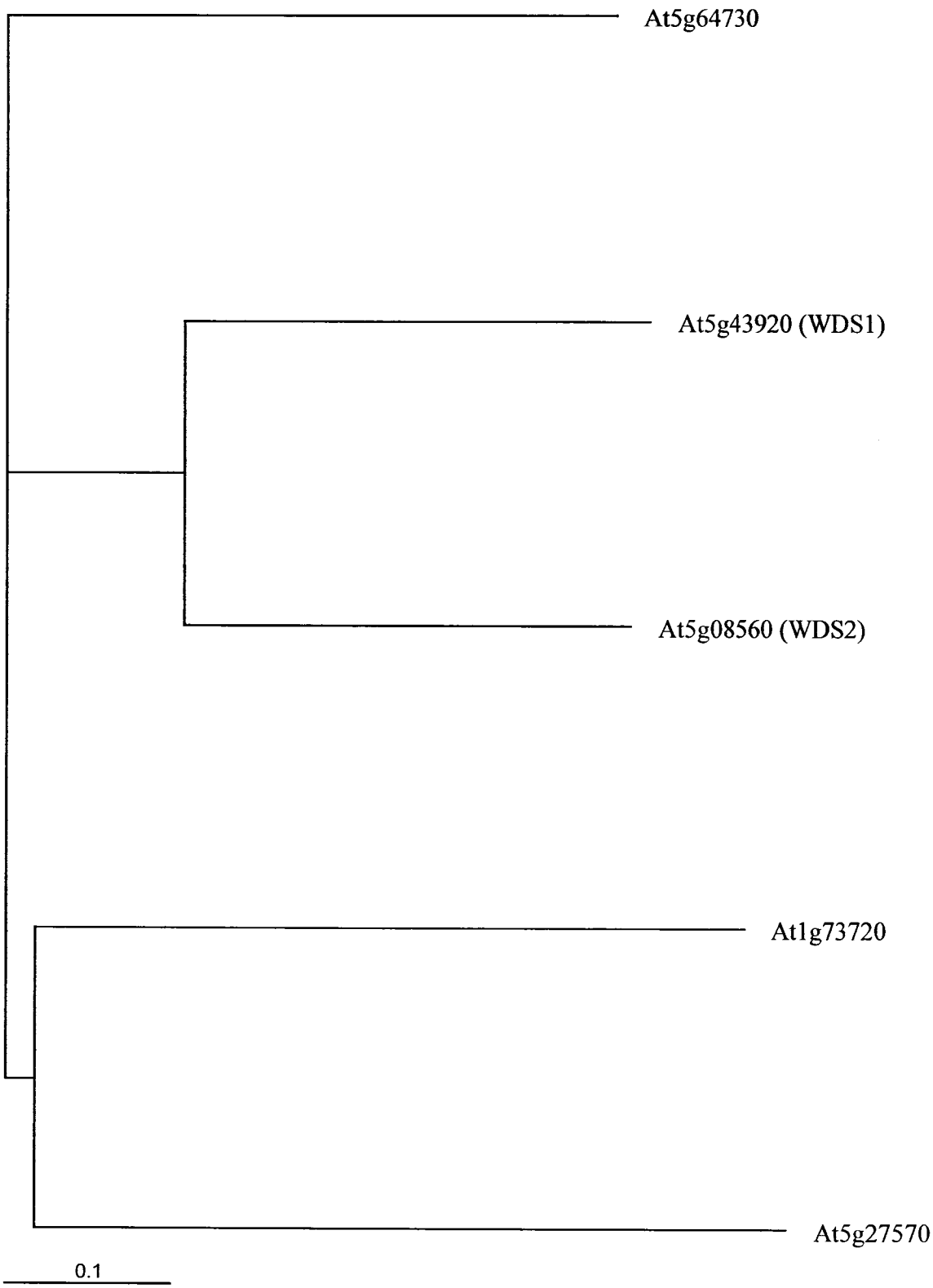


Figure 2-5: Multiple sequence alignment of the five WDS family members in *Arabidopsis* using ClustalW version 1.83. The WD40 repeats were highlighted by pink.

```
AT5G08560.1(WDS2)SCVFHNTLDSDLSLYSDHQCGKHQIPSQTAQILES-----HTDEVWFLQFSHNGKYLASS 292
AT5G43920.1(WDS1)TCMYHNSCD-AVSLYKDHCCGRDQIPSETVQILVA-----HKNEVWFVQFSNSGKYLATA 242
AT5G64730.1      ------------------MSATELPTKEAHILKG-----HEGAVLAARFNGDGNYALTC 36
AT1G73720.1      QGLLP--PGTQFDLFRGTAAMKQDVEDTHPNVLTHTIKFGKKSHAECARFSPDGQFLASS 231
AT5G27570.1      --------------------PRRYIPQNSERVLDAPG---IADDFYLNLLDWGSSNVLAI 108
                                      :     .:*         .      :. ...   :

AT5G08560.1(WDS2)SKDQTAIIWE-----ISADGHISLKHTLVGHHKPVIAILWSPDDRQVLTCGAEEVIRRWD 347
AT5G43920.1(WDS1)SSDCTAIIWK-----VLDDNKVELKHTLESHQNPVSFVSWSPDDTKLLTCGNAEVLKLWD 297
AT5G64730.1      GKDRTIRLWN-----PHRG---ILIKTYKSHGREVRDVHVTSDNAKFCSCGGDRQVYYWD 88
AT1G73720.1      SVDGFIEVWDYISGKLKKDLQYQADESFMMHDDPVLCIDFSRDSEMLASGSQDGKIKIWR 291
AT5G27570.1      ALGDTVYLWD------ASSGSTYKLVTIDEEEGPVTSINWTQDGLDLAIGLDNSEVQLWD 162
                 . .    :*.        .       :   .   *  :  : *.  .        :  *

AT5G08560.1(WDS2)VDSGDCVHMYEKG--GISPISCGWYPDGQGIIAGMTD--RSICMWDLDGREKECWKGQRT 403
AT5G43920.1(WDS1)VDTGVLRHTFGNNNTGFTVSSCAWFPDSTRLVCGSSDPERGIVMWDTDGNEIKAWRGTRI 357
AT5G64730.1      VSTGRVIRKFRGH--DGEVNAVKFNDSSSVVVSAGFD--RSLRVWDCRSHSVEPVQIIDT 144
AT1G73720.1      IRTGVCIRRFDAH--SQGVTSLSFSRDGSQLLSTSFD--QTARIHGLKSGKLLKEFRGHT 347
AT5G27570.1      CVSNRQVRTLRGG-HESRVGSLAWN--NHILTTGGMDGKIVNNDVRIRSSIVETYLG-HT 218
                   :.   :            :  :   .  :     *           .

AT5G08560.1(WDS2)QKVSDIAMTDDGKWLVSVCKDSVISLFDREATVERLIEEEDM---------ITSFSLSND 454
AT5G43920.1(WDS1)PKVVDLAVTPDGESMITVFSDKEIRILNLETKVERVISEEQP---------ITSLSISGD 408
AT5G64730.1      FLDTVMSVVLTKTEIIGGSVDGTVRTFDMRIGREMSDNLGQP---------VNCISISND 195
AT1G73720.1      SYVNHAIFTSDGSRIITASSDCTVKVWDSKTTDCLQTFKPPPP-LRGTDASVNSIHLFPK 406
AT5G27570.1      EEVCGLKWSESGKKLASGGNDNVVHIWDHRSVASSNPTRQWLHRFEEHTAAVRALAWCPF 278
                              :     *  :   : .                    : .:

AT5G08560.1(WDS2)NKYILVN---LLNQEIRLWNIEGDPKIVSRYKGHKRSRFIIRSCFGGYKQAFIASGSEDS 511
AT5G43920.1(WDS1)GKFFIVN---LSCQEIHLWDLAGEWKQPLKFSGHRQSKYVIRSCFGGLDSSFIASGSEDS 465
AT5G64730.1      GNCVLAG---CLDSTLRLLDRTTG-ELLQVYKGHISKSFKTDCCLTNSDAHVIG-GSEDG 250
AT1G73720.1      NTEHIVV---CNKTSSIYIMTLQGQVVKSFSSGNREGGDFVAACVS-TKGDWIYCIGEDK 462
AT5G27570.1      QASLLATGGGVGDGKIKFWNTHTGACLNSVETGSQVCSLLWS---KSERELLSSHGFTQN 335
                     :.                          .*                       :

AT5G08560.1(WDS2)QVYIWHRSTGKLIVELPGHAGAVNCVSWSPTNLHMLASASDDGTIRIWGLDRINQQNQKK 571
AT5G43920.1(WDS1)QVYIWNLKNTKPLEVLSGHSMTVNCVSWNPKNPRMLASASDDQTIRIWGPGKPNKPLN-- 523
AT5G64730.1      LVFFWDLVDAKVLSKFRAHDLVVTSVSYHPKEDCMLTSSVDG-TIRVWKK---------- 299
AT1G73720.1      KLYCFNYQSGGLEHFMMVHEKDVIGITHHPHRN-LLATYSEDCTMKLWKP---------- 511
AT5G27570.1      QLTLWKYPSMVKMAELNGHTSRVLFMAQS-PDGCTVASAAGDETLRLWNVFGEPPKTTKK 394
                  :  :.         :  *   *  ::        :::   . *:::*
```

Figure 2-6: Phylogeny of the five *WDS* family members in *Arabidopsis*. The phylogenetic tree in phylogram was constructed from an alignment of proteins using ClustalW version 1.83. The scale bar represents estimated number of mutations per nucleotide.



2.2.5 Analyses of the phylogeny and structure of *WDS1* homologies

To further investigate the structure and evolutionary connections of the *WDS1* gene, we performed PSI-BLAST searches using as the query the predicted protein sequence of At5g43920 (GenBank identifier GI:15240036). The first iteration of this search against NCBI databases of all organisms retrieved apparent homologs of the *WDS1* from more than ten other organisms, such as *Homo sapiens, Mus musculus* and *Drosophila melanogaster*. However, only one of these organisms belongs to the plant species, *Oryza sativa*. Therefore, further blastings were performed by limiting the search against the databases of UniProt plant proteins. Three more plant species were retrieved with several homologies of the WDS1 in the second search. All of these matches are in common that they are confined to a repetitive motif -WD repeats (Figure 2-7), which is characteristic of a number of regulatory proteins (Neer *et al.*, 1994; Temple *et al.*, 1999).

Table 2-1 presents the homologs of WDS1 as well as the pairwise similarity for these protein sequences compared with the *Arabidopsis* WDS1. The amino acid similarity that is observed between the homolog from NCBI database and WDS1 over their entire length is about 40% identical residues plus a 15%-22% conservative amino acid replacement, indicating the highly conserved nature of this protein. Most of the homologies that show high identity to the WDS1 are WDR26 or predicted WD-repeat protein 26. The putative expressed WD-repeat protein 26 in rice shows the highest similarity with WDS1. On the contrary, homologs that are indicated by the NCBI comment as 'will die slowly' (NP_524684 in fly) or 'putative will die slowly' (ABF98634 in rice) both showed relatively lower identity to the WDS1 protein. The amino acid similarity observed between the homologs from the left three plant species and WDS1 (25% identity) is much lower as compared to that seen for the animal homologs (30%-44% identity).

A global alignment (Figure 2-7) was carried out on the homologies to WDS1 representing thirteen species together with the query WDS1 and phylogeny based on this gene was studied (Figure 2-8). There is a high degree of sequence identity and similarity among the fourteen proteins except for five sequences (frog, dog, rat, mouse and cow) which lack the LisH domain. The proteins are related to one another both in their seven WD repeats as well as in the CHTL regions. Within the WD repeats this is

best shown by those ten positions where the repeats in WDS1 deviate from the consensus (Figure 2-7, asterisks). In these positions all sequences show closely related or even identical exchanges (one example is an asparagine in the fifth WD repeat). In addition, at those positions where variability is allowed, many amino acids are identical of conservation between the corresponding repeats of all proteins. Since there is also a high degree in the variable regions of sequence similarity between the WD repeats it is most likely that all these proteins are true homologues.

Table 2-1: Homologues of *WDS1* detected in the GenBank and UniProt plant databases

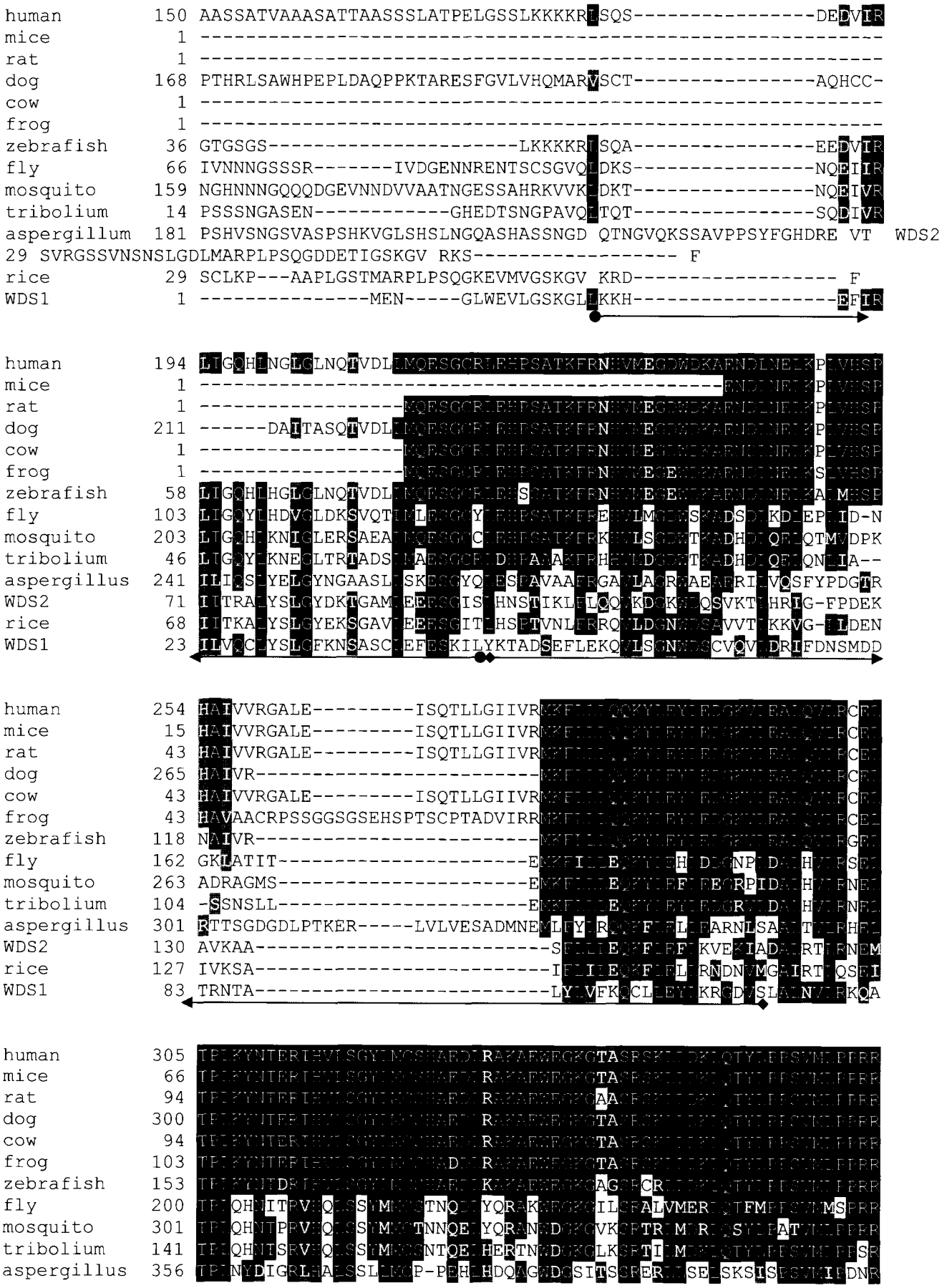
Species	Common name	Genbank accession number	Identities %	Similarity %	homologous segment (amino acid positions)	comments
Drosophila melanogaster	Fly	NP_649326 and NP_730650[a,b]	35	54	98-607	CG7611-PA
		NP_524984	29	53	60-357	will die slowly
Homo sapiens	Human	BAD93124[a]	37	55	190-708	WD repeat protein 26 variant
		AAH31471[b]	44	61	2-356	WDR26 protein
Mus musculus	Mice	AAH58601[a]	41	60	38-469	WDR26 protein
		AAH20044[b]	43	61	32-386	WDR26 protein
Rattus norvegicus	Rat	XP_001062709[a,b]	41	60	66-497	PREDICTED: similar to WD-repeat protein 26
		XP_573529	41	60	30-461	PREDICTED: similar to WD-repeat protein 26
Bos taurus	Cow	NP_001070527 and AAI09898[a,b]	41	59	66-497	hypothetical protein LOC767999
		Q2KIG2	29	51	41-330	WD repeat protein 5
Canis familiaris	Dog	XP_537237[a,b]	39	57	238-703	PREDICTED: similar to WD-repeat protein 26
Gallus gallus	Chicken	XP_419389[b]	37	55	101-619	PREDICTED: similar to WD repeat domain 26

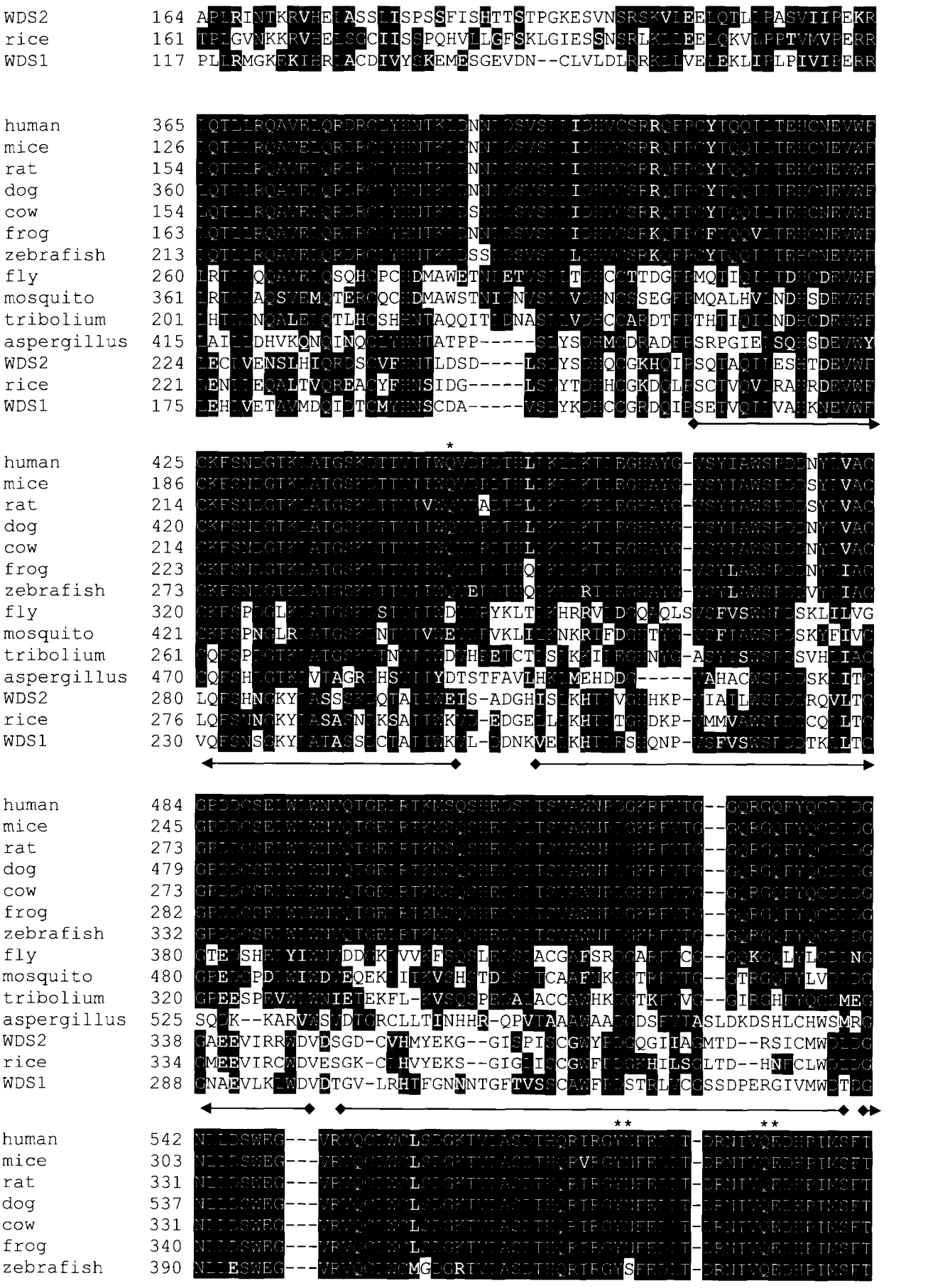
Danio rerio	Zebrafi-sh	Q5SP67 and XP_693216[a,b]	37	56	52-556	WD repeat protein 26
Apis mellifera	Honey bee	XP_001120876[b]	30	47	51-503	PREDICTED: similar to CG7611-PA
Xenopus tropicalis	Frog	Q28D01	36	53	70-597	WD repeat protein 26
		NP_001039080 and CAJ81593[a,b]	41	59	75-506	WD repeat domain 26
Tribolium castaneum	Tribolium	XP_973492[a,b]	34	52	41-542	PREDICTED: similar to WD-repeat protein 26
Aedes aegypiti	Mosquito	EAT41843[a,b]	34	54	198-707	wd-repeat protein
Aspergillum oryzae	Aspergil-lum	BAE61413[a,b]	33	50	235-747	unnamed protein product
Arabidopsis thaliana	Arabido-psis	NP_196473[a]	44	63	55-566	at5g08560, similar to will die slowly in Drosophila
Oryza sativa	Rice	NP_001046605[b]	44	66	53-510	Os02g0294600/ similar to Q9FND4 at5g43920
		AAP54181	55	70	2-233	WD-repeat protein 26, putative, expressed
		ABF98634	28	48	15-314	Will die slowly protein, putative, expressed
		XP_465062[a]	44	66	53-510	putative WD repeat protein/superseded by a new assembly of the genome
Medicago truncatula	Barrel medic	Q6UZ79[b]	25	47	275-447	B-type cell cycle switch protein ccs52
Chlamydo-monas reinhardtii	Chlamy-domonas	Q3Y8L7[b]	25	38	97-416	WD repeat protein
Solanum chacoense	Chaco potato	Q6EE07[b]	25	39	113-438	Notchless-like protein/WD40 repeat protein

a: homologous proteins of *WDS1* chosen for multiple alignment

b: homologous proteins of *WDS1* chosen for construction of phylogenetic tree representing eighteen species.

Figure 2-7: Multiple alignment of the homologies of WDS1. Comparison of the amino acid sequences of WDS1 and its homologues: human, BAD93124; mice, AAH58601; rat, XP_001062709; dog, XP_537237; cow, AAI09898; frog, CAJ81593; zebrafish, XP_693216; fly, NP_730650; mosquito, EAT41843; tribolium, XP_973492; aspergillum, BAE61413; WDS2 (*Arabidopsis*), NP_196473; rice, XP_465062; WDS1 (*Arabidopsis*), NP_199205. Residues neither identical nor similar to the consensus were printed in white background; residues that were not identical but at least similar to the column-consensus were printed on gray and those identical to the column-consensus were on black._____, LisH; _____, CTLH;_____, WD40.





