IDENTIFICATION AND PARTIAL CHARACTERISATION OF A PUTATIVE NOVEL PLANT STRESS RESPONSE GENE FROM *Arabidopsis thaliana*.

By

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PREFACE

"I declare that, IDENTIFICATION AND PARTIAL CHARACTERISATION OF A PUTATIVE NOVEL PLANT STRESS RESPONSE GENE FROM *Arabidopsis thaliana*, is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references."

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December 2000.

ABSTRACT

Exposure of plants to stress results in the expression of plant genes whose products play a role in defence responses against the stress. Stress stimuli to which plants may be exposed are wounding, drought, salinity, excessive light intensity, heat stress, pathogens, biotic and abiotic factors that lead to the accumulation of H_2O_2, salicylic acid and plant hormones *e.g* absiscic acid.

This study describes the identification and partial characterisation of a putative novel gene from *Arabidopsis thaliana*, named the *DWNN* gene. The sequence of the predicted protein encoded by the *DWNN* gene shows similarity to a gene isolated from Chinese hamster ovary cells that were resistant to chemically induced programmed cell death. Since programmed cell death is one of the processes involved in plant defence responses to stress, it is hypothesised that the *DWNN* gene may also play a role in plant programmed cell death.

The protein product encoded by the *DWNN* gene, DWNN, shows homology to proteins from diverse species and phyla. Plants transformed to over-express *DWNN* show severely stunted growth and abnormal developmental patterns while plants in which *DWNN* has been knocked out show an accelerated growth rate. Analysis of the expression pattern of the *DWNN* gene using the GUS gene reporter system suggests that the *DWNN* gene is expressed in secondary lignification during xylogenesis and in wounded plant tissue. Both xylogenesis and wounding are processes known to involve programmed cell death and the regulation of protein turnover.

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ABBREVIATIONS

ATP:	Adenosine triphosphate
Avr:	Avirulence
BLAST:	Basic Local Alignment Search Tool
bp:	Base pairs
CaMV:	Cauliflower Mosaic Virus
CDPK:	Calcium-Dependent Protein Kinase
CHO:	Chinese Hamster Ovary
Ds:	Dissociation
DWNN:	Domain With No Name
EST:	Expressed Sequence Tag
GTP:	Guanosine triphosphate
GUS:	β-glucuronidase
HR:	Hypersensitive response
IL-1R:	Interleukin-1 receptor
Kb:	Kilobase pairs
LRR:	Leucine-Rich Repeat
MS30:	Murashige and Skoog medium with 3% sucrose
NADPH:	Nicotinamide adenine dinucleotide phosphtate reduced form.
NBS:	Nucleotide Binding Site

PCD: Programmed Cell Death

PCR: Polymerase Chain Reaction

R: Resistance

RACE-PCR: Rapid Amplification of cDNA Ends-Polymerase Reaction

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcription-Polymerase Chain Reaction

SDS: sodium dodecyl sulphate

SDS-PAGE: SDS-polyacrylaminde gel electrophoresis

SIPK: Salicylic acid-Induced Protein Kinase

Tris: 2-amino-2-(hydroxymethyl)-1,3-propandiol

UWC: University of the Western Cape

WIPK: Wounding-induced Protein Kinase

X-GlcA: 5-bromo-4-chloro-3-indolyl- β-D-glucuronide

1. Introduction

The role of programmed cell death (PCD) in development and stress responses

Programmed cell death is a genetically controlled biological process by which selected cells undergo a series of metabolic changes leading to cell death. This process has been observed only in eukaryotes. Cell death is selective in that only specific target cells die in the process and their death is beneficial or even necessary for the normal functioning and survival of the organism (Evan and Littlewood, 1998; Green and Reed, 1998; Raff, 1992; Raff *et al.*, 1993; Thornberry and Lazebnik, 1998).

Programmed cell is also required for normal development of multicellular eukaryotes, in particular during cell differentiation into specific tissues or organs. It may also occur in response to environmental stimuli that are harmful to the organism and in response to pathogens that may be a potential cause of disease (Stellar, 1995; Thompson, 1995; Wyllie, 1995; Wyllie *et al.*, 1980).

In animals, characteristic cellular processes in programmed cell death are calcium influx, activation of specific proteases, protein kinase activation and nuclear DNA fragmentation into large 50 kilobase (kb) fragments which are then broken into smaller fragments that form the diagnostic nucleosomal ladders when viewed under ultraviolet light on ethidium bromide-stained agarose gels. Morphological characteristics of the dying cells include cytoplasmic shrinkage, chromatin condensation and membrane blebbing

(Raff, 1992; Wyllie, 1995; Wyllie *et al.*, 1980).