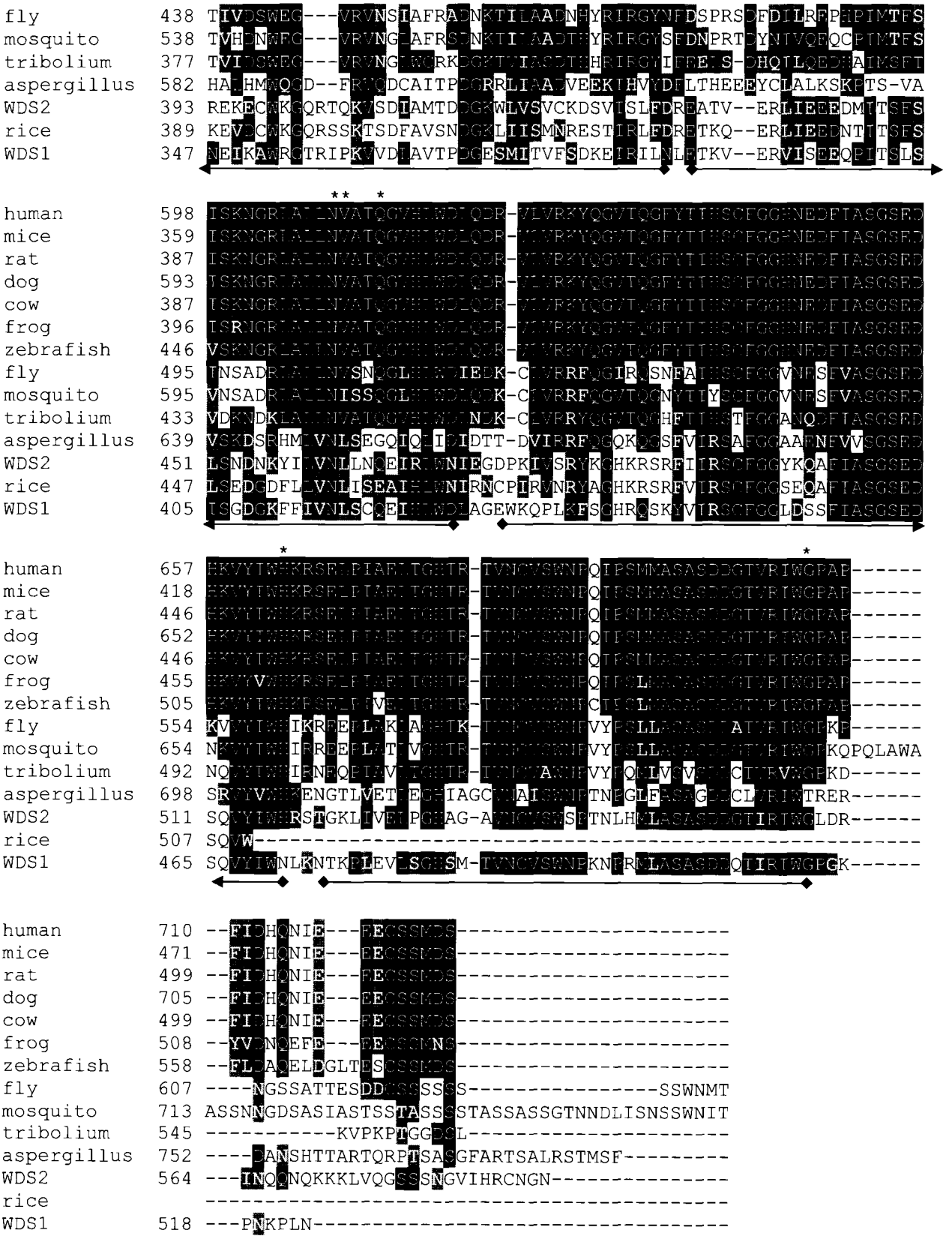
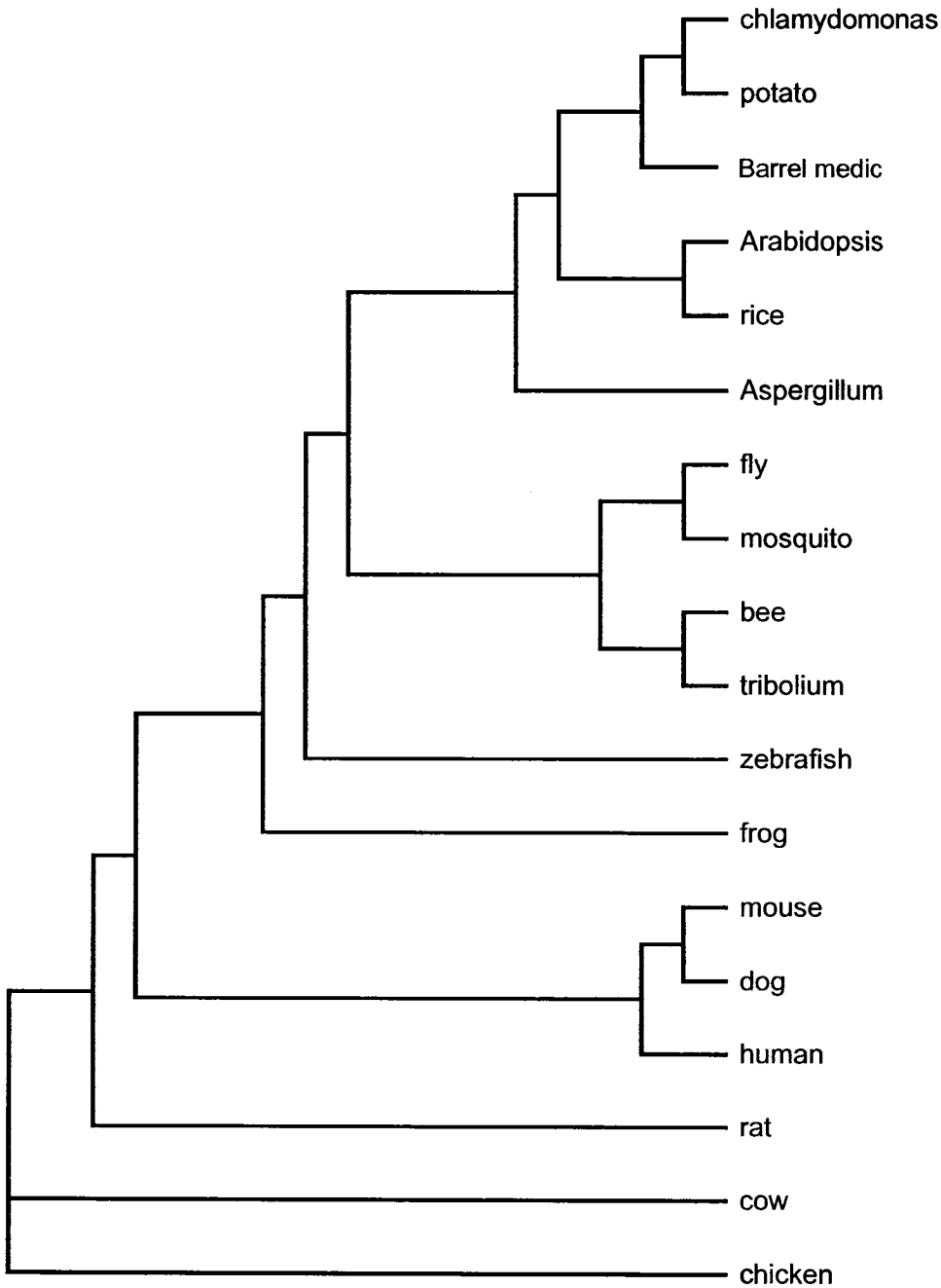


Figure 2-8: Phylogenetic tree of amino acid sequences homologous to WDS1. The consensus tree in cladogram was constructed from an optimal alignment of proteins using ClustalW program.



To carry out the phylogenetic analysis, proteins representing eighteen species were picked out according to their similarity to the WDS1 protein. A consensus tree based on these sequences is shown on Figure 2-8. The overall topology of the resulting tree is consistent with current phylogeny. Homologous sequences from species belonging to the same taxonomic family clustered together. In the phylogenetic tree, sequences from insects and plants species formed separate monophyletic groups. The branching order of sequences in Figure 2-8 indicates that *WDS1* homologs that are present in the plant species showed greater affinity for each other than the homologs from animals, even though three plant homologs share a lower sequence identity with the WDS1 compared to the animal proteins. The phylogenetic tree also reveals a closer relationship between plant and fungi species.

2.2.6 Comparison with G-beta proteins

The pairwise alignments show sequences identities in the range of 18-25% between WDS1 and G beta proteins from several other species and no significant similarity between WDS1 and the rat G beta protein.

Table 2-2: Pairwise alignments were performed between WDS1 and several G beta or G beta-like proteins from several species.

species	accession no.	identity%	similarity%	comments
Botryotinia fuckeliana	BAD93278	18	41	G protein beta subunit
Pisum sativum (pea)	AAM97354	24	41	G protein beta subunit
Homo sapiens	AAP97225	23	52	G protein beta subunit-like protein
Homo sapiens	AAF04308	25	42	G beta 6; G beta-like protein
Homo sapiens	AAA35922	21	39	G protein beta subunit
Rattus norvegicus	AAA62620	22	40	G-protein beta-subunit
Mus musculus	CAA44500	no significant similarity found		G-protein beta subunit
Fusarium oxysporum f. sp. lycopersici	AAO91808	18	41	G protein beta subunit
Setosphaeria turcica	ABN54456	20	42	G protein beta subunit

2.3 Discussion

WD40 repeat proteins comprise a family with a highly conserved structural motif but a diversity of functions. In this report, we described the identification of a novel protein, WDS1. The protein contains seven WD-40 repeats that have no more than five mismatches relative to the defined consensus sequence (Neer *et al.*, 1994). The WDS1 protein resembles the beta-subunits of G proteins and its structure is very similar to the known structural model of G beta protein. Based on the crystal structure of G beta, WD repeats are all predicted to form a circular bladed beta-propeller structure. Each propeller blade consists of four-stranded anti-parallel beta sheets and each WD 40 sequence repeat contains the last strand of one blade and the first three strands in the next. The final blade of G beta requires N-terminal residues to complete its beta sheet and to close the ring. This arrangement defines an overlapping structure of the WD repeats (Chen *et al.*, 2004). However, since the WDS1 sequence only showed a range of 18-25% similarity to G beta proteins as listed on table. It is proposed that WDS1 is not homologous to any known mammalian G beta subunit. This is also supported by the study on WDR26 proteins indicating that WDR26 belong to a new subfamily differing from G beta proteins (Zhu *et al.*, 2004). The *WDS1* family in *Arabidopsis* might be the counterpart proteins of the *WDR26* in humans considering the high sequence similarity between WDS1 and WDR26 proteins (Table 2-2). On the other hand, function of a particular gene will also be regulated at transitional and post-translational level. An example is the Drosophila ESC-C(Z) protein, assembly of which is proceeded by the phosphorylation of WD40 protein ESC that contributes to the function or regulation of the complexes (Joyce *et al.*, 2000).

2.4 Material and Methods

Phylogenetic Analysis

Homologous sequences of WDS were searched against the non-redundant database of proteins sequences (National Center for Biotechnology Information, NIH, Bethesda) using BLASTP (Altschu SF *et al.*, 1997). The search was also performed against the UniProt plant database for homologies in plant species. Multiple alignments of chosen sequences were constructed using the ClustalW version 1.83 (European Bioinformatics Institute) and then formatted using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Search of family members of the WDS1 were carried out against the *Arabidopsis* database using the amino acid sequences as the query. ClustalW version 1.83 was used to do multiple alignments of the homologous genes.
Protein structure studies were performed on SMART using normal mode (http://smart.embl-heidelberg.de/), while phylogenetic analyses of both of the two groups of sequences were performed on the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw/index.html).

G beta proteins were searched on NCBI databases and pairwise alignments were carried out using the Align program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) with default parameters.

Chapter III

Changes of WDS1 transcript levels during *Arabidopsis* development & Characterization of *WDS1*-knockout mutant

3.1 Introduction

Programmed cell death (PCD) is recognized as a physiologically and genetically controlled process of cell death necessary for the proper development of the organism (Pennell and Lamb, 1997; Madeo *et al.*, 2004). The programmed death can be activated by endogenous or exogenous triggers, which lead to sculpting of structures, deleting unneeded structures, controlling cell numbers, eliminating abnormal or harmful cells, and producing differentiated cells without organelles (Gilchrist, 1998). Both animal and plant PCD share hallmark features including plasma membrane blebbing, cellular and nuclear condensation and fragmentation, and cleavage of DNA into oligonucleosomes (Swidzinski *et al.*, 2002). Cell death signaling molecules such as ceramides can also regulate cell death in both animal and plant cells (Watanabe and Lam, 2006). These evidences argue for conserved cell death signaling mechanisms in eukaryotes, which provides plant researchers a way to study plant PCD by analogy to animal PCD molecules.

Leaf senescence is a developmentally programmed degeneration process responding to external and internal signals (Kong *et al.*, 2006). It is thought to be a regulated PCD process because, the final stage of leaf development, leaf senescence is fine-tuned by a complex regulatory network for plant fitness, which includes both de novo gene expression and protein synthesis (Morris *et al.*, 2000). A number of senescence-associated regulatory genes have so far been identified, such as *WRKY6* from *Arabidopsis* that is predicted to encode a transcription factor and *SARK* from bean, a receptor-like kinase (Kong *et al.*, 2006). It has been reported that leaf senescence can be influenced by a set of plant hormones. For example, treatment with jasmonates (JAs) causes premature senescence in leaves in wild-type

Arabidopsis (He *et al.* 2001). However, despite such advances the intricate nature of leaf senescence is still a mystery and little is known about the regulatory signals and pathways that control this event.

As well as being a developmental process, senescence can also be induced prematurely by environmental conditions, a mechanism that they have evolved to cope with unfavourable environmental conditions (Buchanan-Wollaston *et al.*, 2003). Plant cell death also occurs in senescence as a consequence of plant-pathogen interactions in both compatible and incompatible relationships and under abiotic stress (Kangasjarvi *et al.*, 2005). Recent analysis of the signaling pathways involved with different stress responses has indicated that these pathways have considerable cross-talk with senescence related gene expression (Buchanan-Wollaston *et al.*, 2003). Genes identified as being senescence-enhanced have been shown to be expressed in leaves exposed to many different stresses (Butt *et al.*, 1998; Pontier *et al.*, 1999; Miller *et al.*, 1999; John *et al.*, 2001), but they did not all show the same patterns of expression (Park *et al,* 1998; Weaver *et al.*, 1998; Buchanan-Wollaston *et al.*, 2003). For instance, Morris *et al.* (2000) found that the salicylic acid (SA) pathway may be involved in both senescence-related cell death and pathogen or ozone induced death.

The phenolic compound SA has been identified as a key signal component for the induction of plant resistance (Durrant and Dong, 2004). It plays an important role in numerous plant responses to stress, including pathogen invasion, exposure to ozone and UV-B (Morris *et al.*, 2000). Application of exogenous SA or SA analog activates the expression of a variety of pathogenesis-related (PR) genes and enhances resistance to a variety of pathogens. A number of plant mutants compromised in SA accumulation or perception show enhanced susceptibility to biotrophic pathogens, whereas mutants that overproduce SA display enhanced resistance (Gil *et al.*, 2005). Besides, SA has also been discovered to play a role in gene expression control during developmental senescence (Morris *et al.*, 2000). A set of genes are involved in SA-dependent pathways, such as *EDS5, PAD4, NPR1* and *NahG* (Glazebrook *et al.*, 2003), yet further work are required to study the details concerning the SA pathways, especially on the downstream regulation.

Fumonisin B1 (FB1) is produced by *Fusarium moniliforme* (Desjardins *et al.*, 1995), and has been found to elicit an apoptotic form of PCD in both plants and animals (Asai *et al.*, 2000; Tolleson *et al.*, 1999; Wang *et al.*, 1996) and inhibit growth in yeast (Stone *et al.*, 2005). This fungal toxin disrupts sphingolipid metabolism in eukaryotes by acting as a competitive inhibitor of ceramide synthase (Desai *et al.*, 2002), a key enzyme in sphingolipid biosynthesis (Abbas *et al.*, 1994; Gilchrist *et al.*, 1995; Yoo *et al.*, 1996; Stone *et al.*, 2000). Sphingolipids play diverse roles in many cellular processes, functioning both as anchors for membrane proteins (Futerman, 1995) and as second messengers regulating various cellular functions, including differentiation, growth, and apoptosis (Spiegel and Merrill, 1996; Stone *et al.*, 2000). The sphingolipid ceramide is a key component of the mammalian stress response pathway, activating several stress-activated protein kinases and phosphatases (Nickels and Broach, 1996; Zhang *et al.*, 1997; Stone *et al.*, 2000). In *Arabidopsis thaliana*, FB1 treatment initiates nuclear DNA fragmentation preceding the loss of membrane integrity, which resembles apoptosis typically associated with PCD in animal cells. FB1-induced cell death is light-dependent, and also dependent on active transcription and translation, as well as reversible protein phosphorylation (Asai *et al.*, 2000). It has been demonstrated that FB1 killing of *Arabidopsis* requires SA-, JA-, and ET-mediated signaling pathways as well as one or more unidentified factors activated by FB1 and the *acd2-2* mutation (Asai *et al.*, 2000).

In this report, we describe the isolation and characterization of the *WDS1* gene. The *WDS1* transcript level varies during different developmental stages of wild type *Arabidopsis*, which implies that the *WDS1* gene may function in senescence. This is further confirmed by the accelerated induction of the age specific senescence marker, Senescence Associate Gene 12 (*SAG12*), in the loss-of-function mutant *wds1-1* compared to the wild type plants. On the other hand, the *WDS1* expression is significantly up-regulated in SA treatment, suggesting that *WDS1* may play a role in the SA-mediated pathway.

3.2 Results

3.2.1 Expression of *WDS1* in plant development

The sequence similarity between WDS1 and the WDS protein in *Drosophila* implies that *WDS1* may also play a role in the development of *Arabidopsis*. To test this hypothesis, we began with monitoring the expression of the *WDS1* gene at different growth stages of *Arabidopsis* defined by Boyes *et al.* (2001) (Table 3-3).

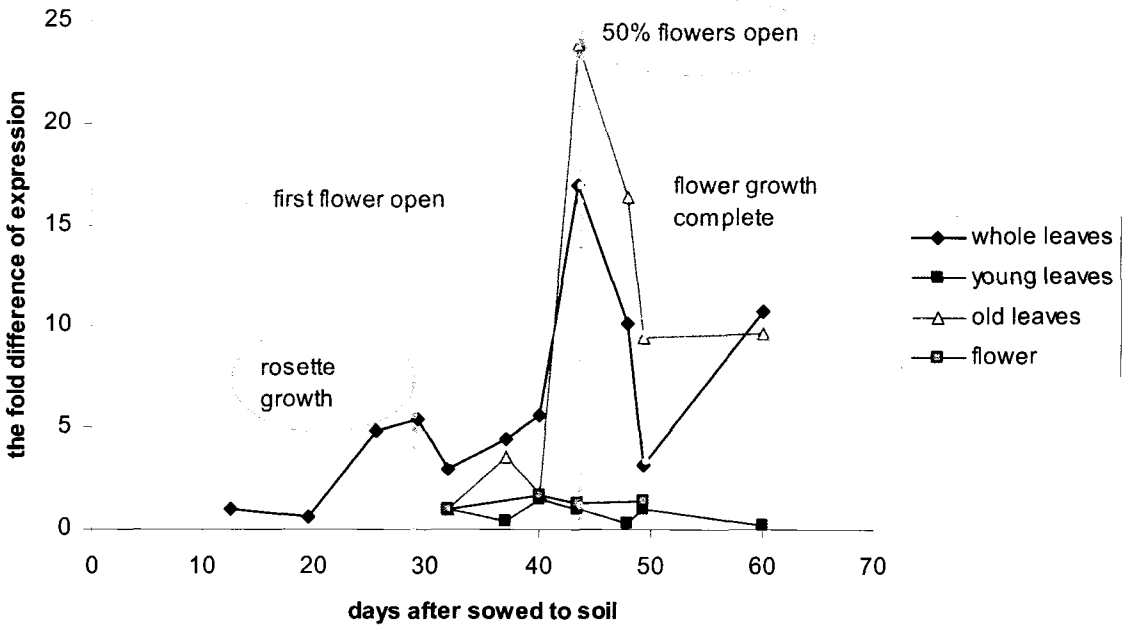
Similar to the natural plant senescence (Quirino *et al.*, 2000), the leaf yellowing of *Arabidopsis* starts at the leaf margins and progresses to the interior of the leaf blade. The results from real-time PCR revealed that the expression of the *WDS1* gene in the leaves changes considerably during the development of *Arabidopsis*. Based on the data from the whole-leaf samples, the *WDS1* gene increased transcriptionally along the growth of rosette leaves until the rosette leave growth was completed (29.3 days old), at which stage the gene was up-regulated by approximately five fold. Afterwards, the expression of the *WDS1* gene declined slightly when the first flower opened (31.8 days old), but quickly climbed to the peak of 16-fold up-regulated when 50% of flowers that are to be produced are opened (43.5 days old). Thereafter, the transcription dropped to a minimum at the completion of flower growth before its up-regulation during the senescence of the plants. The expression level of *WDS1* gene in 60 days' old plants was 10 fold greater than that in the 12 days' old ones.

Study of the *WDS1* transcription in old leaves also indicates a dramatic increase prior to the stage when 50% flowers that are to be produced are opened. The gene was around 24 fold up-regulated at the peak compared to the stage when the first flower opened. Afterwards, the transcription of *WDS1* dropped significantly till the flower growth completed, and then remained to a level which was of 10 fold greater.

The *WDS1* gene showed little change in expression in both young leaves and the flowers when compared to that in the whole leaves and old leaves (Figure 3-1), suggesting that *WDS1* was mainly expressed in leaves that were undergoing senescence.

Figure 3-1: Change of the expression levels of *WDS1* gene in the development of *Arabidopsis*. The experiment was conducted once and needs to be repeated in the future work.

Expression pattern of *WDS1* gene in the development of *Arabidopsis*



The fluctuation of the expression level at different growth stages indicates that *WDS1* might be associated with the development of *Arabidopsis*. Therefore, the knockout lines of *WDS1* were isolated as stated below and phenotype analysis was first carried out between the wild-type and knockout plants.

3.2.2 Microarray data on developmental senescence

The NASCArrays databases were surveyed for experiments on expression profiles of *Arabidopsis* development with both At5g43920 (*WDS1*) and At5g08560 (*WDS2*, the closest member of *WDS1* according to the phylogeny analysis) as the query, respectively. Only three sets of experiments were retrieved for At5g43920, whereas more than 100 experiments were found when using at5g08560 as the query (data not shown). The AtGenExpress experiment on developmental series of leaves (NASCARRAYS-150) was chosen for analysis in consideration of the procedure of sample collection. The averaged signal values from triplicate slides for both *WDS* genes and *SAG12* gene remained constantly low through young leaves to adult leaves, with the transcriptional level in the order of *WDS2>WDS1>SAG12*. In the senescing leaves as bolded in the Table 3-1, both *WDS1* and *WDS2* were up-regulated by 1.65 and 3.29 fold respectively, in comparison with the significant up-regulation of *SAG12*. This suggests that *WDS1* and *WDS2* may function in leaf senescence during the plant development.

Table 3-1: Comparison of *WDS1, WDS2* and *SAG12* gene expression on developmental samples. Signal values listed were the average of raw data from triplicate slides for each time point. Up-regulated values from senescing leaves were in bold.

Tair accession No.	Age(d) / Protein	7, leaves 1+2	17, leaf2	17, leaf4	17, leaf6	17, leaf8	17, leaf10	17, leaf12	35, senescing leaves	Ratio*
At5g08560	*WDS2*	114.9	158.9	169.2	142.1	136.5	129.6	133.2	**377.8**	**3.29**
At5g45890	*SAG12*	5.5	5.9	0.7	5.6	3.6	3.5	4.9	**3307.4**	**601.35**
At5g43920	*WDS1*	49.3	52.3	54.3	50.5	37.6	37.0	41.0	**81.2**	**1.65**

*: Calculation=signal values in 35-day-old senescing leaves/signal values in 7-day-old leaves.

3.2.3 Isolation and molecular characterization of homozygous T-DNA knockout *wds* mutants

To identify the potential role of *WDS1* on *Arabidopsis*, three transgenic lines with T-DNA insertion in the Col-0 background were obtained. The SALK lines SALK_106274, SALK_022240, and SALK_039908 were designated as *wds1-1, wds1-2* and *wds1-3* respectively (Figure 3-4). All the insertions were confirmed by using PCR-based markers that can discriminate between the wild-type and mutant alleles (Figure 3-2). Only the mutant *wds1-1* contains a T-DNA insertion in the first exon, +319 bp from the translation initiate site. T-DNA insertion is located 223 bp upstream from the translation initiate site in the *wds1-2* mutant and 36 bp downstream from the stop codon in the *wds1-3* (Figure 3-4).

Seeds for the SALK insertion lines were obtained from *Arabidopsis* Biological Resource Centre (ABRC). The ~4 kb T-DNA inserted into the gene is designed to contain the *NTPII* as a marker (kanamycin resistance). However after several generations of growth, some of the lines show silencing of this gene. Thus, sometimes a mutant line may not be able to express the drug resistance phenotype. The left border sequence of the T-DNA was used for PCR amplification of plant flanking sequences and two sets of primers (LBa1 and LBb1) for the left border sequence were designed. PCR methods are available for recovery of insertion site flanking sequences (Sieber *et al.*, 1995). Based on insertion location and the genomic sequences, left primer (LP) and right primer (RP) by the insertion site were designed.

To identify the homozygous mutant, standard PCR were carried out with all the three primers, LP, RP, LBa1 or LBb1, using the genomic DNA extracted from the mutant line. If the T-DNA has inserted in the plant genomic sequence, amplification with LP and RP would be impossible due to the large size of T-DNA. On the other hand, sequences between RP and LBa1 or LBb1 would be amplified. If there is no insertion, amplification of sequence between LP and RP will occur, which will result in a product of larger size. PCR amplification using the homozygous mutant or wild-type plant as the template would each give one product, but of different size. And PCR amplification on heterozygous mutant would contain each of the two bands after agarose gel electrophoresis. In this way, the T-DNA homozygous

Figure 3-2: PCR amplification for identification of homozygous mutant lines. The 1 kb ladders are indicated at the right of the gel. Leaves from *wds1-1, wds1-2* and *wds1-3* were collected and the PCR amplification with the corresponding primers was performed on the corresponding genomic DNA. From the gel, homologous lines are from lane 1, 2, 7, 9, 13, 15, 19 and 21-30.

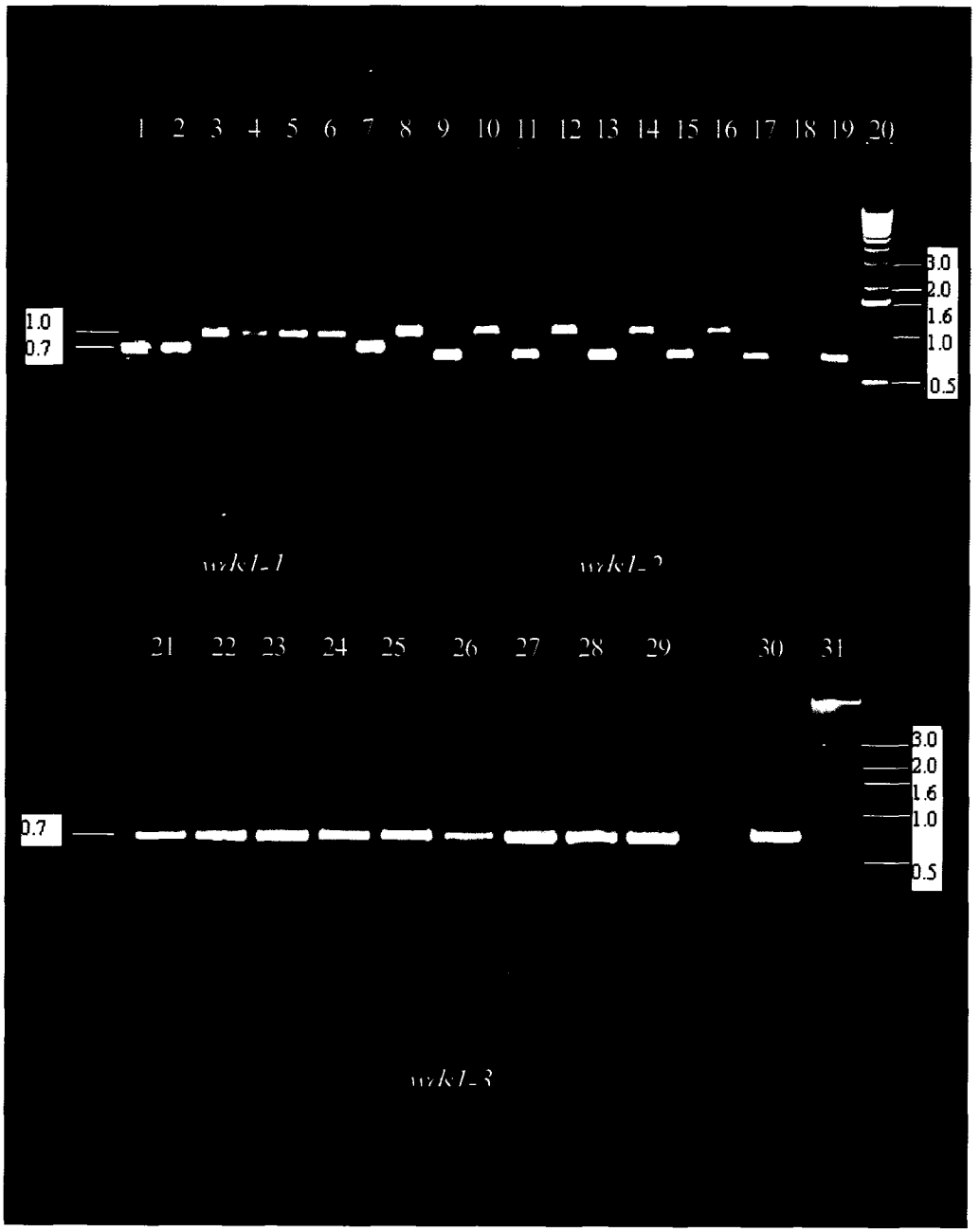


Figure 3-3: PCR amplification identifies T-DNA insertion. In the figure: Lba1 or LBb1, left border a1 or b1 (LBa1 locates 200 bp left of LBb1); LP, left primer; RP, right primer.

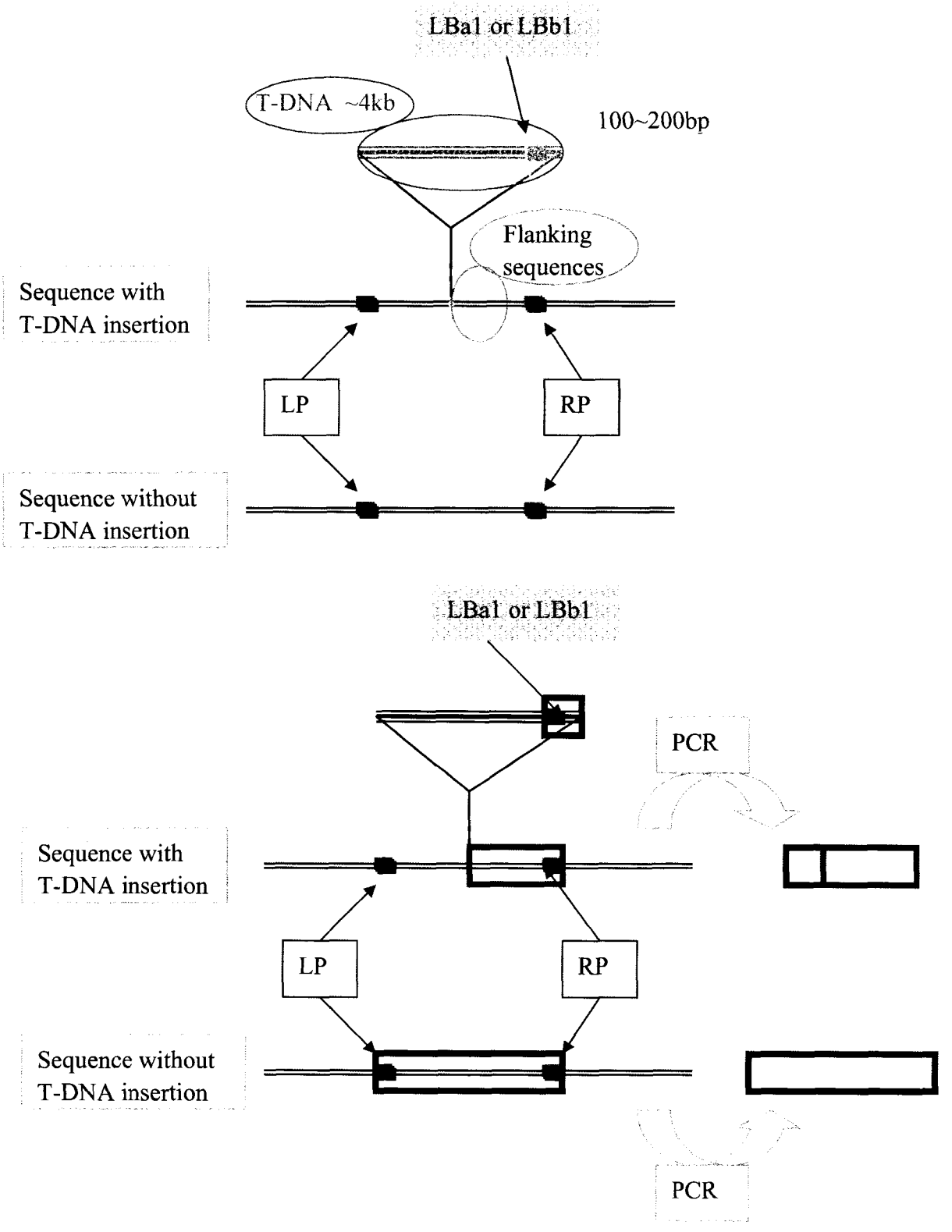


Figure 3-4. Scheme of the genomic regions corresponding to *WDS1* constructed from information available from the *Arabidopsis* Information Resource (TAIR; www.*arabidopsis*.org). Closed boxes represent exons. The positions of the T-DNA insertions, the start codon ATG and stop codon TGA are indicated.

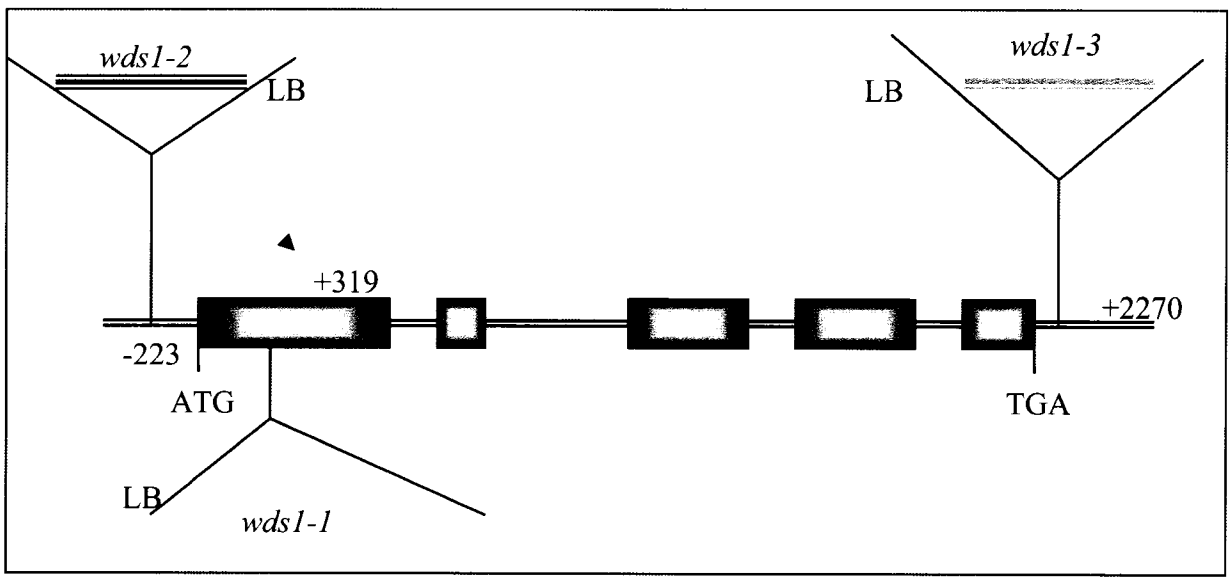
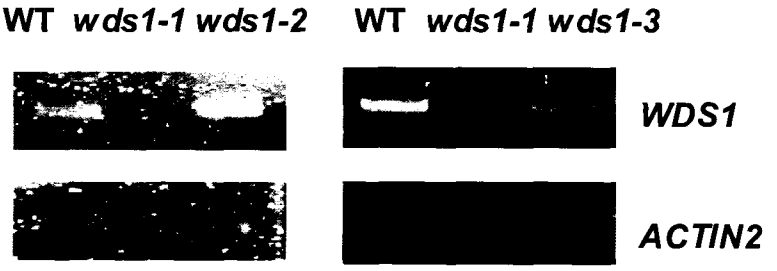


Figure 3-5: Expression of *WDS1* in the wild type and three *wds1* mutants.

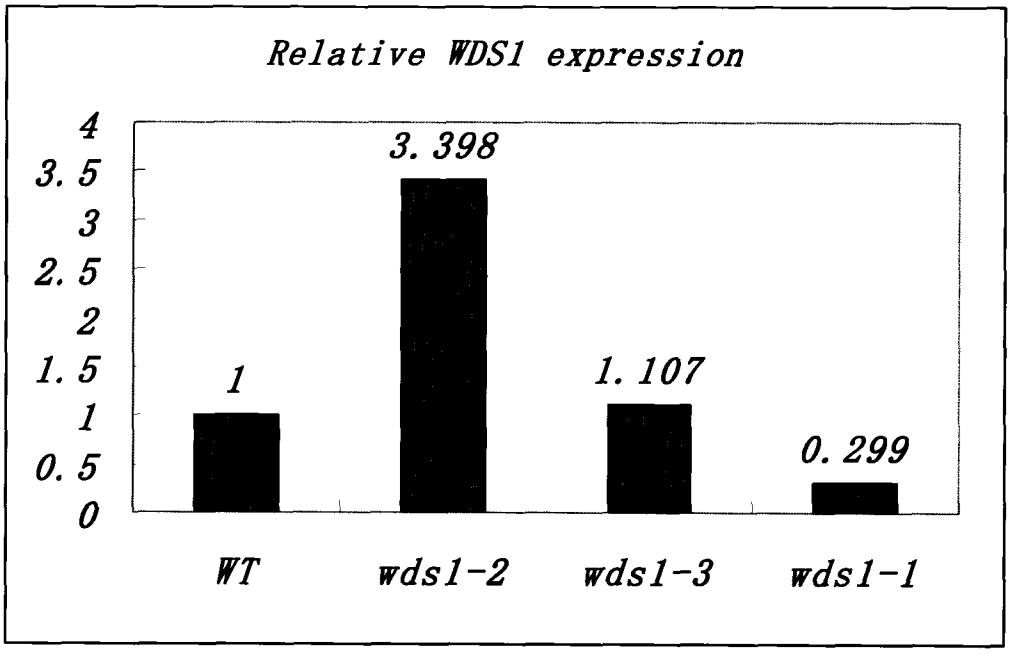
a) RT-PCR analysis of mRNA accumulation of *WDS1*. The mRNA control of *Actin2* for equal loading are shown at the bottom.

b) Real-time RT-PCR analysis of mRNA accumulation of *WDS1*. Expression of *Actin2* (ACTIN2) was used to normalize cDNA input from the different genetic backgrounds. Both kinds of PCR were conducted with the same reaction conditions as indicated in the materials and methods section using the same primers.

a)



b)



mutant is identified (Figure 3-3). As shown in the Figure 3-5, the level of *WDS1* transcript in *wds1-1* was considerably low while that in the *wds1-2* displayed a slight increase when compared with that in the wild-type plant. It is possible that the insertion of T-DNA in the 5'-UTR of *WDS1* gene sequence helps to release the repression of the transcription initiation, therefore resulted in abundant *WDS1* transcripts. The expression of *WDS1* in *wds1-3* is similar to that in wild-type.

3.2.4 Comparison of the phenotypes of the three knockout mutants with wild-type

Both the wild type and three *wds1* mutants were grown under the same conditions for phenotypic analyses. Potential morphological defects on flowering time, leaf development and root elongation were recorded for comparison in five independent experiments. No significant difference was detected on leaf development between the *WDS1*-knockout lines and the wild type. The transition to flowering is a phase change in plant ontogeny leading from vegetavie to reproductive growth (Pouteau *et al.*, 2006). For practical reasons, flowering time is usually recorded at later stages based on macroscopic morphogenetic changes. It can be measured by direct temporal indicators such as the time to first floral bud opening or the number of days to bolting (Pouteau *et al.*, 2006). Flowering time can also be estimated by indirect morphometric indicators such as the total number of nodes bearing leaves, i.e. the sum total of rosette leaf number and cauline leaf number below the secondary inflorescence bearing flowers without

Table 3-2: Comparison of flowering time and silique length between the three mutants and wild-type plants.

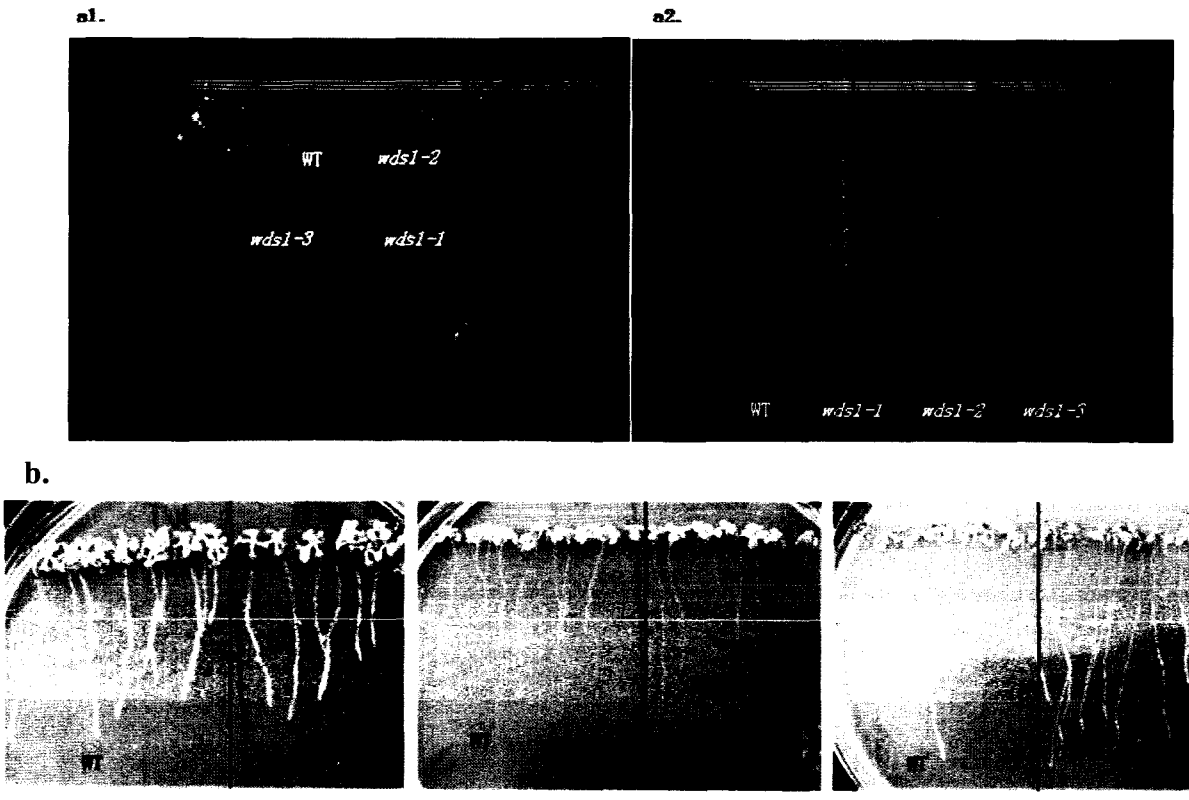
plants	Rosette leave number[a,c]	Cauline leave number[a, c]	silique length[b,c] (cm)
WT	14.67±1.83	3.25±0.87	14.00±1.22
wds1-1	14.92±1.31	3.17±0.58	11.97±1.16
wds1-2	14.33±1.15	3.08±0.79	12.33±1.00
wds1-3	14.58±1.78	3.58±0.51	13.73±1.20

[a]: Data based on five repeats of experiments

[b]: Data based on the one group of experiment

[c]: Mean±standard error.

Figure 3-6: photographs illustrating the phenotypes of the three mutants *wds1-1, wds1-2* and *wds1-3.* No significant difference was identified in both the size and shape of seeds (a1) and the root (b) growth after comparison of the mutants and wild-type plants. Three independent experiments were conducted under the same condition.



bracts (Pouteau *et al.*, 2006). Here we measured the flowering time through by measuring the rosette and cauline leaf numbers. The deletion of *WDS1* had little effect on the growth of rosette and cauline leaves as shown in Table 3-2. Siliques produced by the three *wds1* plants in one group appeared shorter than those from the wild-type (Table 3-2 and Figure 3-6). Generally speaking, the silence of *WDS1* in *Arabidopsis* had no significant influence on leaf, flower or root on both the morphological and developmental levels.

3.2.5. FB1 treatment led to earlier induction of *SAG12* in *wds1-1* than wild-type

Leaf senescence may be induced by both internal factors, such as age and hormone (Gan and Amasino, 1995; Wingler *et al.*, 1998; Masterrer *et al.*, 2002; Grbic and Bleecder, 1995; Oh *et al.*, 1997), and external factors, including biotic and abiotic stresses (Pic *et al.*, 2002; Buchanan-Wollaston *et al.*, 2003). The marked up-regulation of *WDS1* in senesces leaves from *Arabidopsis* implicated that it may be associated with biotic stress-induced senescence. Therefore, to further investigate the role of *WDS1* in PCD during senescence, we measured the expression of a senescence specific marker viz *SAG12* (Gan and Amasino, 1995; Lim *et al.*, 2003; Yoshida, 2003) in the wild-type and *wds1-1* mutants using the fungal toxin fumonisin B1 (FB1) as the biotic cell death inducer of *Arabidopsis* leaf. FB1 is naturally produced by *Fusarium spp*, including *F. moniliforme* that may play a role in virulence (Asai *et al.*, 2000; Dutton, 1996; Gilchrist, 1997; Jardine and Leslie, 1999), and elicits an apoptotic form of PCD in both plants and animal tissue culture cells (Tolleson *et al.*, 1996; Wang *et al.*, 1996a, 1996b; Yoo *et al.*, 1996; Gilchrist, 1997; Stone *et al.*, 2000). Infiltration with FB1 can trigger both necrotic lesion formation and a characteristic PCD response in *Arabidopsis* leaves (Stone *et al.*, 2000; Watanabe and Lam, 2006).

In two independent experiments, FB1 elicited similar lesion formation in both wild type and mutant leaves (Figure 3-8). As displayed in Figure 3-8, lesions appeared on both kinds of samples within 60 h when a 5 μM FB1 solution was infiltrated into mature leaves of 3-week-old wild-type soil-grown *Arabidopsis* plants under controlled environmental growth conditions. Different from the typical leaf senescence spreading from the edge to the interior, FB1 treated leaves displayed a uniform chlorosis over the entire leaf lamina.

In addition to the yellowing of leaves that appeared on the third day after FB1 infiltration, *SAG12* level was induced in both the wild type and *wds1* mutant leaves, suggesting that FB1 most likely elicits cell death by activating a controlled program. In contrast to the synchronous chlorosis of wild-type and *wds1* plants, expression of the *SAG12* gene was detected 12 h earlier in *wds1* leaves than in the wild type under FB1 treatment. Real-time PCR analysis revealed that, expression of *SAG12* stayed at a low level in both mutant and control within the early two days of the treatment. In the first group of experiment, *SAG12* transcripts started to accumulate in the mutant leaves from the third day (at 60 h time point) and reached maximum level 12 hours later, which generally paralleled the yellowing progress. After a rapid decrease, *SAG12* transcription started to rise again in the last 12 hours of FB1 treatment, resulting in a 5 fold up-regulation of *SAG12* at 96 h compared the 0 h time point (Figure 3-7). In the wild-type samples, rapid transcript accumulation of *SAG12* was detected at 72 h, followed by an immediate drop to the same low level as prior to FB1 treatment. In general, induction of *SAG12* occurred 12 hours earlier in *wds1* mutant, but the maximum level was six fold smaller than in the wild type. In both cases, the induction of *SAG12* transcripts was followed by a rapid decrease 12 hours later. The second group of experiments exhibited a similar trend of SAG12 expression change, but its onset started from 36 hours. This is probably due to the inconsistent operation of vacuum infiltration.

Elevated expression of the *SAG12* gene has been suggested to correlate with the manifestation of age-dependent senescence but not that induced by environmental stressors (Gan and Amasino, 1997; Lim *et al.*, 2003; Yoshida, 2003). Therefore, the accelerated expression of *SAG12* gene in the *wds1* mutant suggests that *WDS1* may play a positive role in the age-dependent senescence.