Similar events occur in plants during programmed cell death. However, unlike in animals, the mechanisms underlying programmed cell death in plants are less well understood. In plants, programmed cell death has been observed during xylogenesis (Van der Mijnsbrugge *et al.*, 2000; Roberts and McCann, 2000) and senescence (Perez-Amador *et al.*, 2000), the hypersensitive response to incompatible pathogens and in response to biotic and abiotic stresses (Seo *et al.*, 1995, Seo *et al.*, 1999).

The hypersensitive response is a programmed cell death pathway that leads to the formation of dehydrated lesions that can be observed on the surface of the plant tissue at the site of pathogen entry (Bennet *et al.*, 1996). Initiation of the hypersensitive response involves a series of complex interactions, signals and signal transduction pathways. The hypersensitive response can also result in specific defence reactions that hinder the penetration of the pathogen (e.g. through secondary lignification) or inhibit the pathogen (e.g. through the synthesis of protease inhibitors or chitinases) (for review see Dangl *et al.*, 1996; Hammond-Kosack and Jones, 1996).

Events that cause programmed cell death in plants result in signal transduction events that lead to the up-regulation of the expression of pathogenesis-related proteins and other defence genes. These in turn contribute to disease resistance, defence against tissue injury and the establishment of systemic acquired resistance (for review see Hammond-Kosack and Jones, 1996; Ryals *et al.*, 1996; Shirasu *et al.*, 1996).

Host responses to pathogens - the role of PCD

It is desirable and often vital for plant survival to recognise invading

pathogens in order to rapidly (and systemically) activate their defence

mechanisms. Such recognition depends on pathogen-encoded ligand-like

elicitors and specific plant-encoded receptor-like proteins. The elicitors are

products of avirulence (*avr*) genes in the pathogen and are integrated in the

cellular surface of the pathogen (see Figure 1.1). The receptors are

resistance proteins on the plant's cell surface and are encoded by resistance

(*R*) genes in the plant. In gene-for-gene interactions, the plant can recognise

the pathogen only when the plant's *R* gene encodes a receptor that can bind

the pathogen's *avr*-encoded elicitor (Flor, 1971; De Wit, 1997; Staskawicz

et al., 1995). An example of an extensively characterised *avr-R* interaction

is seen during resistance of tomato to *Cladosporium fulvum*, the causative

fungus for leaf mould. This interaction occurs between the tomato-encoded

Cf-9 receptor from the *Cf-9* resistance gene and the *C. fulvum*-encoded Avr9

elicitor from the *Avr9* gene (De Wit, 1997; Jones *et al.*, 1994). Cf-9 is an

extracellular protein with an N terminal signal peptide sequence for

transport across the cell membrane. The C terminus of Cf-9 contains a

transmembrane domain and 28 amino acids that form a cytoplasmic peptide.

It is this type of interaction that leads to the generation of the hypersensitive

response (HR) and consequent disease resistance. Such an interaction is

known as an incompatible interaction. The plant fails to initiate the HR if

the elicitor is not recognised by the receptor and disease may occur as a

result of a compatible interaction.
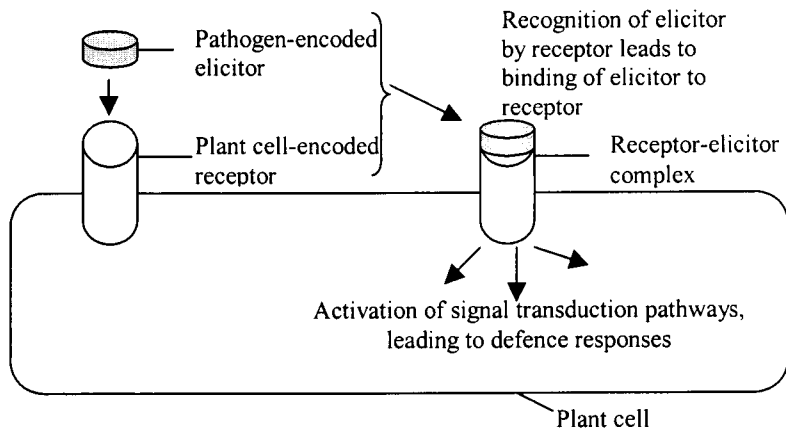
Figure 1.1. Schematic representation of elicitor receptor interactions.