3.2.6 Up-regulation of *WDS1* transcripts by SA

The considerable overlap between pathogenesis-related and senescence-related genes (Weaver *et al.*, 1998; Quirino *et al.*, 1999) suggests that *WDS1* expression might be able to respond to senescence-inducing stresses or hormones. Salicylic acid has been known as a crucial signal molecule leading to systemic acquired resistance (SAR) and pathogenesis-related (PR) gene induction (Delaney *et al.*, 1994; Gaffney *et al.*, 1993), and also plays a role in regulation of gene expression in the course of leaf senescence (Morris *et al.*, 2000). To determine whether *WDS1* is associated with SA-dependent

pathway during leaf senescence, we carried out real-time PCR to monitor the expression level of *WDS1* gene under SA treatment.

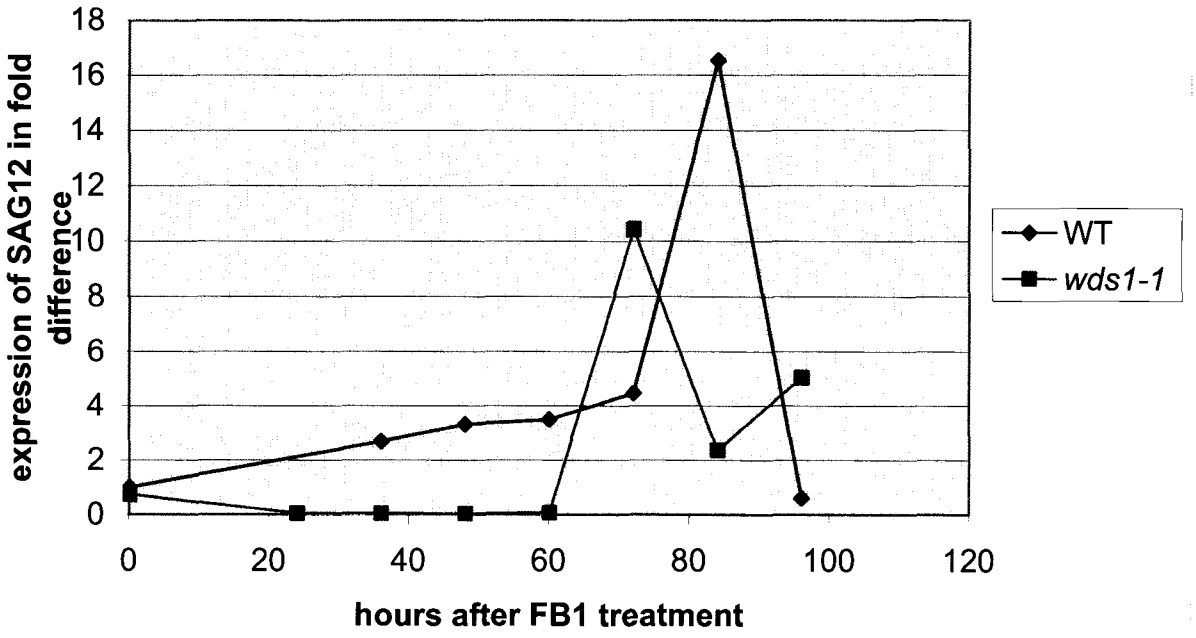
Similar to natural senescence (Betania *et al.*, 2000), four weeks old mature leaves under SA treatment exhibited a loss of chlorophyll starting at the leaf margins (preferably from the cutting sites) progressing to the interior of leaf center. This chlorosis was rapidly followed by necrosis. In contrast to the gradual color change displayed by the mock control, SA-treated leaves showed a more rapid yellowing process turning from green to pale (Figure 3-9). Lesions started to appear on the leaves infiltrated with either 0.5 mM or 2 mM SA solution within 24 hours (Figure 3-10). By 48 hours, all the leaves have turned pale after infiltration of 2 mM SA solution.

Real-time PCR analysis revealed that at 48 h and 72 h 2 mM SA infiltration, *WDS1* expression was dramatically up-regulated (~35 fold) compared to water treatment (~5 fold up-regulation). The *WDS1* transcription also rose to a high level by 0.5 mM SA infiltration, though the increase is smaller than that treated with 2 mM SA (Figure 3-10).

The expression level of *WDS1* correlated with appearance of lesions. At each of the intervals, it was the leaves treated with 2 mM SA infiltration that showed the most serious lesions, followed by those treated with 0.5 mM SA. Lesion formation on the leaves under mock treatment was much weaker compared with SA treatment, which implied an accelerated cell death progression by SA in the leaves. Also, in water treatment, cell death was eventually observed as shown at 72 hours. This occurs and is normal.

Figure 3-7: Expression pattern of *SAG12* in FB1-treated wild-type and *wds1-1* mutant leaves. Two independent experiments were conducted. As shown in both graph, the onset of SAG12 expression in the *wds1-1* mutant was twelve hours earlier than in the wild-type in both repeats.

SAG12 expression in WT and *wds1-1* after FB1 treatment in Gp1



SAG12 expression in WT and *wds1-1* after FB1 treatment in Gp2

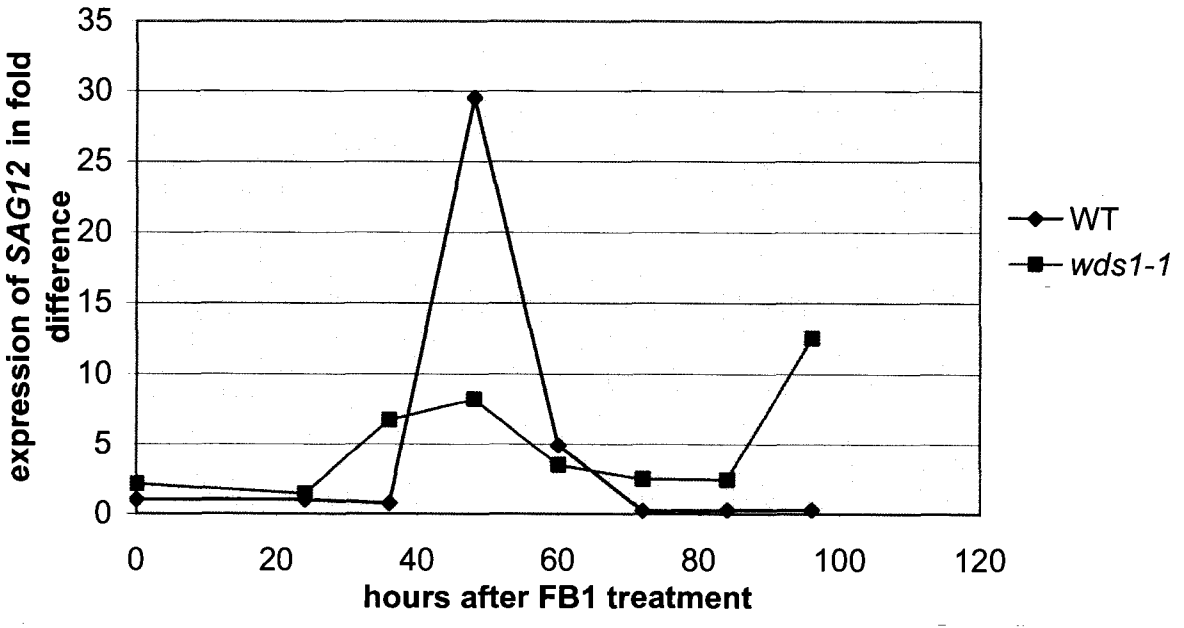


Figure 3-8: Comparison of phenotypic alternation between the wild-type and *wds1-1* mutant leaves during 5μM FB1 inoculation in both repeats.

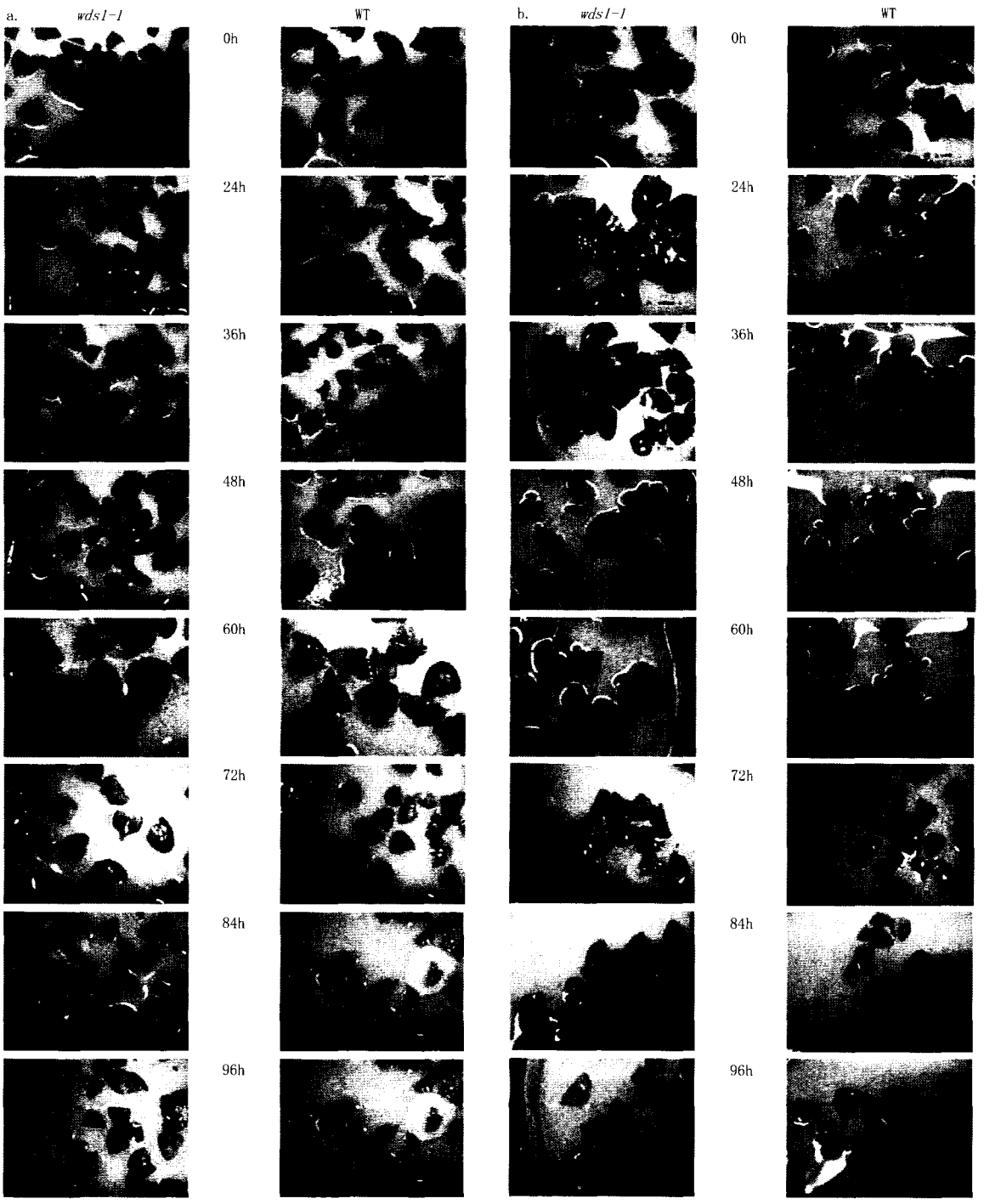


Figure 3-9. Wild-type leaves at 0 h, 24 h, 48 h and 72 h water, 0.5 mM SA, 2 mM SA infiltration.

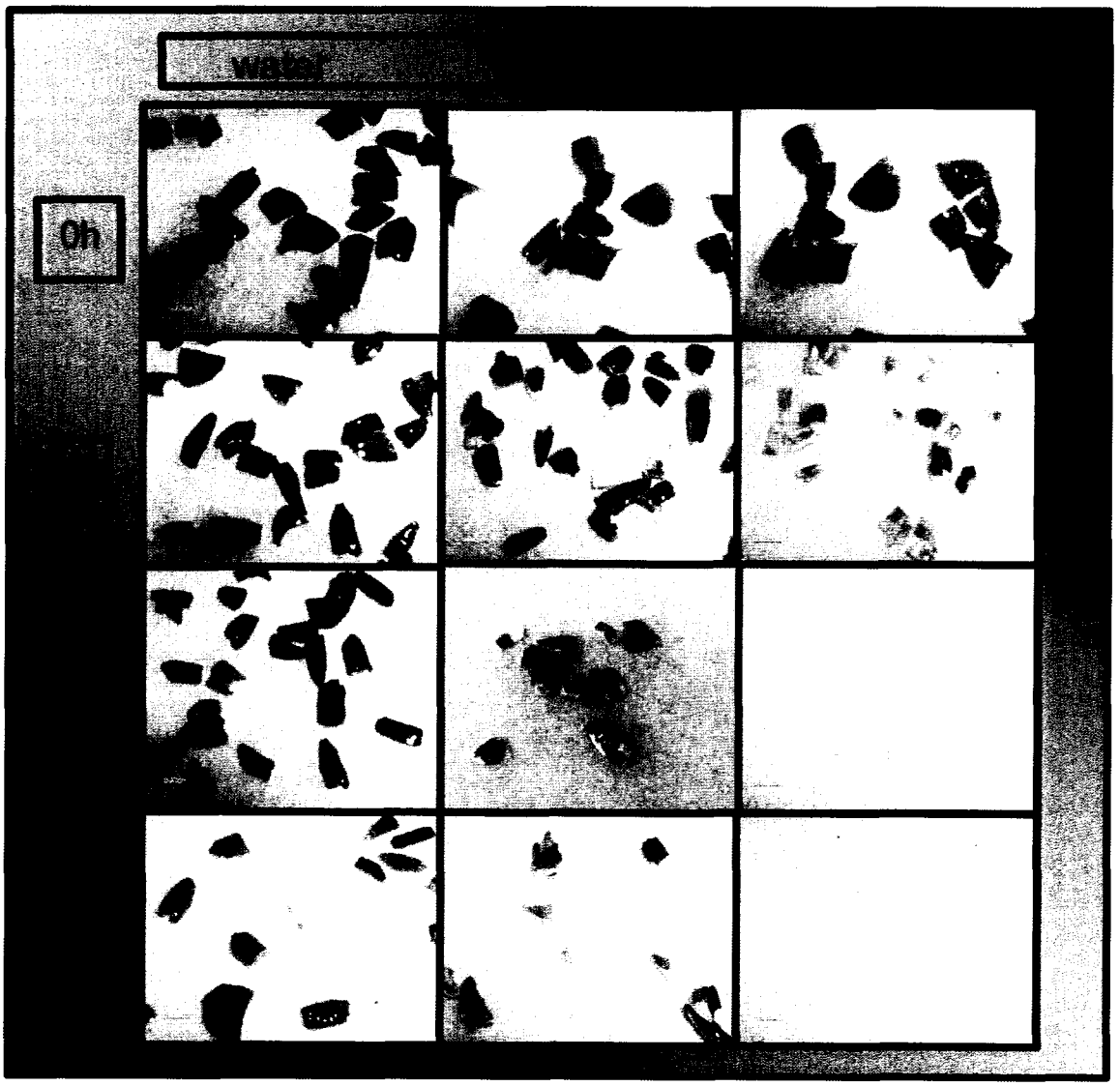
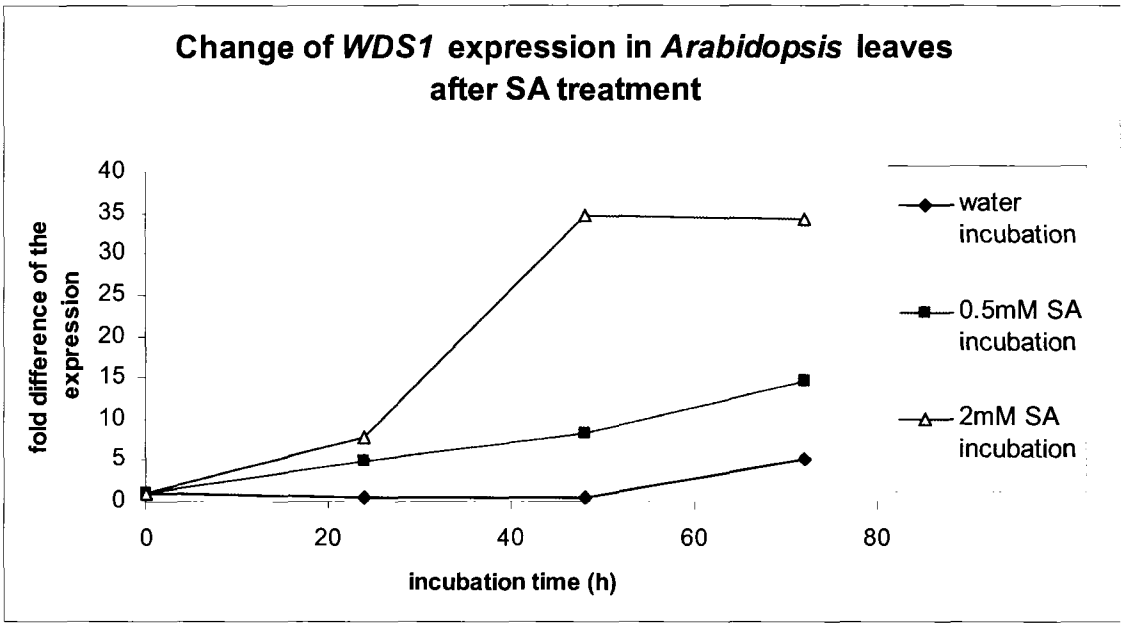


Figure 3-10. Up-regulation of *WDS1* gene expression in wild-type *Arabidopsis* leaves by SA treatment in one experiment. Repeated experiment need to be conducted in the future work.



3.3 Discussion

3.3.1 *WDS1* modulates the development of *Arabidopsis* plant

Plant development is a dynamic process in which the topology and geometry change over time in a complex manner under the control of gene action (Lars *et al.*, 2005). Senescence, as the last stage of development, has been viewed as a form of programmed cell death (PCD), which is both highly regulated and genetically programmed (Betania *et al.*, 2000). The ordered progress involves the degradation of chlorophyll and many other macromolecules, such as protein, lipid and nucleic acid, and the mobilization of nutrients, including nitrogen, phosphorus, metal ions and minerals (Lin and Wu, 2004; Hortensteiner and Feller, 2002; Takamiya *et al.*, 2000). Although it means the loss of vitality of leaf tissue, leaf senescence is tightly controlled during the developmental program to increase the fitness of the whole plant (Betania *et al.*, 2000; Lin and Wu, 2004). Research over the last few decades has shown that the process can be induced by both internal and external factors and it requires the expression of novel genes and the synthesis of proteins (Lin and Wu, 2004; Buchanan-Wollaston *et al.*, 2005; Nooden *et al.*, 1997). Several different groups have identified genes that show increased expression during the onset of senescence in experiments conducted with *Arabidopsis* (Buchanan-Wollaston *et al.*, 2003; Betania *et al.*, 2000; Buchanan-Wollaston *et al.*, 2005). Transcriptome analysis carried out by Guo *et al.* (2004) showed a collection of over 2000 expressed sequence tags clones that represent genes that are expressed in *Arabidopsis* senescing leaves. These up-regulated senescence-associate genes (*SAGs*) include genes encoding proteins involved in the breakdown of cellular components, such as ribonucleases, polyubiquitin proteases and cell wall hydrolases, and genes whose products are associated with the mobilization of nutrients and minerals (Bleecker and Patterson, 1997; Nooden *et al.*, 1997; Quirino *et al.*, 2000). *SAG*s of particular interest include *SAG12*, which is specifically expressed in senescing tissues at high levels (Lohman *et al.*, 1994). The protein encoded by *SAG12* has been suggested to be located in cytoplasm due to its lack of a canonical signal sequence (Weaver *et al.*, 1997). It is a member of the cathepsin superfamily of proteases, which includes a CED-3 (for cell death) and ICE (for interleukin-1 β-converting enzyme). These proteases trigger programmed cell death in animals (White, 1996). However, given the large number of up-regulated

genes in senescence, only a small portion of *SAGs* have been isolated and functions of the others remain to be illustrated.

The monitor of *WDS1* expression in different developmental stages of *Arabidopsis* suggests that *WDS1* may play a role in the regulation of leaf senescence. The mRNA abundance of the gene was relatively constant in young plants until early flowering stages when it started to increase and reached its maximum value within a short period in senescing plants. The *WDS1* transcripts remained at a relatively high level in later senescence (Figure 3-1). This expression pattern is similar to that of *LSC210*, which encodes a putative metallothionein expressed at all stages of leaf development but the level of transcript increased steadily as senescence progressed (Buchanan-Wollaston and Ainsworth, 1997). It has been suggested that the presence of metallothionein proteins may protect the nuclear DNA from oxidative damage caused by free radicals, thus allowing expression of senescence-specific genes required for the process to take place (Buchanan-Wollaston and Ainsworth, 1997).

The enhanced expression of *WDS1* in senescing leaves is also supported by the microarray data downloaded from the NASCArrays experiment, but the up-regulation is smaller. This may be explained by the slight differences in sample collection between the two sets of experiment. Leaves in the present work are collected with regard of the seedling age, ranging from 12 to 60 days of the plant; on the contrary, samples used in the public microarray experiments were collected based on the age of a particular leaf in study. Future experiment will adopt the latter system.

In the analyses, signal values from microarray experiments greater than 40 were regarded as reliable in this study given that the NASCArrays tutorial has suggested that a signal value lower than 100 are generally not being expressed. This is done for two reasons. First, most of the three duplicates for each time point present similar signal values (data not shown), supporting the reliability of the microarray system. Second, the *SAG12* transcription was extremely low in young leaves, which agrees with the previous conclusion that no *SAG12* transcripts can be detected in green leaf tissues (Lohman *et al.*, 1994).

The microarray data exhibited a greater expression of *WDS1* than *SAG12* in young leaves; however, the small signal values (<100) indicated a lower transcriptional level of *WDS1* compared to functional genes at early developmental age. Another interesting discovery from the microarray profiles is the abundant expression of another *WDS* family member, *WDS2*, transcripts of which succeed that of *WDS1* in young *Arabidopsis* leaves by more than two fold. The *WDS2* gene also displayed a greater up-regulation of transcription in senescing leaves, indicating a similar or even more significant function in comparison with *WDS1*.

3.3.2 *wds1* do not cause alteration in *Arabidopsis* phenotype

Although the expression profile of *WDS1* was proposed to monitor the development of *Arabidopsis*, none of the three *wds1* mutants exhibit any significant phenotypic alteration. A possible explanation is the potential functional homologues of the *WDS1* gene. As indicated in Chapter one, four other putative *WDS* genes shared considerable sequence identity with *WDS1*, which suggests that they may possess similar function in regulation of plant development. The sequencing of the *Arabidopsis* genome carried out by Blanc *et al.* (2000) revealed large areas of duplication that may contain genes with the same function. Insertion knockouts of one of these duplicates may not show any phenotype (Buchanan-Wollaston *et al.*, 2003). The shatterproof genes SHP1 and SHP2 may serve as an example (Vision *et al.*, 2000). Being in a chromosomal block that duplicated around 100 million years ago, the two genes must be removed simultaneously before the nodehiscence phenotype is observed (Liliegren *et al.*, 2000).

3.3.3 *WDS1* plays a role in FB1-induced leaf senescence

Premature senescence in a plant can be induced by several internal and external factors. The internal factors include developmental age and changes in hormone levels (Park *et al.*, 1998). The external factors include a number of different environmental stresses such as pathogen infection, nutrient or water stress, or oxidative stresses induced by ozone or UV-B. The PCD-eliciting mycotoxins such as AAL toxin or fumonisin B1 have been used in model systems for the study of plant cell death in pathogen response pathways (Gilchrist, 1998). In this work, the loss of *WDS1* expression resulted in

accelerated expression of *SAG12* gene under FB1 treatment, indicating that *WDS1* functions as a cell death attenuator in FB1-induced senescence.

To facilitate the infiltration of FB1, we cut the leaves into small pieces. As shown in Figure-8, FB1 induced a visible senescence on both wild type and *wds1* mutant leaves, yet the lesions formed are not significant compared to the results obtained by Asai *et al.* (2000). This is probably because a smaller concentration (5 μM) of FB1 solution was used in the present work. Stone *et al.* (2000) have detected in their work that macroscopic lesion formation was dosage dependent and was evident at concentrations no less than 0.1 μM FB1. Different from the lesion formation pattern caused by SA, lesions appeared synchronously in all the areas of the FB1 treated leaves, indicating a similar accumulation level of FB1 in most cells of individual leaf sample. This suggests either that FB1 is being transported from the margin to the interior of the blades through the vasculature or that it induces a systemic signal that causes the formation of lesions throughout the leaf. A similar phenomenon has been observed by Stone *et al.* (2000) that smaller punctuate lesions had formed on upper leaves that had not been infiltrated when the FB1 solution was only infiltrated into two lower leaves of the *Arabidopsis* plants grown in soil.

As shown in figure 3-7, the *wds1* mutation resulted in earlier onset of *SAG12* accumulation but the degree of increase is smaller than the control. FB1 induced accelerated up-regulation of *SAG12* expression in the *wds1* mutant compared to the wild-type. The detectable *SAG12* transcripts are tightly correlated with the visible senescence, an increase of the gene started at the third day when leaves began to show signs of yellowing. This is consistent with the previous work of *SAG12* as an age-specific senescence enhanced gene (Gan and Amasino, 1997; Morris *et al.*, 2000). The up-regulation of *SAG12* in leaves displaying the systemic FB1-treated lesions sheds light to the conclusion that FB1 induces PCD in detached *Arabidopsis* leaves. Interestingly, the *SAG12* accumulation declined at the late senescence stage in FB1-treated leaves. This disagrees with the previous report by Lohman *et al.* (1994) that the levels of *SAG12* mRNA increase throughout the progression of senescence and reach maximal levels at the last stage with almost complete loss of chlorophyll. This may be explained by the different senescing progress between the FB1 induced and natural senescence. As stated above,

senescing leaves treated with FB1 displayed a lesion formation in all the areas, indicating a uniform cell death on the leaves; therefore, they need less time to accomplish the senescence process. In this case, the *SAG12* expression level was low in FB1 treated leaves that showed a complete yellow color at the final stage when senescence is almost completed.

SAG12 encodes a cysteine protease, an enzyme that might be expected to have a role in protein degradation prior to mobilization (Morris *et al*, 2000). Its expression appears to be closely linked to senescence and chlorosis and is minimally regulated by environmental factors (Weaver *et al.*, 1998; Bleecker and Patterson, 1997; Lohman *et al.*, 1994). The acceleration of *SAG12* induction in *wds1* mutant suggests *WDS1* may function as a negative regulator in leaf senescence.

The slight difference between the induction of *SAG12* and the chlorosis in FB1-treated wild-type sample might be due to the similarity between the lesions and chlorotic sites of the leaf. It has been reported that lesioned phenotype occurred in mature leaves, which made it difficult to distinguish lesions from senescence. This is especially true when leaves were quite chlorotic (Weymann *et al.*, 1995).

3.3.4 *WDS1* is involved in SA-mediated pathway

Asai *et al.* (2000) have proposed a model for cell death signaling in *Arabidopsis* protoplasts induced by FB1. They demonstrated that FB1-elicited PCD requires SA, JA and ET-dependent signaling pathways as well as one or more unknown factors activated by FB1 and the *acd2* mutation. *WDS1* probably functions through one or more of the pathways during the PCD. The work we carried out suggested that *WDS1* expression will be regulated by the SA-signaling pathway during developmental or stress-induced senescence.

Salicylic acid (SA) has been identified as a key signaling component in numerous plant responses to both biotic and abiotic stresses (Gaffney *et al.*, 1993; Glazebrook, 1999; Rao and Davis, 1998; Surplus *et al.*, 1998) and also plays a role in regulating gene expression during leaf senescence (Morris *et al.*, 2000). Several senescence-enhanced genes showed a dependence on the accumulation of SA for

maximal expression, including *LSC94* (*PR1a*), *LSC222* (a class IV chitinase) and *LSC460* (cytosolic glutamine synthetase) (Morris *et al.*, 2000; Hanfrey *et al.*, 1996). Figure 3-10 shows that the mRNA level of *WDS1* was strongly induced after SA inoculation, indicating the role of SA pathway in controlling its expression during stress-induced senescence.

Morris *et al.* (2000) have reported that the presence of SA in senescing leaves is essential for the normal expression of the *SAG12* gene. Nevertheless, the *SAG12* gene can not be induced by spraying green, healthy leaves with SA, indicating that the expression of *SAG12* cannot be solely dependent on SA. The author proposed the requirement of a putative age-related factor in addition to SA for the induction of *SAG12* gene during the senescence. The stimulation of *SAG12* in FB1-treated young mature leaves suggest that the unknown factor may be stimulated by FB1, leading to the up-regulation of *SAG12* level through the SA pathway.

As illustrated in Figure 3-10, the transcripts of *WDS1* increased and remained at a much higher level compared to un-treated leaves (at 0 h time point), which contradicts the decrease of total mRNA mass in leaves at late senescence stage. This increase of *WDS1* during the senescence may be a result from the up-regulation of gene transcription, paralleled with the decline of many previously accumulated mRNA (Lohman *et al.*, 1994; Nooden *et al.*, 1997; Drake *et al.*, 1996). *WDS1* transcripts may actually decrease and still appear to be increasing if they decrease at a smaller rate than the total RNA (Drake *et al.*, 1996). Buchanan-Wollaston *et al.* (2005) have suggested that transcript abundance may not necessarily represent increased rates of transcription of individual genes as post-transcriptional regulation will vary between different genes. Also, transcript levels do not necessarily reflect the amount of final active protein product. However, for simplicity, increased transcript abundance is often referred to as induced gene expression (Buchanan-Wollaston *et al.*, 2005).

3.4 Material and Methods

3.4.1 Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* ecotypes Col-0 were originally obtained from the *Arabidopsis* stock center at Ohio State University. Plants were grown in growth chambers for 8 h of light, 16 h of darkness at 22 °C in growth chambers. Seeds were surface sterilized for 2 min in 70% ethanol and 8 min in 30% bleach and 0.005% TritonX-100, rinsed ten times with autoclaved water, and vernalized for 3~5 days at 4 °C refrigerator. In development experiments, seeds were sowed directly to autoclaved soil (Metro-Mix 360; The Scotts Company, Marysville, OH, USA) after sterilization. For phenotypic analyses between the wild-type and mutant plants, seeds were germinated on 1× Murashige and Skoog (MS) basal medium (Sigma, St Louis, MO, USA) with 3.0% sucrose in parafilm-sealed Petri dishes (Fisher Scientific, Ottawa, Canada), and then moved to autoclaved soil at 2-rosette leaves stage (10~12 days of growth). The medium was solidified with 0.8% agar (EMS Chemicals Inc. Damstadt, Germany) and pH adjusted to 5.7 with NaOH.

3.4.2 Sample collection

To analyze the regulation of *WDS1* transcripts in plant development, rosette leaves were collected from *A. thaliana* Col-0 plants at different developmental stages defined by Boyes *et al.* (2001). Details about the collected samples are listed in Table 3-3.

Salicylic acid treatment – Three to four fully expanded leaves harvested from the 4-week-old plants were cut to ~1 cm^2 pieces and incubated in 0.5 mM and 2 mM SA solution at the same growth condition after infiltration. Leaf discs were stretched upward on the filter paper in Petri dish so that they were just covered by the solution. Control leaf discs were treated in the same way with an equal volume of distilled water. Samples after 24 h, 48 h and 72 h incubation were collected and frozen in liquid nitrogen after picture taken. The cut discs without any treatment were taken as the sample at 0 h for both SA and water treatment.

Fumonisin B1(FB1,* Sigma*) treatment – The three *wds1* mutants (*wds1-1, wds1-2, wds1-3*) and wild-type plant was grown to 3-week old before harvesting their medium-aged leaves (leaves 5 through 8). Detached leaves were cut to ~1 cm^2 pieces before being infiltrated with FB1 (5 μM) as an elicitor to trigger cell death. After infiltration, leaves were incubated in FB1 solution at 22 °C under 8-h-light/16-h-dark condition. Samples were collected at 0 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h and 96 h after treatment. Pictures of the samples were taken at each time point before collection.

All the tissues were collected in pre-cooled RNase-free tubes and snap-frozen in liquid nitrogen. They were stored at -80 °C and used for RNA extraction within 3 months.

3.4.3 RNA isolation, reverse transcription and real-time PCR reaction

Arabidopsis RNA was extracted from frozen plant tissue using the RNeasy plant extraction mini kit (Qiagen). The purity and yields of the resulting RNA preparation were examined by dissolving the sample in RNase-free water and measuring the absorbance at 280 and 260 nm. 1μg RNA was pretreated with Deoxyribonuclease I, Amplification Grade (Invitrogen) to digest any single- and double-stranded DNA following the manufacturer's instruction. A total of 1μg RNA after the treatment was then used as the RNA template in the 20μl reaction system with iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instruction. The generated cDNA were 20 times diluted before used in the real-time PCR with iQ™ SYBR Green Supermix (Bio-Rad). In FB1 treatment experiments, the iScript™ cDNA Synthesis Kit and iQ™ SYBR Green Supermix from Bio-Rad were replaced by the Cloned AMV First-Strand cDNA Synthesis Kit and Platinum SYBR Green qPCR SuperMix-UDG (invitrogen). The cDNA were synthesized as suggested by the manufacturer using random hexamer as the primers. In real-time PCR reaction, the primers used for amplification of the *WDS1* gene were 5'-ATCGCCAGTGGAAGTGAGGATTC-3' and 5'-AGTTCAGTGGTTTGTTCGGTTTGC-3'; the primers for *SAG12* gene were 5'-CGGTTAGCGTTGGAATTGAAGGAG-3' and 5'-TCCGTTAGTAGATTCGCCGTATCC-3'. Control primers to amplify the *ACTIN2* gene (TAIR Accession No. At3g18780) were 5'-CCTCATGCCATCCTCCGTCTTG-3' and 5'-TTCCATCTCCTGCTCGTAGTCAAC-3'. PCR was carried out under following conditions: 94 °C for 3 min; 30s at 94 °C, 30s at 55 °C, 30s at 72 °C for 40

cycles; 5 min at 72 °C. All the primers were designed to amplify products in the range of 100 to 200 bp and where appropriate, to span an exon-intron boundary using Beacon primer design.

Table 3-3: Different stages for sample collection (Boyes *et al.*, 2001)

Stage number	Approx. number of days	Organs collected	Description
1.02	12.5	Whole leaves from one seedling	2 rosette leaves are greater than 1mm in length
1.07	19.4	Whole leaves from one seedling	7 rosette leaves are greater than 1mm
1.14	25.5	Whole leaves from one seedling	14 rosette leaves are greater than 1mm
3.9	29.3	Whole leaves from one seedling	Rosette growth is complete[e]
6	31.8	Young leaves[c]; old leaves[d]; whole leaves from one seedling; flowers	First flower is open, petals are at 90 degree angle to the pistil
6.3	40.1	Young leaves; old leaves; whole leaves from one seedling; flowers	30% flowers to be produced are open
6.5	43.5	Young leaves; old leaves; whole leaves from one seedling; flowers	50% flowers to be produced are open
6.9	49.4	Young leaves; old leaves; whole leaves from one seedling; flowers	Flowering complete, flowers are no longer produced
8	37[a]	Young leaves; old leaves; whole leaves from one seedling	First green silique appear, ~0.5cm
8	48	Young leaves; old leaves; whole leaves from one seedling	First silique or seed pod shatters
9.7	60[b]	Young leaves; old leaves; whole leaves from one seedling	Senescence complete

a: The day was counted based on the analysis of my record on the approximate time between the appearance of the first open flower and that of the first green silique.

b: The seedling was still alive but all the siliques on the major stem were all shattered.

c: Leaves that were no longer than 1cm or the youngest 3~4 leaves of the seedlings were collected as young leaves.

d: The first appeared four leaves were labeled when the seedlings were still in their young stages; they were collected as the old leaves.

e: The plants with the major stem no longer than 0.8cm and that had not yet bolted were collected as the samples in this stage.

3.4.4 Identification of homozygous knockout lines

The *Arabidopsis wds1* mutants (lines *wds1-1, wds1-2 and wds1-3*) were identified by screening the database of T-DNA insertional mutants from the *Arabidopsis* Biological Resource Center (Ohio State University, USA). These lines, produced by *Agrobacterium*-mediated transformation, were transformed with a T-DNA construct (*pROK2* vector) that carries *NPTII* selection marker (Baulcombe *et al.*, 1986). Primary transformants were self-pollinated to obtain plants homozygous for the insertion. To identify homozygous plants, PCR analyses were carried out on genomic DNA from three knockout lines. All the three pairs of primers for identification of the homozygous mutants were designed using SIGnAL iSect Primer Design (Salk Institute Genomic Analysis Laboratory). Standard PCR was carried out under following conditions for both *wds1-1* and *wds1-2*: 94 °C for 3 min; 30s at 94 °C, 30s at 55 °C, 30s at 72 °C for 40 cycles; 5 min at 72 °C. PCR reactions for identification of *wds1-3* homozygous were performed as 3min at 94 °C, followed by 30s at 94 °C, 1min at 55 °C, 45s at 72 °C for 40 cycles, and then 72 °C for 5min. The primer sequences are: *wds1-1*, 5'-AGGATCGTTTTCAAAGTTGCC-3' (LP in Figure 2-4) and 5'- CGAATTCCAGACAAGAAGCAG-3' (RP in Figure 2-4); *wds1-2*, 5'-TTTTCCACCTCACAGCGTG-3' (LP in Figure 2-4) and 5'- AGATGGAGAATGGGTTATGGG-3' (RP in Figure 2-4); *wds1-3*, 5'- AGGTGAAATACTTCTTATGTATGCAC-3' (LP in Figure 2-4) and 5'- GATACGGTCATGTTTTGGTGG-3' (RP in Figure 2-4).

3.4.5 Comparison of phenotypes between the three knockout mutants and wild-type plants

A total of five experiments were carried out to study the phenotypes of the three knockout mutants, *wds1-1, wds1-2* and *wds1-3*. In two of the five experiments, plants were first grown on MS mediate plates and then moved to the soil as introduced earlier about growth conditions; the other three had the seeds sown directly into the soil after germination. In each group of experiment, at least 20 plants were used to examine the flowering time and silique length of each genotype. Two temporal indicators, the time to bolting (DtB) and the number of leaves (TLN), were analyzed to measure the flowering time(Pouteau *et al.*, 2006). DtB was measured as the number of days from sowing to the open of first flower based on one set of experiment. The number of true leaves (RLN, CLN, TLN) produced by the

apical meristem was recorded on bolted plants from another five independent repeats, each of which comprised of 12 seedlings.

3.4.6 Microarray analysis

Microarray data were downloaded from Nottingham *Arabidopsis* Stock Centre's microarray database (NASCArrays). For developmental senescence study, signal values were collected from the experiment 'AtGenExpress: Developmental series (leaves)' (NASCARRAYS-150). The raw data were from triplicate slides for each time point. The twenty-four slides were: ATGE_5_A, ATGE_5_B, ATGE_5_C, ATGE_12_A, ATGE_12_B, ATGE_12_C, ATGE_13_A, ATGE_13_B, ATGE_13_C, ATGE_14_A, ATGE_14_B, ATGE_14_C, ATGE_15_A, ATGE_15_B, ATGE_15_C, ATGE_16_A, ATGE_16_B, ATGE_16_C, ATGE_17_A, ATGE_17_B, ATGE_17_C, ATGE_25_A, ATGE_25_B, ATGE_25_C.

Chapter IV

Phenotypic and Functional Analysis of *WDS1* in Overexpression Lines

4.1 Introduction

Promoters and enhancers have been identified in plants using a variety of T-DNA vectors that lack promoters or possess only a minimal *CaMV 35S* promoter linked to T-DNA border repeats (Goldsbrough and Bevan. 1991; Sivanandan *et al.*, 2005). T-DNA insertions within or immediately adjacent to genes result in the activation of promoter-less or enhancer-less marker genes, which can be tagged and identified with promoter and enhancer trapping (Wei *et al.*, 1997; Alvarado *et al.*, 2004). In plants, these trap strategies have been used to find cryptic regulatory sequences, which are inactive at their native locations in the genome but become activated as promoters or regulatory elements when positioned with genes (Fobert *et al.*, 1994, Sivanandan *et al.*, 2005). For example, tCUP promoter has been shown to contain many separate gene regulatory elements including core promoter elements, transcriptional enhancers and a translational enhance that are essential for constitutive *GUS* reporter gene expression and the binding of nuclear proteins (Wu *et al*, 2003).

A number of physiological and metabolic adaptive processes are initiated in plants under these environmental stresses, including sequestration of ions, the accumulation of sugars, and regulation of genes involved in protein breakdown mechanisms (Galaud *et al.*, 1997; Seki *et al.*, 2002; Kreps *et al.*, 2002; Ndimba *et al.*, 2005). Mannitol, the most widely distributed sugar alcohol in nature, has been found to accumulate in response to abiotic stress and confers salt tolerance in various plants (Stoop *et al.*, 1996; Zamski *et al.*, 2001). It is referred to as a compound of compatible solutes, a group of metabolites that alone or in combination accumulate in a number of stress-tolerant plants but do not interfere with normal metabolic reactions even at high concentrations (Hanson *et al.*, 1994; Shen *et al*, 1997). There is growing evidence that mannitol can also play a key role in a number environmental and

developmental responses in plants (Zamski *et al.*, 2001). For example, as a potent antioxidant, mannitol could quench the pathogen-induced reactive oxygen that mediates many plant defense responses (Shen *et al.*, 1994; Zamski *et al.*, 2001).

An alternative to studies of mutant phenotypes to discern gene functions is to investigate phenotypes generated by the overexpression of a given gene. To test the hypothesis on *WDS1* function in plant development and stress resistance, *Arabidopsis* plants were transformed with a construct driving *WDS1* expression with the constitutive *tCUP* promoter. Overexpression of *WDS1* resulted in aberrant phenotypes, including the delay of flowering time and a higher survival rate under hyperosmotic condition than the wild type.

4.2 Results

4.2.1 Generation of the constitutive overexpression lines

The *Agrobacterium* strain containing the binary vector *pCAMBIA1300-tCUP-WDS1* was obtained as described in the Material and Methods section. The overlapping 5' and 3' *WDS1* were amplified by PCR using genomic DNA as the template (Figure 4-1). Primers were designed to introduce *BamH*I site at the 5' and 3' ends of the ORF and the unique *Hind*III site in the *WDS1* coding region was used. As shown in Figure 4-2, the amplified fragments for the 3' and 5' half of *WDS1* were 1357 bp and 1316 bp respectively. The 5'*WDS1* or 3'*WDS1* was then ligated into the *T-Easy* vector. The positive plasmids of *T-Easy-5'WDS1* and *T-Easy-3'WDS1* were identified by *Eco*RI restriction digestion (Figure 4-3 and Figure 4-4) before plasmids were sequenced. After digestion with *Pst*I and *Hind*III, the *T-Easy* vectors with *5'WDS1* or *3'WDS1* inserted in clockwise direction were determined; ligation of the small fragment from *T-Easy-3'WDS1* and large fragment from *T-Easy-5'WDS1* resulted in the plasmid *T-Easy-WDS1* (Figure 4-5).

The *EntCUP2*, derivative from deletion of original *tCUP* sequence, was released from *pUC19* by *Eco*RI/*Hind*III digestion and moved into the corresponding sites of *pCAMBIA1300* to generate plasmid *pCAMBIA1300-tCUP* (Figure 4-6), which was identified by *Xho*I/*Pst*I restriction digestion (Figure 4-7). The final *pCAMBIA1300-tCUP-WDS1* was generated by cloning the *WDS1* isolated from the *T-Easy-WDS1* with *BamH*I into the *BamH*I-digested *pCAMBIA1300-tCUP* (Figure 4-8). The PCR product of the final plasmid was around 1.3kb using the primers for *5'WDS1* (Figure 3-9), indicating successful insertion of *WDS1*. The insertion of *WDS1* was also verified with *Eco*RI/*Nhe*I digestion (Figure 4-9) and identified as in the same direction of *tCUP* with *Nhe*I restriction digestion (Figure 4-10).

The constructed plasmid *pCAMBIA1300-tCUP-WDS1* was amplified in *E.coli* and then introduced into *Agrobacterium* as described in Materials and Methods section. The *A. tumefaciens* strain with transformed binary plasmid was determined by PCR identification using primers for *5'WDS1* (Figure 4-9).

Figure 4-1: The isolation procedure of the complete open reading frame of *WDS1* gene. The 3' and 5' half of *WDS1* was cloned separately into the *T-Easy* vector and then ligated to produce the complete ORF after *Pst*I/*Hind*III digestion. The black boxes are exons.

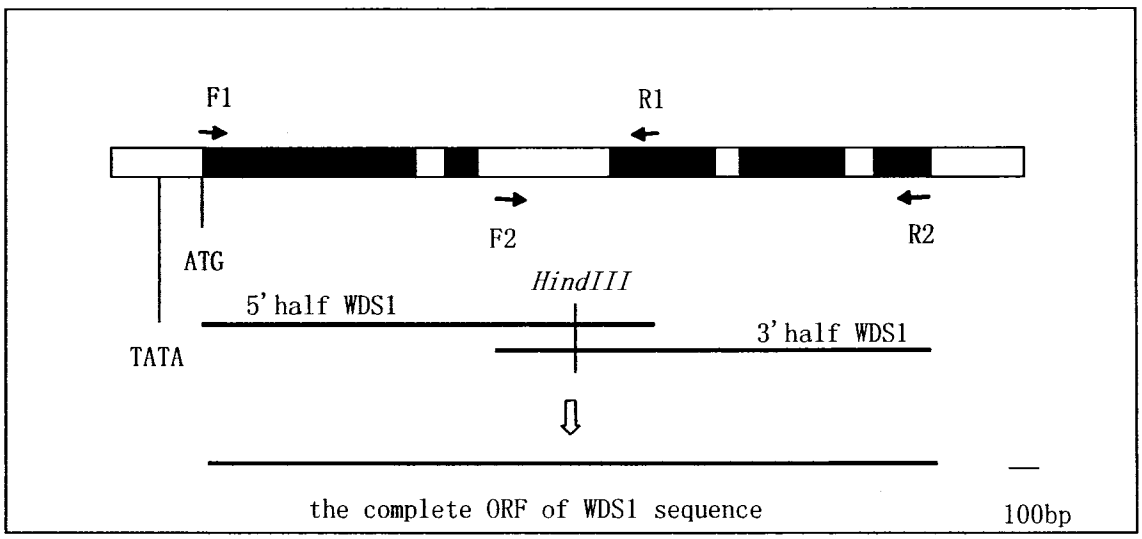


Figure 4-2: PCR-amplified fragments for each half of the *WDS1* gene. The amplified fragments for the 3' and 5' half of *WDS1* were 1357 bp and 1316 bp respectively. The 1 kb ladder was loaded in the middle lane and indicated on the right side of the photograph.

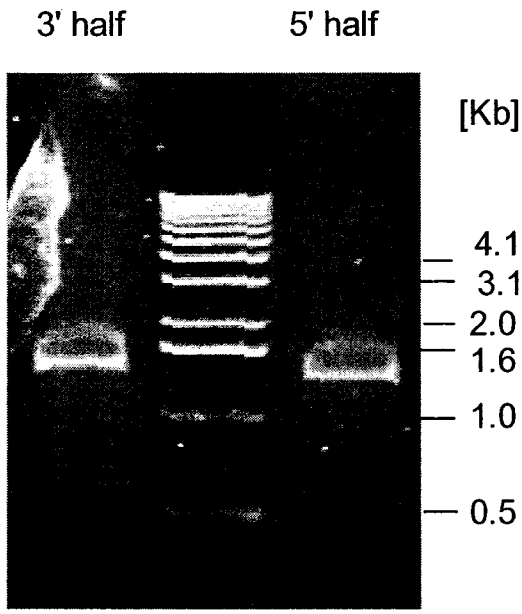
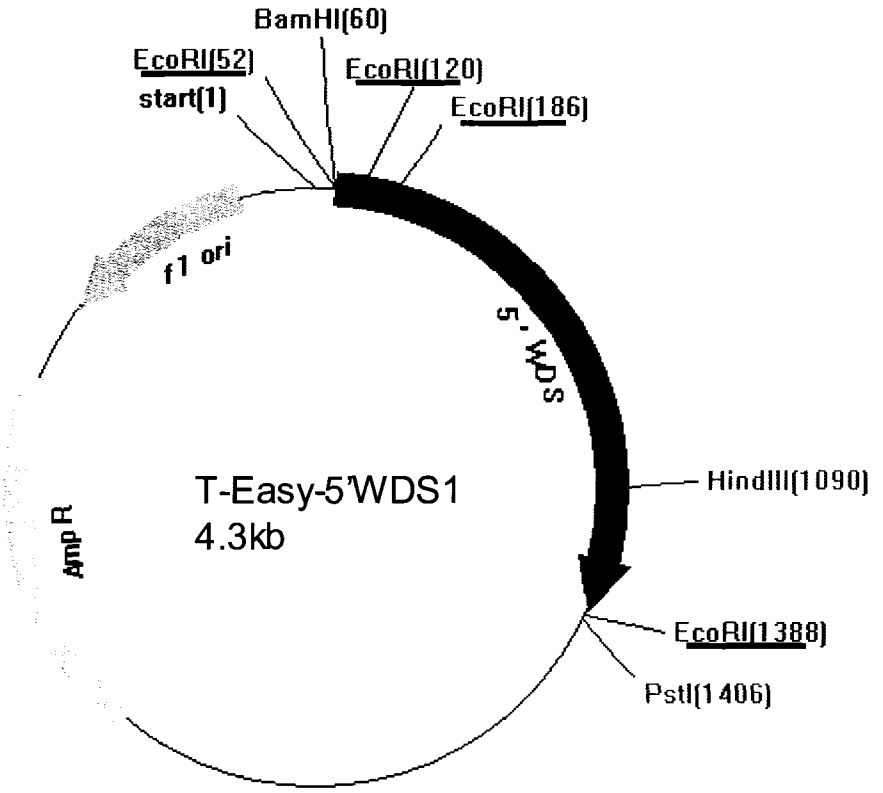


Figure 4-3: Map of *T-Easy-5'WDS1* construct and its identification by endonuclease restriction analysis.

a) *T-Easy* vector (3015 bp) with insert of 5' *WDS1* (1316 bp).

b) Construct was identified by *EcoR*I restriction digestion. The positive and negative plasmids were indicated as '+' and ' − ' on the top of the lane. The 1kb ladder was indicated on the left side of the gel photo.

a)



b)

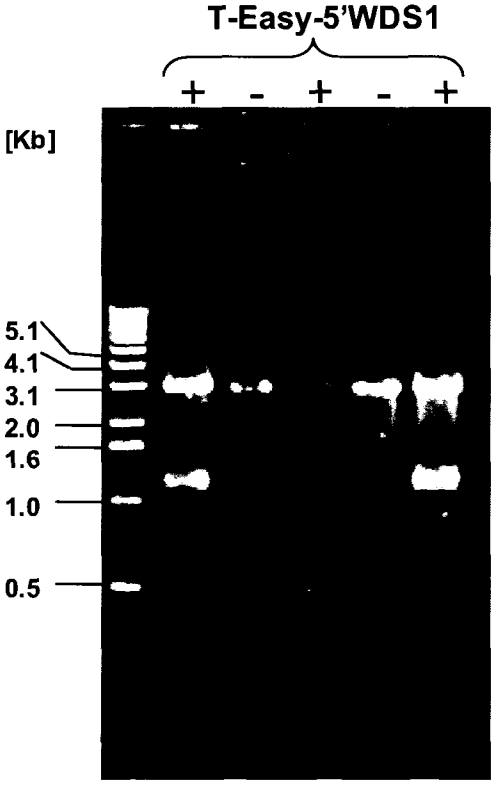
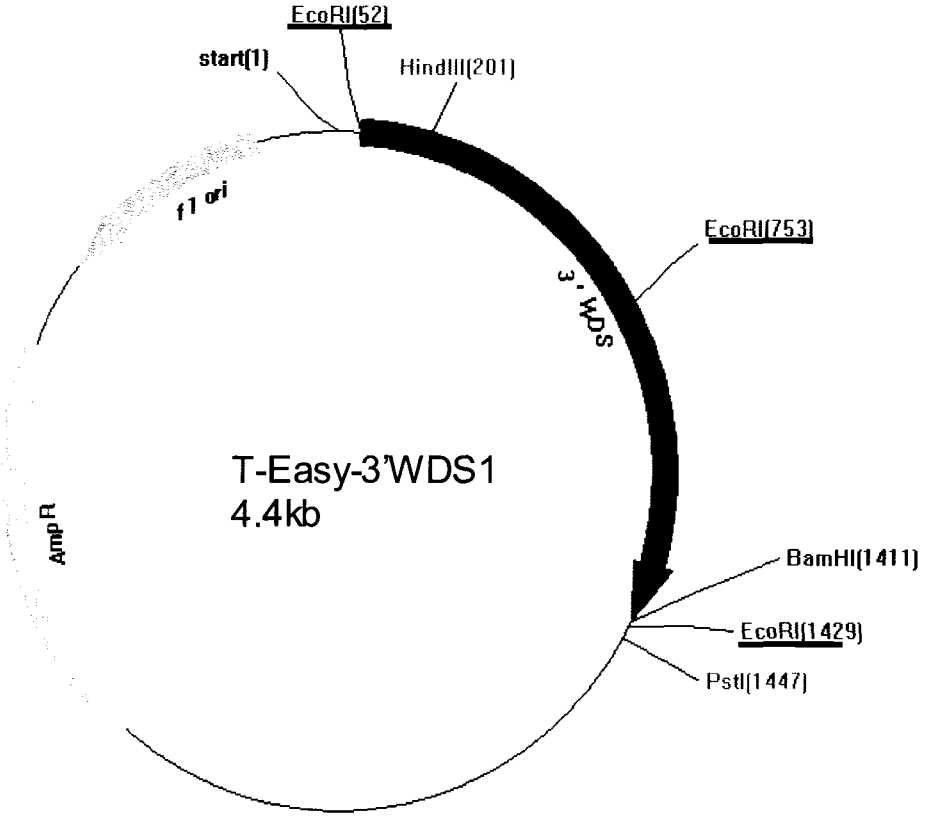


Figure 4-4: Map of *T-Easy-3'WDS1* construct and its identification by endonuclease restriction analysis.

a) *T-Easy* vector (3015 bp) with insert of 5'*WDS1* (1357bp).

b) Construct were identified by *EcoR*I restriction digestion; the positive and negative plasmids were indicated as '+' and ' - ' on the top of the lane. The 1kb ladder was indicated on the right side of the gel photo.

a)



b)

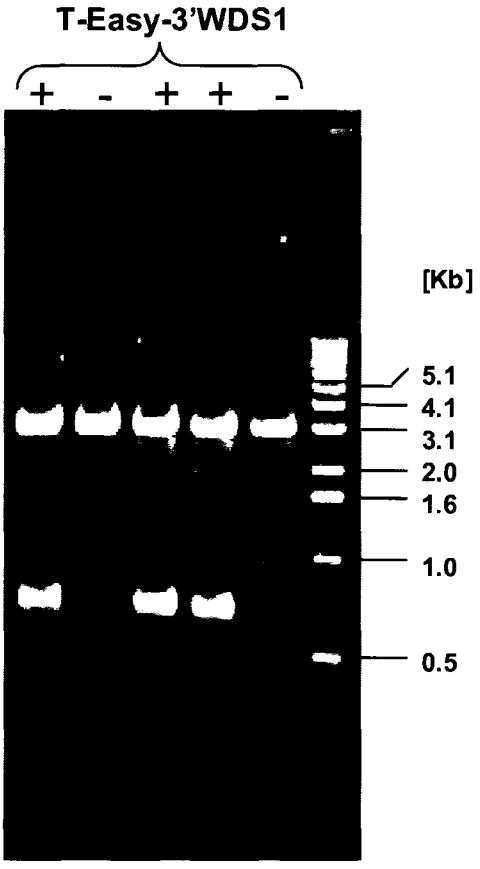
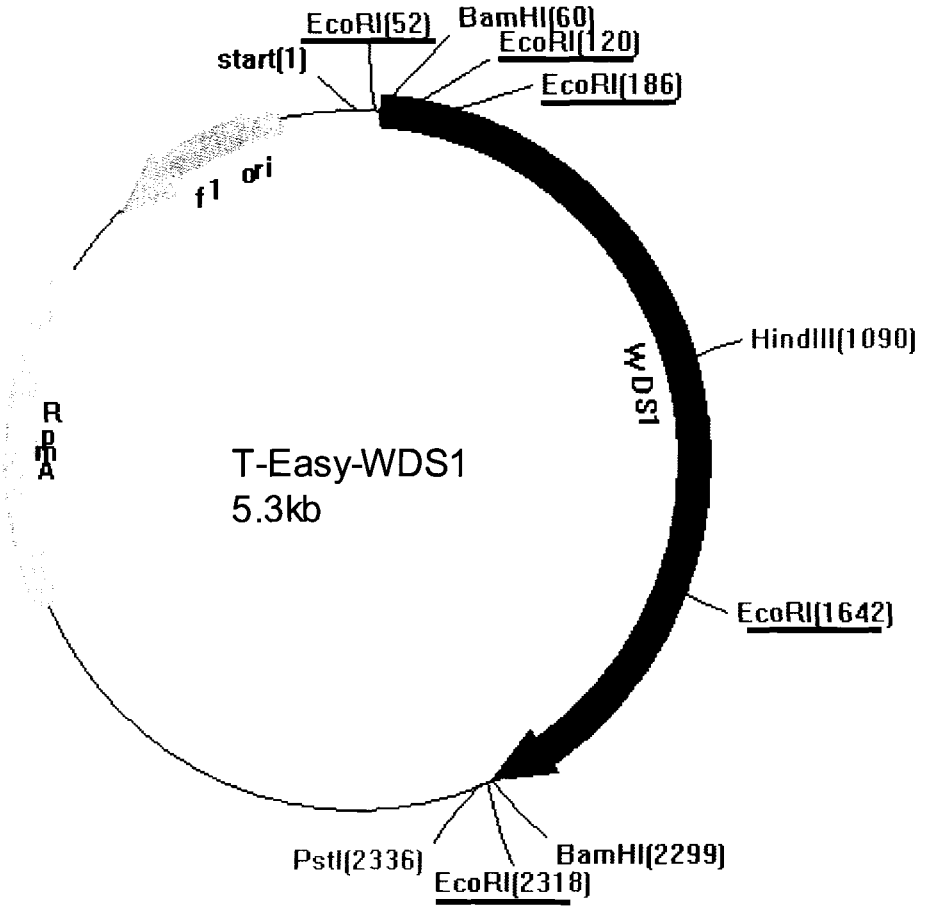


Figure 4-5: Map of *T-Easy-WDS1* construct and its identification by endonuclease restriction analysis.

a) *T-Easy* vector (3015 bp) with insert of ORF of *WDS1* (2234 bp).

b) Construct was identified by *EcoR*I restriction digestion. The positive plasmids were indicated as '+' on the top of the lanes. The 1 kb ladder was indicated on the left side of the gel photo.

a)



b)

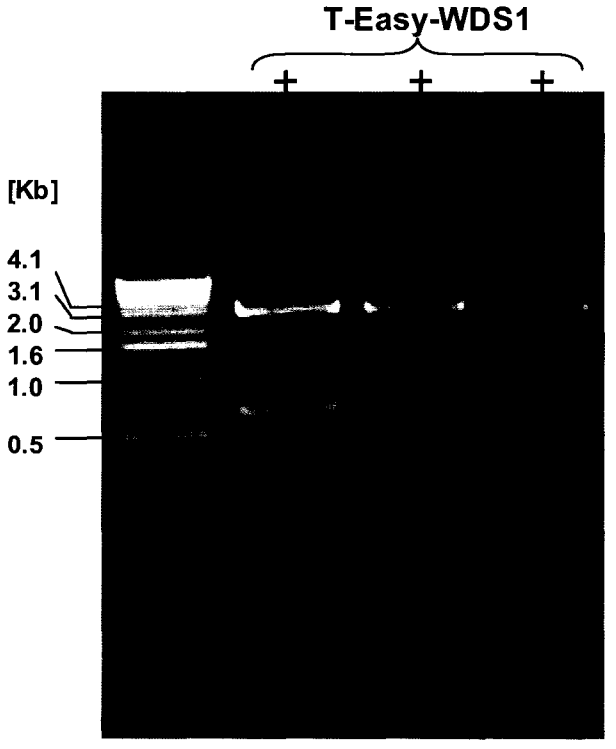


Figure 4-6: The *tCUP-GUS-nos* is ligated into *pCAMBIA1300* in the same direction of *CaMV35S* promoter after digestion with *EcoR*I and *Hind*III.

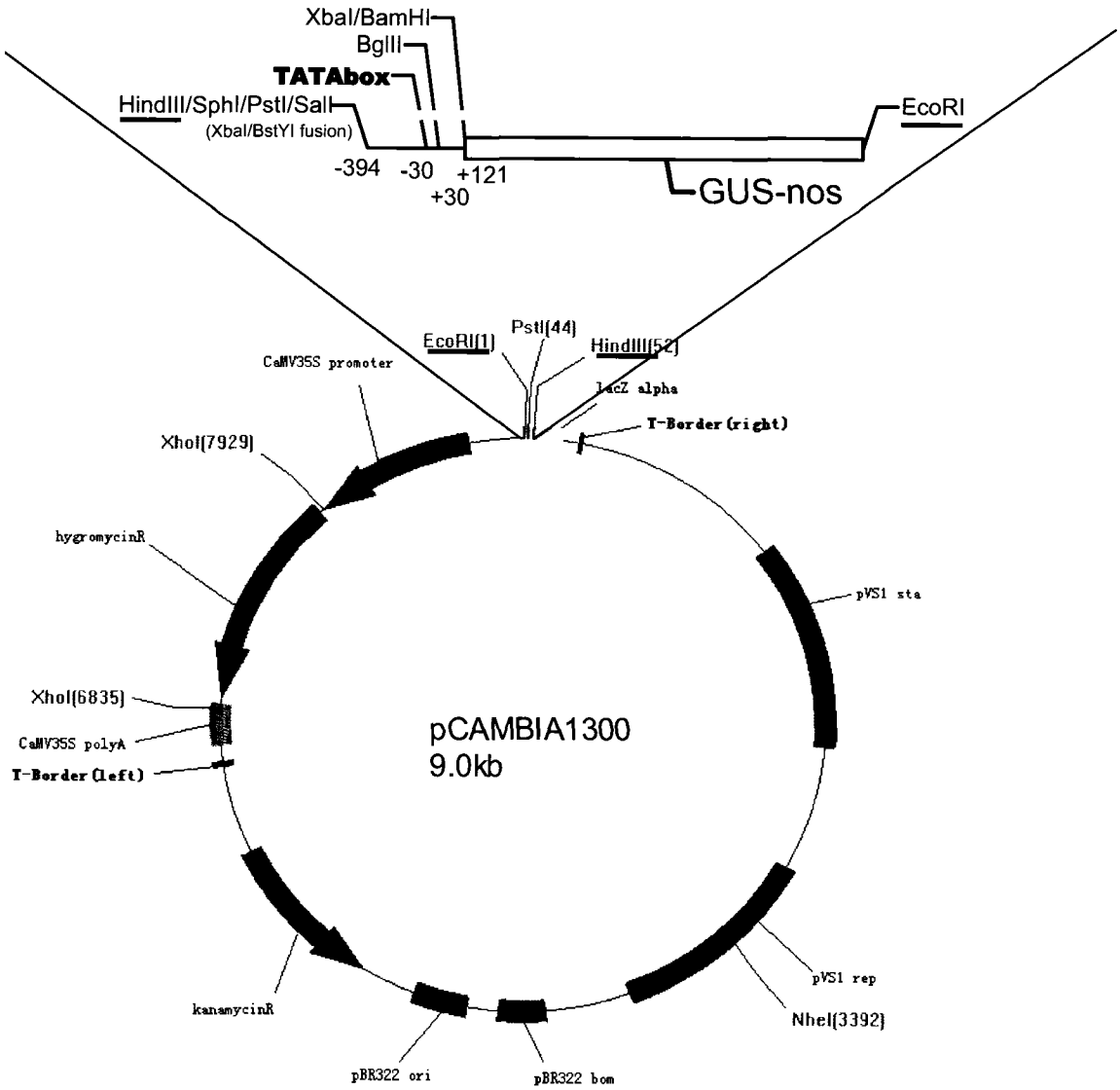
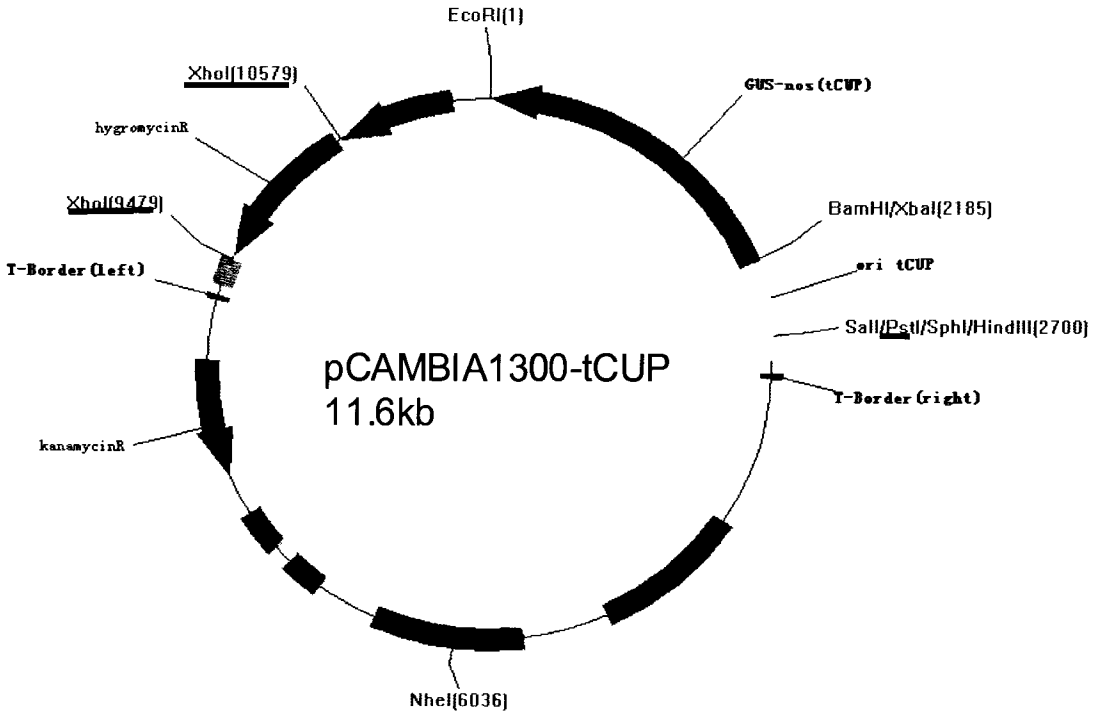


Figure 4-7: Map of *pCAMBIA1300-tCUP* construct and its identification by endonuclease restriction analysis.

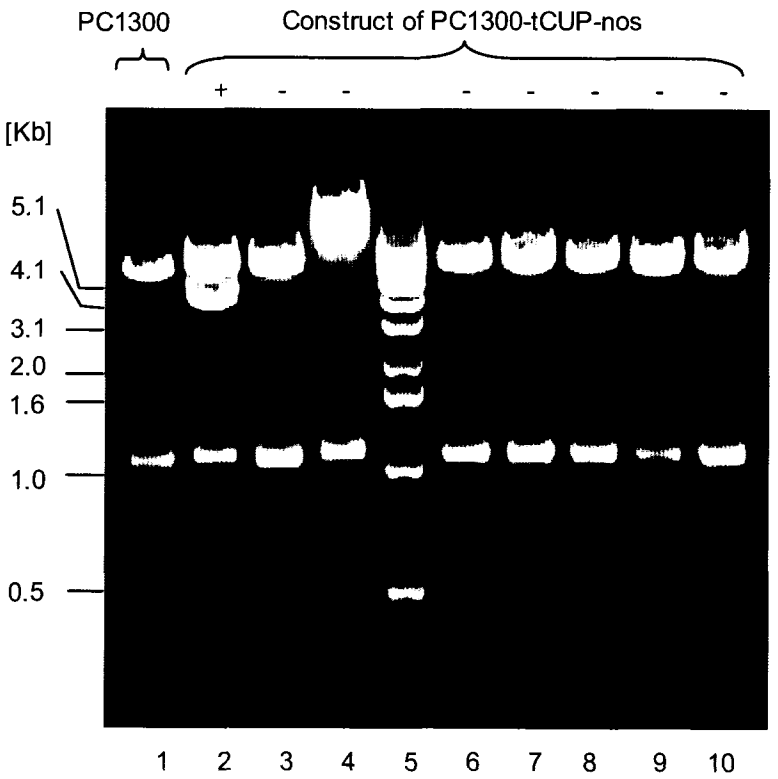
a) *pCAMBIA1300* (8958 bp) with insert of ORF of *tCUP* (2700 bp).

b) Construct was identified by restriction digestion with *Pst*I/*Xho*I. The positive and negative plasmids were indicated as '+' and ' - ' on the top of the lanes. The vector *pCAMBIA1300* was used as the negative control and the 1kb ladder was indicated on the left side of the gel photograph.

a)



b)



Firgure 4-8: The *WDS1* (2.2 kb) is inserted into the *pCAMBIA1300-tCUP* (11.6 kb) in the same direction of *GUS-nos* after digestion with *BamH*I.

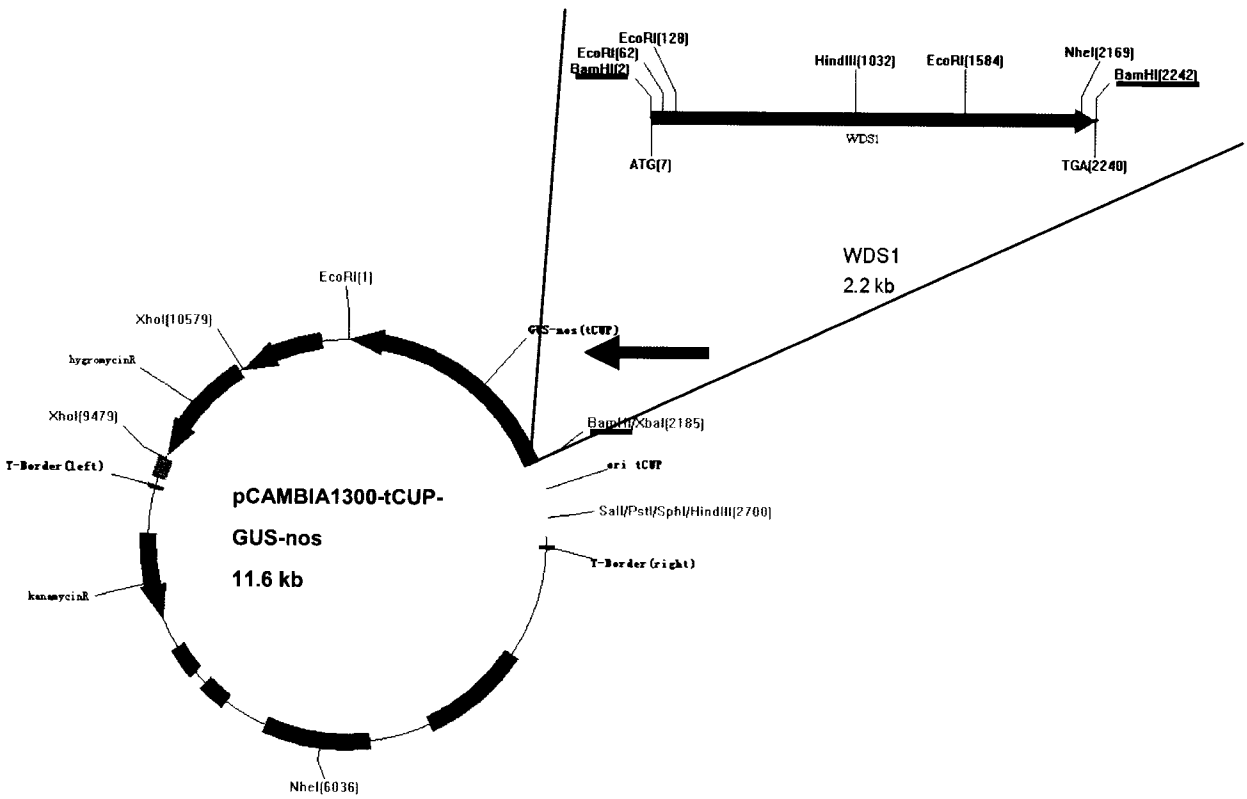
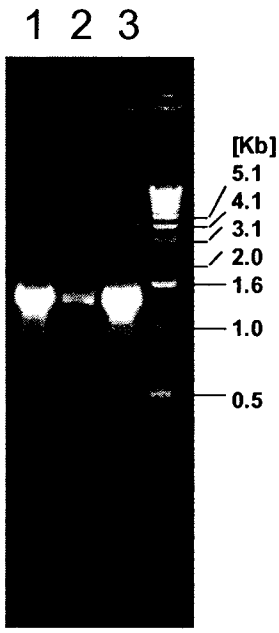


Figure 4-9: Identification of *pCAMBIA1300-tCUP-WDS1*.

a) Construct was identified using PCR method with primers for *5'WDS1* amplification. Templates in each lane were as follows: lane 1, *pCAMBIA1300-tCUP-WDS1* plasmids; lane 2, *Agrobacterium* cell culture transformed with putative *pCAMBIA1300-tCUP-WDS1*; lane 3, *T-Easy-5'WDS1*. The 1 kb ladder was indicated on the right side of the gel photograph.

b) Construct was identified by restriction digestion with *EcoR*I and *Nhe*I. The positive and negative plasmids were indicated as '+' and ' − ' on the top of the lane. The 1 kb ladder was indicated on the left side of the gel photograph.

a)



b)

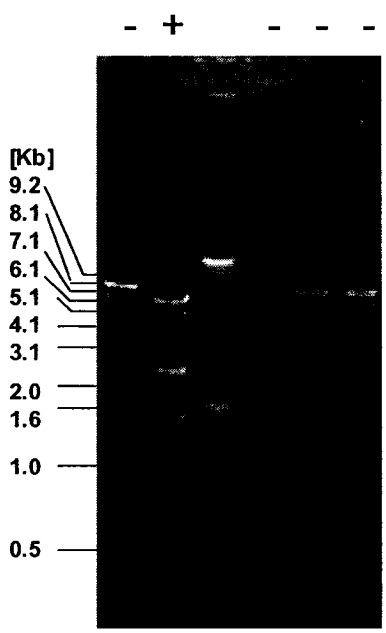
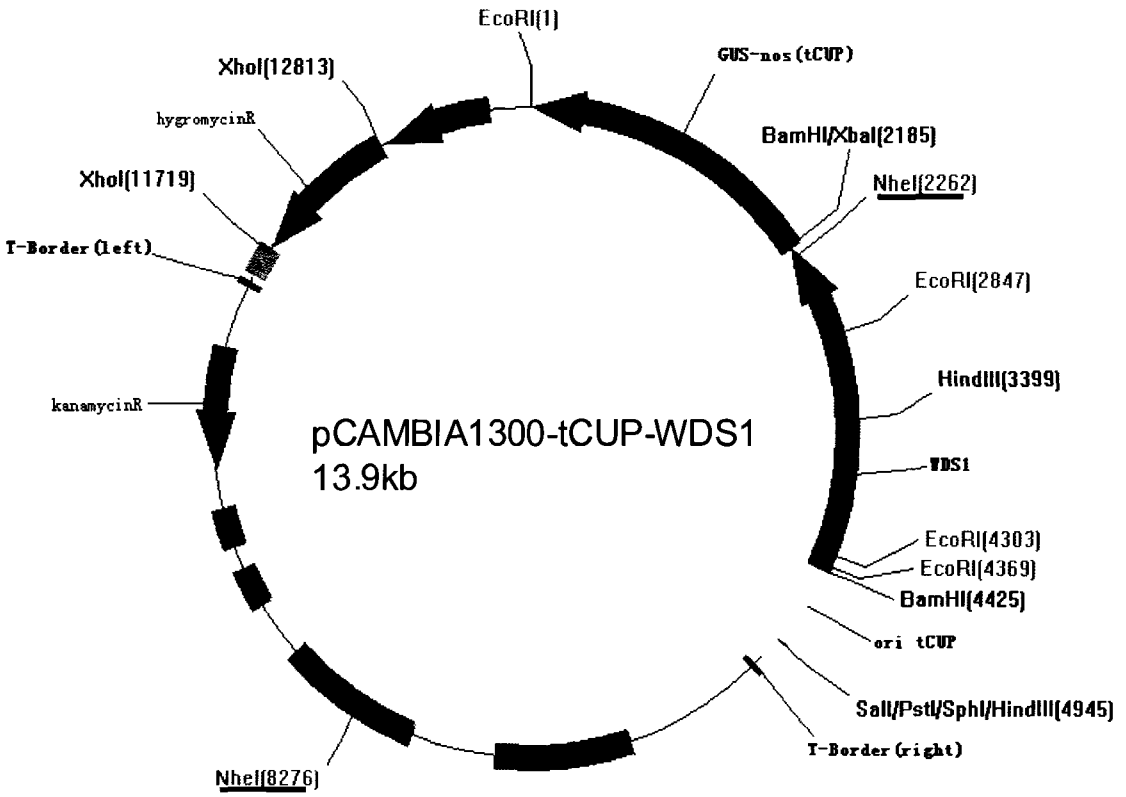


Figure 4-10: Identification of *pCAMBIA1300-tCUP-WDS1* with *WDS1* inserted in the same direction of *tCUP-GUS-nos.*

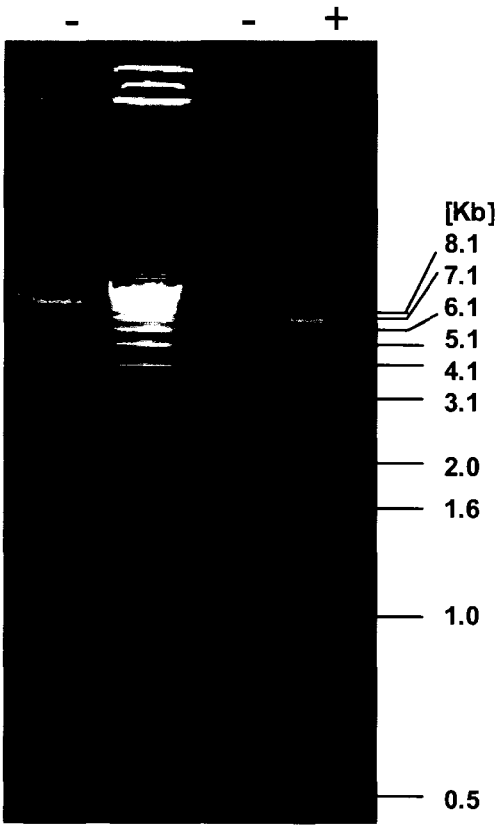
a) *pCAMBIA1300-tCUP* (11.6 kb) with insert of ORF of *WDS1* (2.2 kb).

b) Construct was identified by restriction digestion with *Nhe*I; the positive and negative plasmids were indicated as '+' and ' - ' on the top of the lane. The 1 kb ladder was indicated on the right side of the gel photograph.

a)



b)



4.2.2 Selection of overexpression lines

To elucidate the function of *WDS1*, the corresponding gene was overexpressed in *Arabidopsis* driven by the *tCUP* constitutive promoter. Seeds were collected, dried at room temperature and then sown on sterile media containing 50 μg/ml kanamycin for transformant screening. Eighteen surviving T1 plantlets were retrieved and inspected for aberrant phenotypes. The phenotypes described below were observed in progeny plants of the second generation. Kanamycin selection of seeds was used to identify *tCUP:WDS1* lines with single and multiple T-DNA loci. As shown in Figure 4-11, T2 populations of the overexpression line *WDS1-2* were all kanamycin resistant, suggesting there were multiple copies of the *tCUP: WDS1* transgene. *WDS1-1* and *WDS1-3* were identified to contain single insert since their T2 progeny exhibited a ratio of resistant: susceptible plantlets≈3:1 (46:14 and 45:16 respectively). The smaller size of plants with single insert probably is due to the toxicity of kanamycin (Harst *et al.*, 2000).

4.2.3 Phenotypic analysis of overexpression lines

The transgenic line *WDS1-2* with multiple insert was selected for phenotypic analysis. Seeds of both the wild-type and mutants were sown directly into the soil after sterilization and around 20 seedlings of each genotype were monitored. The flowering time was measured by the number of days to bolting, which is commonly used to divide the vegetative phase into subphases, rosette and primary inflorescence bearing cauline leaves (Haughn *et al.*, 1995; Pouteau *et al.*, 2006). The mutant line flowered 3 days later than the wild-type (Table 4-1). *t*-test analysis proved the difference in flowering time is significant with $P<0.001$. However, no significant difference were observed in root morphology, nor was the growing rate between the mutant and wild-type (Figure 4-18).

Table 4-1: Difference in flowering time between *WDS1-2* and wild-type.

Plants	Days to bolt*
WDS1-2	34.31±1.82
Wild-type	31.18±1.84

*: mean ± standard error

NA: experiments in progress

Figure 4-11. Segregation analyses on T2 progeny using 50 μg/ml kanamycin. The *WDS1-2* line contains multiple inserts and *WDS1-1* and *WDS1-3* were with single copy of *tCUP: WDS1*. Photographs were taken 21 days after germination.

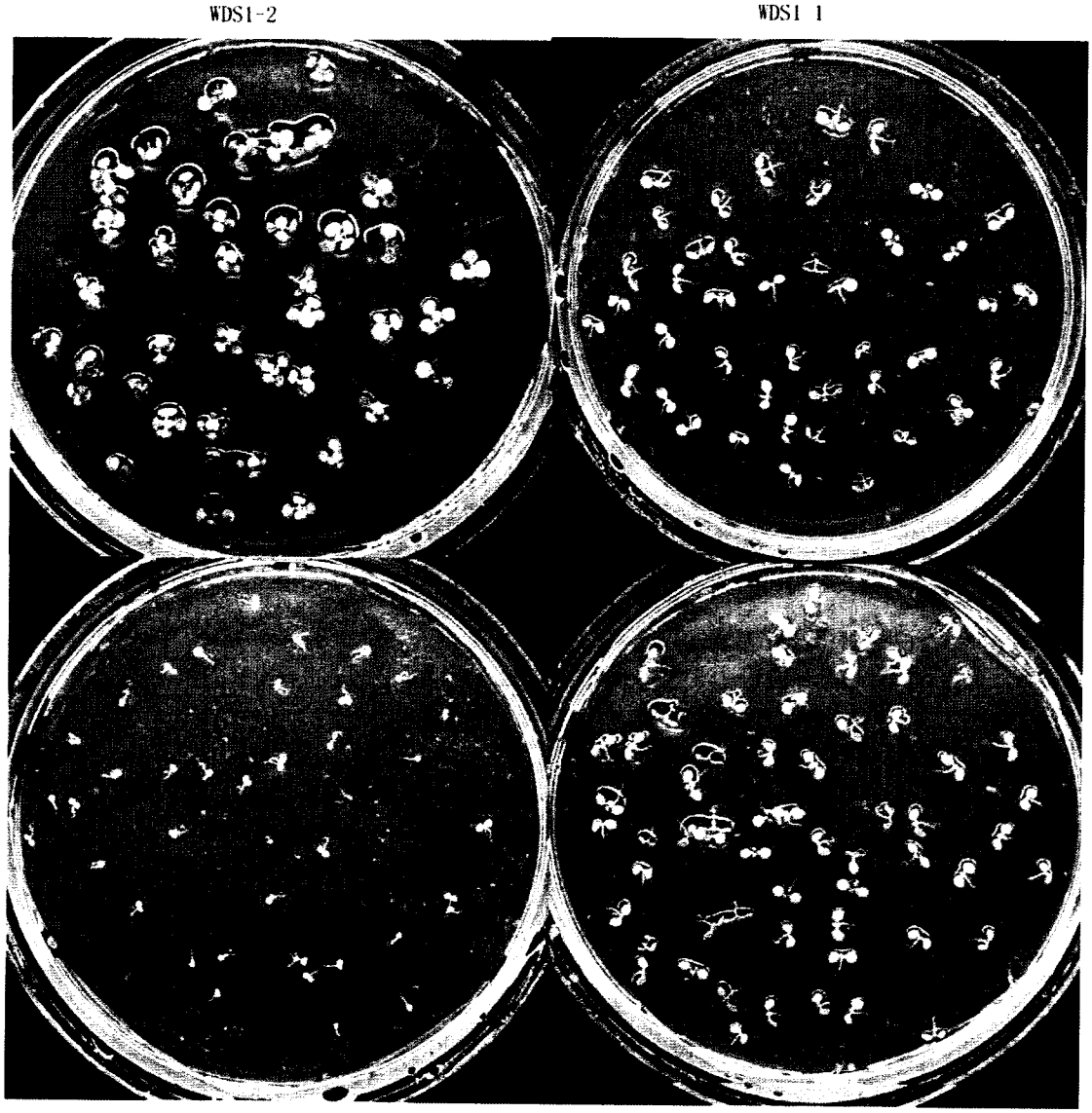


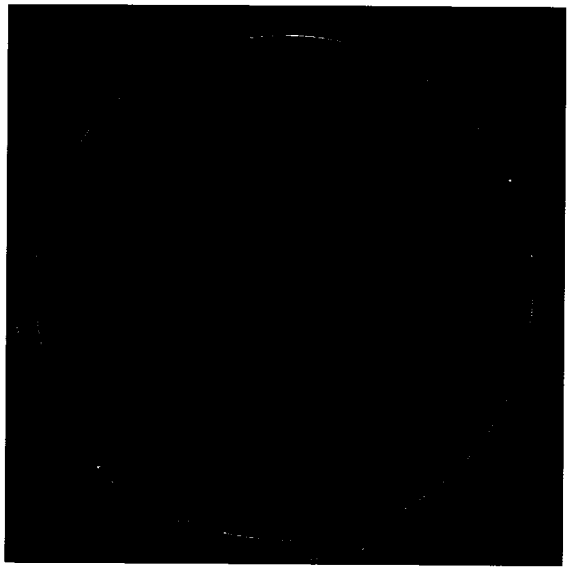
Figure 4-12: Comparison of roots between the *WDS1-2* and wild-type. Photographs were taken 17 days after germination.

4.2.4 Enhanced tolerance of *WDS*1-overexpressing *Arabidopsis* to hyperosmotic stress

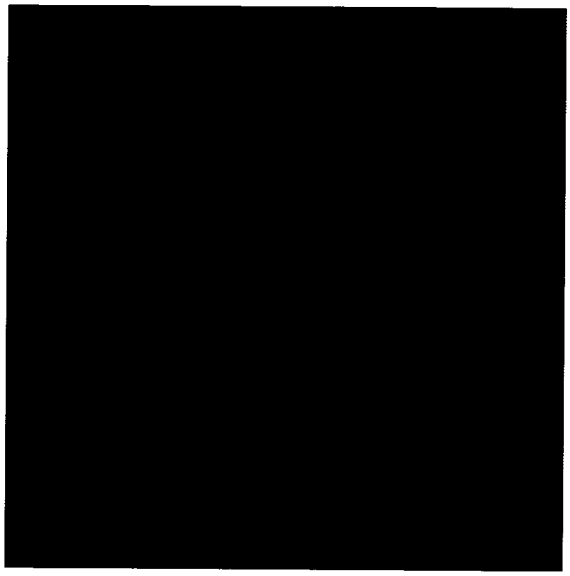
To determine the role of *WDS1* in osmotic stress, we examined the effect of mannitol during seedling development of the *WDS1*-overexpressing transgenic plants. *Arabidopsis* seedlings 10 days after germination were transferred to plates containing 200 mM, 500 mM, or 800 mM mannitol and plant survival monitored. As shown in Figure 4-13, there was a similar effect of growth inhibition on both transgenic and control plants. Exposure to increased concentrations of mannitol, both 500 mM and 800 mM, produced a decrease in plant size in both transgenic and non-transgenic plants, and a gradual increase in seeding death of the wild-type. The survival rates of transgenic seedlings were not affected when treated with 500 mM mannitol (100% survived), but decreased to 80% as the concentration of mannitol increased to 800 mM. Only one out of five wild-type seedlings survived when treated with 800 mM mannitol. These results indicate that the transformed plants have an enhanced ability to transiently survive osmotic-stress conditions.

Figure 4-13: Effect of osmotic challenge on *in vitro* cultured *WDS1-2* transgenic *Arabidopsis* plants. *Arabidopsis* plants grown in standard culture medium for 10 days were transferred to culture plates containing 200 mM, 500 mM or 800 mM mannitol. The photograph was taken 11 days after transplantation, when the survival rate of *WDS1* transgenic seedlings is higher compared to the control.

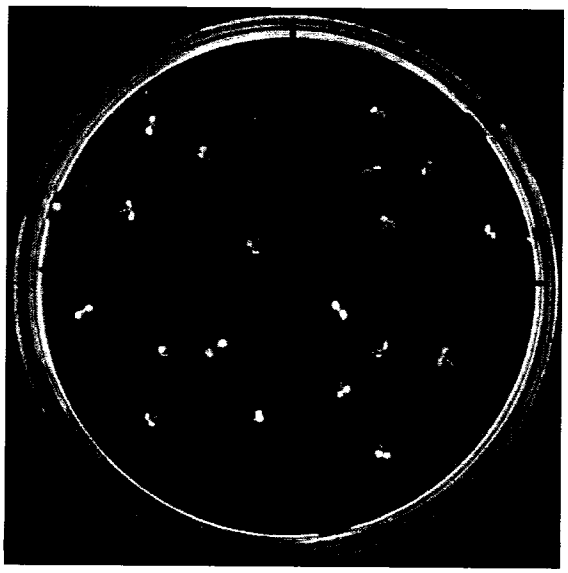
200 mM
mannitol



500 mM
mannitol



800 mM
mannitol



4.3 Discussion

In this chapter we have analysed the effect of overexpression of *WDS1* in *Arabidopsis* vegetative tissues. *WDS1*-overexpressing transgenic plants *WDS1-2* show a delay in flowering time and an improved tolerance to hyperosmotic stress.

4.3.1 Delayed flowering time in *WDS1*-overexpressing transgenic plants

The transition from the vegetative to the reproductive and to the final developmental senescence phase is of vital importance for the survival of flowering plants. Flowering is controlled by the developmental age of the plant and environmental signals, including photoperiod, vernalization, light quality, and the availability of water and nutrients (Bernier *et al.*, 1993; Barth *et al.*, 2006). So far four genetic pathways of flowering have been identified through the isolation of flowering time mutants, the long day (light-dependent) pathway, the gibberellin (GA) pathway, the autonomous pathway, and the vernalization pathway (Komeda, 2004; Corbesier and Coupland, 2005; Barth *et al.*, 2006). The delay in flowering time in *WDS1*-overexpressing *Arabidopsis* grown under long-day conditions suggests that *WDS1* may play a role in the long day pathway in floral induction. CONSTANS (CO) has been reported to be proportional to the earliness of flowering in transgenic plants and be rate limiting for flowering in the light pathway (Komeda, 2004). The delayed flowering phenotype may be explained by a defect in CO-mediated flowering pathway that induces flowering under long days. It is possible that *WDS1* functions as a negative regulator in the CO-mediated pathway, thus represses the floral transition when overexpressed. The possibility also remains that *WDS1* might be involved in the other three flowering pathways. Further experiments conducted in short-day conditions and cold temperatures are needed to identify the function of *WDS1* in flowering at the molecular level.

4.3.2 Enhanced tolerance of *WDS1-2* to hyperosmotic stress

The molecular and genetic basis of plant tolerance or sensitivity to drought and osmotic stress is poorly understood. The identification and characterization of *Arabidopsis* mutants have contributed greatly to the understanding of plant growth and development, and responses to some environmental stresses such as high salinity (Chen *et al.*, 2005). However, it has been difficult to identify mutants with strong

phenotypes in drought and osmotic stress tolerance or sensitivity. Our results show that *WDS1-2* plants are more tolerant to hyperosmotic stress. Transgenic lines overexpressing *WDS1* were found to exhibit no significant change in osmotic tolerance when treated with 200 mM mannitol, suggesting that *WDS1* is not a critical component in plant responses to this stress condition. In contrast, we demonstrated that changes in *WDS1* transcript levels are sufficient to modify plant tolerance to hyperosmotic stresses. The constitutive expression of *WDS1* resulted in a higher survival rate of the transgenic lines under hyperosmotic conditions compared to the wild type. This suggests that *WDS1* may be considered as a positive regulator of stress tolerance under hyperosmotic conditions. It would be interesting to repeat to work on plants in soil to confirm the function of *WDS1* in osmotic stress tolerance of plants.

4.4 Materials and Methods

4.4.1 Construction of the binary vector for overexpression

To construct the vector for *WDS1* overexpression in *Arabidopsis* Col-0 ecotype, the open reading frame (ORF) of *WDS1* was amplified using two sets of PCR reaction. Total DNA was isolated from approximately 3-week-old seedlings using Sigma *Extract-N-Amp*^{TM} Plant PCR kit following the manufacturer's instruction. Two separate PCR amplifications were carried out to obtain the *WDS1* gene. The reaction mixtures were performed as 3 min at 94 °C, followed by 30s at 94 °C, 33s at 55 °C, 2min at 72 °C for 37 cycles, and then 72 °C for 7min. Two pairs of primers, F1 (5'-GGATCCATGGAGAATGGGTTATGGGAGGT-3') and R1 (5'-CTCTCAAGTGTGTGCTTCAGTTCG-3'), F2 (5'-TCCCTAAAGTCATTGAGAAAGAGACC-3') and R2 (5'- GGATCCTCAGTTCAGTGGTTTGTTCGGT-3') were used to amplify the 5' and 3' half of the *WDS1* gene sequence respectively, which were then ligated to the *T-Easy* vector following the manufacturer's instruction (Promega), resulting in the plasmids *T-Easy-5'WDS1* and *T-Easy-3'WDS1*. Positive clones were identified by restriction enzyme digestion with *Eco*RI and nucleotide sequences were determined. After *Pst*I/*Hind*III digestions, the small fragment of *T-Easy-3'WDS1* and the large fragment of *T-Easy-5'WDS1* were recovered and ligated to produce the complete *WDS1* gene.

The binary vector *pCAMBIA1300-tCUP* used for constitutive expression of *WDS1* was generated by cloning the *tCUP-GUS-nos* fusion (*EntCUP2*) from the *pUC19* plasmid into the *pCAMBIA1300* (Both plasmids were kindly provided by Dr. Brian Miki) after digestion of both plasmids with *Eco*RI and *Hind*III. The *WDS*1 from the *BamH*I-digested *T-Easy-WDS1* was inserted into the corresponding site of the construct to generate *pCAMBIA1300-tCUP-WDS1*, which was then overproduced in *E.coli*. Plasmid DNA from *E.coli* cells was extracted and purified using Wizard Plus Minipreps DNA purification kit following the manufacturer's protocol. The resulting plasmid was designated *tCUP:WDS1*, in which the *Arabidopsis WDS1* gene is under the control of the *tCUP* promoter.

4.4.2 Transformation of *Agrobacterium tumefaciens*

The complete construct was introduced into *A. tumefaciens* using a modified freeze-thaw method (www.molbiol.net, Detlef and Jane, 2002). *Agrobacterium GV 3101* were grown overnight with kanamycin (50 μg/ml) at 27-28°C in 5ml TYNG medium (10 g/l bacto-tryptone, 5 g/l yeast extract, 5 g/l NaCl, 0.2 g/l $MgSO_4$ and adjusted to pH 7.5). Early the next day, the bacterial culture was vortexed to break up clumps. Following further incubation for 6-8 hours, 0.5 ml of this starter culture was inoculated in 60 ml TYNG (with kanamycin) at 28°C and at 225-250 rpm on a shaker for overnight. Around midday of the third day, the culture was placed on ice for 10 minutes and then spun down at 4800 rpm for 6 minutes at 4 °C in pre-cooled rotar. Cells were rinsed with 1ml 20 mM $CaCl_2$ (ice-cold) and spun briefly again followed by suspension in 1 ml 20 mM $CaCl_2$. Aliquots of these competent cells were then frozen at -80 °C.

For transformation, 150 μl of competent *Agro* cells were thawed on ice, and 1 μl of the construct plasmids were added to mix and freeze in liquid nitrogen for 5 minutes. Then remove tube and thaw, which will take 5-10 minutes on the bench top. Afterwards, add thawed cells to 1 ml LB in 20 ml sterilin tube, incubate with shaking (200 rpm) overnight. Pour TYNG plates with the same selection the next day. After 16 hours' incubation into the recovery period, plate the A*gro* on TYNG and incubate at 28°C for 24-48 hours. Visible colonies were found on the plate and positive clone was identified using PCR reaction under the same program with either pair of primers for 3' and 5' half of *WDS1*.

4.4.3 Transformation of *Arabidopsis*

Preparation of Agrobacterium tumefaciens - *A. tumefaciens* strain *GV 3101 (pMP 90)* carrying the binary plasmid construct was grown to stationary phase (OD600 between 0.8 and 1.5) at 28°C, 200 rpm in sterilized LB (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) carrying added kanamycin (50 μg/ml). Cultures were grown by inoculation of 200 μl overnight cultures into 500 ml LB with the same antibiotic selection for 18-24 h. Cells were harvested by centrifugation for 8 min at room temperature at 5000 rpm and were then resuspended in infiltration medium to a final OD600 of 1.0-1.5 prior to use. The floral dip inoculation medium contained 5.0% sucrose and 0.05% (500 μl/l) Silwet L-77.

Plant growth - Both wild type and knockout mutant *Arabidopsis* plants (Col-0) were grown till flowering stage in the condition described in Chapter III and inflorescences were clipped after most plants had formed primary bolts, relieving apical dominance and encouraging synchronized emergence of multiple secondary bolts (Steven and Andrew, 1998). They were typically planted 6-10 per 100 cm^2 pot in autoclaved soil and had been watered the day before floral dipping so that at least the peat discs are saturated. Plants were dipped when most bolts were 1.5-10 cm tall (2-6 days after clipping).

Transformation of plants – The prepared *Agrobacterium* solution were poured into a beaker and plants were inverted into this suspension such that all above-ground tissues were submerged. After 5-10 seconds' inoculation with gentle agitation, the plant tray was then removed and lay on their side on pre-moistened paper tower. Typically, more than 10 peat discs were dipped per transformation. As soon as last plant is dipped, the trays were sealed inside autoclaved bags and then kept in the lab out of direct sunlight overnight. They were moved back to the growth chamber without bags the next day. The developing flowers of these plants were redipped 6 days after the day of first dip without dipping rosette and the dipping was repeated again after another 6 days (3 dips in total).

Selection of putative transformants– Seeds were collected and sterilized as described above and then plated on kanamycin selection plates. Plates and lids were sealed with parafilm, dried for four hours and then grown for 10-14 days in the growth chamber. The selection plates contained 1 x MS medium, 0.8% agar and 50 μg/ml kanamycin monosulfate. Primary transformants were selected on MS medium containing 50 mg/L kanamycin. Transformants (T1) were identified as larger and greener plants with more extended roots compared to non-transformed ones. After the development of 3-5 adult leaves, plants were transplanted into heavily moistened soil.

Analysis of transgenic plants- T1 transformants were grown to maturity, and mature seeds (T2) were harvested from individual plants and further analyzed or propagated. Segregation analyses were performed on T2 plantlets screened on kanamycin to determine whether there were single (expected ratio of resistant:suspectible plantlets=3:1) or multiple copies of the *tCUP:WDS1* transgene. T2 lines

with either single or multiple inserts were compared with the wild type plants and phenotype analyses were performed between them.

4.4.4 Osmotic stress treatment

T2 transgenic lines and the wild type control were grown for 10 days on MS plate when the two first leaves had completely emerged. At this stage, seedlings were transferred to MS agar plates containing 200 mM, 500 mM or 800 mM mannitol and grown under controlled conditions for 11 more days. Plants grown on MS plates were used as controls and pictures were taken after the treatment.

Chapter V

Overall Discussion

5.1 Discussion

5.1.1 Putative structure and function of WDS1

We have characterized the *Arabidopsis WDS1* gene, whose 2.3 kb cDNA encodes a novel 523-amino-acid protein containing seven WD40 repeats plus two other domains on the N terminus, LisH and CTLH. The WDS1 protein is similar to the WDS protein of *Drosophila melanogaster*, whose transcript is able to rescue two different lethal *wds* alleles (Hollmann *et al.*, 2002). Homologues of the WDS1 protein includes proteins from several other species such as *H. sapiens, M. musculus, O. sativa.* Pairwise comparisons revealed the WDS1 protein has higher sequence identity to WDS26 proteins than G beta proteins, a new WD40 repeat subfamily differing from G beta proteins (Zhu *et al.*, 2004). Therefore, it is proposed that WDS1 is not homologous to any known mammalian G beta subunit, but rather homologous to the WDR26 in human in consideration of the high sequence similarity between WDS1 and WDR26 proteins (Table 2-1).

The WD40 repeat [previously referred to as the beta transducin repeat (Duronio *et al.*, 1992)] was first identified in the beta subunit of the heterotrimeric G-protein transducin, G-beta (Adler *et al.*, 1999). WD40 repeats are involved in protein-protein interactions (Neer *et al.*, 1994) and have been identified in a variety of proteins. The WD40 motif typically occurs between four and eight times (Duronio *et al.*, 1992; Van der voorn and Ploegh, 1992; Neer *et al.*, 1994; Adler *et al.*, 1999). In addition to heterotrimeric G proteins, WD40 repeats have been found in regulatory proteins, such as those involved in cell division, cell-fate determination, gene transcription, mRNA modification, transmembrane signaling, and vesicle fusion (Neer *et al.*, 1994).

Crystal structures of two WD-repeat proteins have been reported and shown to have a circularized seven-bladed beta-propeller structure: the beta subunit of the G-protein involved in signaling in mammalian cells and an actin-interacting protein from *S. cerevisiae* (Wall et al, 1995; Voegtli *et al.*, 2003). Each propeller blade consists of a four-stranded anti-parallel beta sheets and each WD 40 sequence repeat contains the last strand of one blade and the first three strands in the next. The final blade of G beta requires N-terminal residues to complete its beta sheet and to close the ring. This arrangement defines an overlapping structure with the seven beta-sheets around a central core (Chen *et al.*, 2004). It is likely that the WDS1 protein also has such a beta-propeller structure, but defining its interacting proteins and interaction mechanisms remains a challenge.

An example of the apoptotic signal transduction mediated by the WD40 repeat domain has been reported by Ferraro *et al.* (2003). Apaf1 is the molecular core of the apoptosome, a multiproteic complex mediating the so-called mitochondrial pathway of cell death. There is growing evidences supporting the role of Apaf1 during both development and mitochondria-dependent apoptosis. Apaf1 possesses an amino-terminal CARD domain, a central CED-4-like domain and a long carboxy-terminal domain extremely rich in WD40 repeats. During apoptosis, the pro-apoptotic factor cytochrome c is released from mitochondria and binds the adapter molecule Apaf1 in the cytosol. This promotes the assembly of a multiproteic complex called apoptomone which, in the presence of ATP/dATP, binds and activates procaspase-9 (Li *et al.*, 1997; Ferraro, 2003). The apoptomone is the executioner of the mitochondria-dependent apoptosis and Apaf1 is the core of the apoptosome. It is suggested that the apoptosome is a wheel-like particle containing seven Apaf1 monomers which, like seven spokes, radiating from a central hub, in which the CARD domain of Apaf1 is located (Acehan *et al.*, 2002; Ferraro, 2003). The cytochrome c would interact with the WD40 domain, which forms the distal part of the spoke. Normally Apaf1 has a compact autoinhibited shape in which the CARD domain binds the WD40 domain. Following its release from mitochondria, the cytochrome c displaces the CARD domain from the WD40 domain taking its place and allowing the CED-4 homology domain to bind dATP/ATP and undergo a conformational change in which Apaf1 has a more extended conformation (Acehan *et al.*, 2002; Ferraro, 2003). This is required for efficient assembly of the apoptosome and procaspase-9 binding to the exposed CARD domain, which consequently activates caspases that play

essential roles in cell death and inflammation (Acehan *et al.*, 2002; Ferraro, 2003). It is possible that WDS1 may mediate signal transduction by interaction with the other proteins through its WD40 domain. But it remains unclear how the CTLH and LisH motifs coordinate the interactions. The LisH motifs are suggested to mediate protein dimerazation or binding during its regulation of microtubule dynamics. Therefore, a dimerazation structure may be formed to mediate the interaction of WDS1 with other proteins and/or small ligands.

5.1.2 WDS1 functions in the regulation of developmental senescence

The modulation of *WDS1* transcripts at different developmental stages of *Arabidopsis* implies that *WDS1* may function as a regulator in leaf senescence. The *WDS1* mRNA is detectable in leaves collected from plants of any age, suggesting that *WDS1* may play a role in maintenance of normal plant physiology. Increased accumulation of *WDS1* transcripts is detected from the early flowering stage, leading to the highest level when 50% of the flowers to be produced were opened. The mRNA abundance of *WDS1* remained at a high level in leaves from senescing plants. Based on the above observation, we therefore propose that *WDS1* may be a senescence associated gene with enhanced transcription along the age-dependent leaf senescence.

An alternative support for the regulatory role of *WDS1* in leaf senescence comes from the analyses of public microarray data. Two *WDS* transcripts, *WDS1* and *WDS2*, were up-regulated 1.7 and 3.3 fold respectively in senescing leaves. A possible explanation for the relatively smaller up-regulation of *WDS1* expression in comparison with the result in our experiments may be the different criteria in sample collection. Leaves in the microarray experiments are collected no later than 35 days after planting, whereas our leaf samples range from the 12 d to 60 d. It is interesting to detect a greater expression and up-regulation of *WDS2* gene than *WDS1*. This provides substantial support for that *WDS2* may possess a similar function as *WDS1*.

During senescence, genes that show enhanced expression may change in different patterns of transcriptional products. Temporal expression patterns may indicate the role of each gene during the various senescence steps from the initiation signal to the terminal phase of cell death (Gepstein *et al.*,

2003). The experiments carried out by Buchanan-Wollaston *et al.* (2003) identified over 1400 genes that show relative changes in expression during leaf development. These genes can be classed into at least four different groups depending on their expression patterns. The first category includes genes that have some degree of basal expression in non-senescent leaves, but their mRNA levels increase markedly during the successive stages of senescence. Among this groups are genes encoding cationic amino acid transporters, amino acid permease and metallothionein. Genes belonging to a second category are expressed during the emergence of the inflorescence stem just prior to or at the onset of senescence and may suggest their participation in the initiation phase of senescence. Among this group are the RING-H2 finger protein and xylose isomerase. Genes whose products are involved in the terminal phase of leaf senescence during which irreversible loss of cell integrity and viability occurs would display a characteristic profile of late expression. An example is the *lethal leaf spot 1* (*lls1*) gene, known to be involved in the HR in corn, which is preferentially expressed at the very late phase of the senescence syndrome. The product of this gene is probably responsible for the irreversible necrosis typically involved in this stage of senescence (Dangl *et al.*, 2000; Gepstein *et al.*, 2003). The final obvious group contains a small number of genes that seem to be expressed early in senescence with expression levels falling as senescence progresses. An example is the chlorophyll a/b-binding (cab) protein expression that displayed the typical temporal pattern of a senescence-downregulated gene reflecting the decline of photosynthesis during senescence (Gepstein *et al.*, 2003).

The expression pattern of *WDS1* resembles that of *LSC210*, which encodes a putative metallothionein expressed at all stages of leaf development but the level of transcript increased steadily as senescence progressed (Buchanan-Wollaston and Ainsworth, 1997). Metallothionein proteins have been suggested to play a possible role in protection of the nuclear DNA from oxidative damage caused by free radicals, thus allowing expression of senescence-specific genes required for the process to take place (Buchanan-Wollaston and Ainsworth, 1997). This implies that *WDS1* might assume a role in the regulation of genes that are induced by reactive oxygen species (ROS).

ROS are products of cellular respiration, and include oxygen ions, free radicals and peroxides both inorganic and organic. They have important roles in cell signaling and are well established as inducers

of PCD in yeast, plants and animals (Jin and Reed, 2002: Lam *et al.*, 2001: Madeo *et al.*, 2004). During times of environmental stress ROS levels can increase dramatically, which can result in significant damage to cell structures. Harmful effects of ROS on the cell are most often the damage of DNA, and oxidations of polydesaturated fatty acids in lipids and amino acids in proteins. Cells under oxidative stress are normally able to defend themselves through the induction of defence genes and mobilization of ion transport systems. Whether and how WDS1 may function in the cell metabolism of ROS, as yet, requires further elucidation.

5.1.3 *WDS1* is not essential for normal growth and development in *Arabidopsis*

Three T-DNA knockout lines, *wds1-1, wds1-2, and wds1-3*, were identified and selected for the characterization in aspect of plant development. Despite the up-regulation of *WDS1* expression in senescing plants, the growth and development of the three *wds1* mutants appeared normal, with no significant phenotypic alternation detected in either the leaf, flower or root development.

This may be explained by the existence of potential functional homologous of the WDS1 protein. In *Arabidopsis*, four addictive putative *WDS* genes were found to share a high sequence identity with *WDS1*, translating products with WD40 repeat domain. Moreover, three of the four genes are also located on the fifth chromosome in *Arabidopsis*. Therefore, they presumably will share a similar transcription and translation pathways as *WDS1* and participate in the regulation of plant development. The sequencing of the *Arabidopsis* genome revealed large areas of duplication that may contain genes with the same function (Blanc *et al.*, 2000), and insertion knockout of one of these duplicates would not show any phenotype (Buchanan-Wollaston *et al.*, 2003). Take the shatterproof genes *SHP1* and *SHP2* for example. The two genes must be removed simultaneously before the nodehiscence phenotype is observed because they both locate in a chromosomal block that duplicated around 100 million years ago (Liliegren *et al.*, 2000; Vision *et al.*, 2000).

Another possibility is that *WDS1* is dispensable at least for the physiological regulation of developmental PCD in *Arabidopsis* plants. Similarly, it was reported that a cell death suppressor, Bax inihibitor-1 (BI1), is not essential for developmental PCD in *Arabidopsis* as the two *atbi1* mutants

were indistinguishable from wild-type plants under normal growth conditional. Rather, these two mutants exhibit increased sensitivity to heat shock-induced cell death (Watanabe and Lam, 2006). Whether *wds1* mutants are sensitive to heat stress-inducing factors remains to be examined.

5.1.4 *WDS1* functions in plant defense response and FB1-induced senescence

Although the *wds1* mutants appeared normal in growth and development, the deletion of *WDS1* resulted in an accelerated expression of *SAG12*, a reliable senescence marker, under FB1 treatment in two duplicate experiments. This suggests that *WDS1* presumably functions as a cell death attenuator in a senescence program triggered by FB1.

The gene *SAG12*, identified from *Arabidopsis*, is specifically controlled by developmental senescence (Noh and Amasino, 1999) and does not rapidly respond to senescence-promoting stresses and hormones (Weaver *et al.*, 1998). The induction of *SAG12* in both transgenic and wild type leaves indicates FB1 treatment has elicited the senescence program. A general model for the initiation of leaf senescence has been proposed in which declining rates of photosynthesis may be the senescence-initiating factor (Hensel *et al.*, 1993). In this model, when the photosynthetic rate drops below a certain threshold level at which the leaf no longer contributes fixed carbon to the rest of the plant, the senescence program and nutrient recycling is initiated. Certain stresses and hormones promote senescence; these include desiccation, darkness, detachment, wounding, pathogenesis, and the hormones abscisic acid (ABA) and ethylene (e.g., Becker and Apel, 1993; Oh *et al.*, 1996; Park *et al.*, 1998; Weaver *et al.*, 1998). It is possible that FB1 incubation first cause a decline in photosynthesis which in turn initiates senescence. Asai *et al.* (2000) suggested that reactive oxygen species produced during photosynthesis and/or light-dependent activation of PAL activity might be related to FB1 activity, the latter because PAL is a key enzyme in salicylic acid biosythesis, which was found to be required for FB1-induced cell death.

FB1-induced cell death are similar to the hypersensitive response in *Arabidopsis* in many respects, including deposition of phenolic compounds and callose, production of ROIs, accumulation of camalexin, and expression of PR genes (Asai *et al.*, 2000). FB1 elicits expression of the PR-1, PR-2,

and PR-5 genes, which are also induced after infection by various pathogens and by treatment with salicylic acid (SA) or its analogs (Yang *et al.*, 1997). In addition, FB1 induces expression of PDF 1.2, a gene that encodes a small, cysteine-rich secreted protein related to insect defensins, which is induced by necrotizing fungal pathogens and abiotic elicitors such as jasmonic acid and ethylene (Penninckx *et al.*, 1996; Penninckx *et al.*, 1998). Thus, FB1 appears to activate directly or indirectly a variety of signaling pathways associated with the defense response to pathogen attack.

The signal molecule SA appears to mediate FB1-induced programmed cell death. Asai *et al.* (2000) have reported that FB1-induced cell death in *Arabidopsis* protoplasts is correlated with the light-dependent accumulation of SA. However, FB1 can induce only relatively small amount of SA accumulation and FB1 susceptibility in mutant protoplasts seems to be reversely correlated to SA signal amplification in many cases. For example, FB1 susceptibility increases in protoplasts isolated from the *cpr1*, *cpr6*, and *acd2* mutants, in which SA is overproduced (Bowling *et al.*, 1994; Greenberg *et al.*, 1994; Clarke *et al.*, 1998; Asai *et al.*, 2000). *WDS1* may play a role in amplification of SA signals in a SA-dependent manner, which results in the decrease of FB1 susceptibility.

FB1 also requires jasmonate (JA) and ethylene (ET) mediated signaling pathways to induce apoptosis-like PCD, but it is less likely that *WDS1* plays a role in JA/ET mediated responses because most of the JA/ET-dependent defense responses analyzed to date are SA independent. For example, induced systemic resistance (ISR) in *Arabidopsis*, triggered by the biocontrol bacterium P. fluorescens in association with *Arabidopsis* roots, depends on both JA and ET but is independent of SA (Pieterse and van Loon, 1999; Asai *et al.*, 2000). Nevertheless, the JA/ET- and SA- dependent resistance pathways share at least one key regulatory component since ISR is dependent on NPR1, a positive regulator that transmits SA signals to downstream components in a signaling pathway (Pieterse et al, 1998; Peterse and van Loon, 1999). Therefore, further studies remain to be conducted to identify the precise pathways *WDS1* may be involved in FB1 elicited cell death.

The *WDS1* expression was strongly induced at an early time following the treatment with salicylic acid. The accumulation of *WDS1* transcripts were accelerated by salicylic acid in a time and dose dependent

pattern. These results suggest that the *WDS1* gene rapidly respond to signaling molecules triggered by biotic and abiotic stresses in the SA-dependent signaling networks (Dong, 1998; Durrant and Dong, 2004).

Salicylic acid has been known to be involved in diverse physiological metabolisms as well as disease resistance responses (Creelman and Mullet, 1997: Kieber, 1997). It has been identified as a key signaling component in numerous plant responses to stress, including pathogen invasion (Gaffney *et al.*, 1993; Glazebrook, 1999); and exposure to ozone (Rao and Davis, 1999; Sharma and Davis, 1997) and UV-B (Surplus *et al.*, 1998). Morris *et al.* (2000) also identified SA as a signaling molecule used by the plant to initiate the expression of a subset of genes during leaf senescence. Their work indicates that several of these senescence associated genes are partially regulated by SA, and also partially regulated by some additional factor present in senescing leaves. For example, the senescence-enhanced cytosolic glutamine synthetase gene LSC460 also shows an increase in transcript during senescence in plants defective in the SA-signalling pathway, but the expression level is considerable reduced (Morris *et al.*, 2000). The functional redundancy of cross-linking signaling pathways may, in part, explain why the *wds1* mutant has no significant influence on *Arabidopsis* development. Genes expressed during senescence may require a combination of pathways for maximal expression, and removal of a single pathway will often not have an obvious effect. Therefore it may be necessary to knock out more than one signaling pathway to make a significant difference to the senescence process itself. It still remains a mystery how SA triggers the rapid increase of *WDS1* expression and whether it is essential for the up-regulation. Further study on the *WDS1* expression pattern using mutants with lesions in SA pathway may help to answer these questions.

The enhanced tolerance of *WDS1*-overexpressing lines in hyperosmotic stress provided further support for the involvement of *WDS1* in plant defense. Osmotic stress activates several protein kinases including mitogene-activated kinases, which may mediate osmotic homeostasis and/or detoxification responses (Zhu, 2002). A number of phospholipids systems are activated by osmotic stress, generating a diverse array of messenger molecules, some of which may function upstream of the osmotic stress-activated protein kinases (Zhu, 2002). Activation of phospholipids signaling and MAP kinase (MAPK)

cascade, and transcription cascade leads to the expression of genes encoding stress tolerance effector proteins (Zhu, 2002).

5.1.5 Overexpression of *WDS1* delay the flowering time

The transition from the vegetative to the reproductive and to the final developmental senescence phase is of vital importance for the survival of flowering plants. Flowering is controlled by the developmental age of the plant and environmental signals, including photoperiod, vernalization, light quality, and the availability of water and nutrients (Bernier *et al.*, 1993; Barth *et al.*, 2006). There are four major pathways that control flowering time: the photoperiodic (long day) pathway, the gibberellin (GA) pathway, the autonomous pathway, and the vernalization pathway (Komeda, 2004; Corbesier and Coupland, 2005; Barth *et al.*, 2006). These four pathways converge to regulate the expression of genes which integrate the information received from the different pathways (Barth *et al.*, 2006). Only the long day pathway controls the response to day length, and specifically promotes flowering in response to LDs (Yanovsky and Kay, 2003; Hayama and Coupland, 2003). Mutations in this pathway can either delay flowering under LDs or accelerate flowering under SDs. The last gene that is specifically involved in this pathway is CONSTANS (CO), which encodes a zinc finger protein that promotes transcription of downstream flowering-time gene (Putterill *et al.*, 1995; Robson *et al.*, 2001).

The four genetic pathways finally converge to regulate the expression of a small group of downstream genes, sometimes described as floral integrator (Mouradov *et al.*, 2002; Simpson and Dean, 2002). This group includes two genes that promote flowering, FLOWERING LOCUS T (FT) and SUPPERSSOR OF OVEREXPRESSION OF CO 1 (SOC1), and LEAFY, a gene encoding a transcription factor required to confer floral identity on developing floral primordial. Overexpression of *WDS1* results in three days' delay in flowering, suggesting that *WDS1* may interfere the genetic pathways that control flowering time in *Arabidopsis*. As the plants in the present work were all grown under long day conditions, it is therefore most likely that the constitutive expression of *WDS1* has a negative effect on the long day pathway. However, since mutations in each of the downstream genes delay flowering, it is also possible that *WDS1* may negatively regulate the expression of SOC1 and FT, which attenuates the signal downstream of the flowering pathways.

5.2 Conclusion

In this report, we described the identification of a novel gene family, *WDS*, which contains five members in *Arabidopsis*. The putative WDS1 protein contains seven WD40 repeats and is conserved across several different species and organisms. Expression of *WDS1* was found in all the developmental stages with the highest in *Arabidopsis* when 50% of the flowers to be produced opened. The *wds1* mutant showed a normal developmental phenotype, but exhibited an earlier onset of *SAG12* expression than the wild-type under FB1 treatment. This indicates that *WDS1* may function as a regulator in *Arabidopsis* senescence, but its role is not essential. Expression of *WDS1* in detached wild-type leaves is up-regulated by SA in a time- and dose-dependent manner, and overexpression of *WDS1* resulted in an enhanced tolerance to hyperosmotic stress, both suggesting an involvement of *WDS1* in plant responses to stresses.

5.3 Future Direction

The *WDS1* gene has displayed a promising role in both *Arabidopsis* development and defense response. However, there is still considerable uncertainty as to which *WDS* family member performs which particular function. It is also unclear how the WD40 repeat domains contribute to the gene function as a signaling molecule. Although the *WDS1* was rapidly up-regulated by SA, it is poorly understood how it is involved in SA pathways. Moreover, it is not clear whether *WDS1* is an interplayer between plant development (e.g. senescence and flowering) and plant responses to stresses. To further delineate the function of *WDS1* in the future, it is probably necessary to use other strategies such as RNAi to determine the exact effect of a particular *WDS* gene. Knockout of multiple members of the *WDS* gene family is critical in evaluating their functions in plant growth and development.

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