R gene products – domain structures and biological functions

R gene products are classified according to structural domains and function

(Ellis et al., 2000). Pto is a tomato R gene that confers resistance to strains

of Pseudomonas syringae pv tomato expressing the avrPto avirulence gene

(Martin et al., 1993). The predicted amino acid sequence of Pto is

characteristic of protein kinases. Regions conserved among serine-threonine

kinases are also found in the sequence of Pto. It has been demonstrated that

Pto exhibits protein kinase activity in vitro (Loh and Martin, 1995; Chandra

et al., 1996). A myristoylation site is found at the N-terminus of Pto and

could act as a membrane anchor for the mainly hydrophobic protein (Martin

et al., 1993). The structure of Pto may suggest that perception of the avrPto

product on the surface of the plant cell membrane by the membrane-

anchored Pto leads to the activation of the serine-threonine kinase domain of

Pto. This activated kinase would then phosphorylate its target proteins in the

cytoplasm, thereby initiating a signal transduction cascade that leads to the

activation of defence responses, including the programmed cell death that occurs during the hypersensitive response of tomato infected by *Pseudomonas syringae* pv tomato.

Leucine-rich repeats (LRRs) are multiple serial repeats of a motif consisting of 24 amino acids (Kobe and Deisenhofer, 1994; Kobe and Deisenhofer, 1995). They contain leucines or other hydrophobic amino acid residues at regular intervals. LRRs may also contain proline and asparagine residues spaced in a regular pattern. LRRs have been shown to mediate protein-protein interactions (e.g. between enzymes and their inhibitors), intracellular components of a signal transduction cascade and the binding of peptide hormones to transmembrane receptors (for review see Kobe and Deisenhofer, 1994). The above example where LRRs play a role as transmembrane receptors of peptides might bear similarity to the elicitor-receptor model. It can be hypothesised that the LRR domain of some *R* gene products may act as a receptor domain to which the *avr* gene product (elicitor) binds. An alternative model for the role of LRR domains in *R* gene products is proposed by Dixon *et al.* (1996). According to this model, LRRs may facilitate the interaction of *R* gene products with other proteins that play a role in defence signal transduction. The *R* gene products containing LRRs fall into a variety of discrete classes and some *R* gene products may be involved in very different functions. Single amino acid changes in the conserved regions of LRRs have been shown to result in non-functional *R* gene products and consequently affect defence responses (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995). This demonstrates that the LRRs

in these *R* gene products are important in plant defence responses to pathogens.

A number of *R* genes encoding LRRs also encode highly conserved motifs that belong to the nucleotide binding site (NBS) domain family of motifs. The NBS domains are found in proteins that have the ability to bind GTP or ATP, most of which have kinase activity (Saraste *et al.*, 1990; Taut, 1994). The presence of highly conserved NBS domains in many *R* gene products suggests that binding of nucleotide triphosphates (ATP and GTP for example) is essential for the functioning of these *R* gene products. Mutations in key residues within the NBS domain of *RPS2* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994) have been reported to eliminate the hypersensitive response inducing function of *RPS2* in *Arabidopsis thaliana* (Bent, 1996). It is conceivable that binding of nucleotide triphosphates by the NBS domain in the LRR affect the interaction of *R* gene products with other proteins participating in a signal transduction pathway that regulates defence responses.

Some *R* gene products, in addition to having the LRR and the NBS domains, have leucine zipper motifs between the N terminus and the NBS and LRR domains. Examples of such *R* gene products are RPS2 (Bent *et al.*, 1994; Mindrinos *et al.*, 1994), RPM1 (Grant *et al.*, 1995) and the tomato *Prf* gene product Prf (Salmeron *et al.*, 1996). Leucine zippers are known to facilitate protein–protein interactions by promoting the formation of coiled coil domains (Alber, 1992). The leucine zippers generally play a role in both homodimerisation and heterodimerisation of transcription factors, but

since salicylic acid plays an important role as a downstream signalling molecule in plant defence responses mediated by *avr-R* gene-for-gene interactions (Ryals *et al.*, 1996; Ryals *et al.*, 1997; discussed further in this review).

A distinct group of leucine rich repeats (LRRs) is represented by Cf-9 and Cf-2, encoded by the by *Cf-9* and *Cf-2 R* genes respectively, from tomato (Jones *et al.*, 1994; Dixon *et al.*, 1996). These LRRs are different from other LRRs in that they do not contain nucleotide binding sites (NBS) domains and are predicted to be extracellular proteins with a signal peptide sequence at the N terminus for transport across the cell membrane. The C terminus of Cf-9 contains a transmembrane domain and 28 amino acids that form a cytoplasmic peptide.

Another class of *R* genes encode proteins that contain both the LRR and protein kinase domains. *Xa21* is an example of this class of *R* genes. *Xa21* encodes an LRR receptor kinase in which the LRR domain is extracellularly located and is linked by a transmembrane domain to a cytoplasmic protein kinase domain (Song *et al.*, 1995). It could be expected that the LRR domain of Xa21 should act as the receptor for the pathogen-encoded elicitor while the protein kinase domain of this protein could possibly become activated when the elicitor is bound to the LRR. Consequently the activated protein kinase domain would phosphorylate another protein, thereby generating a signal transduction pathway that leads to a defence response.

It is possible that separate domains encoded by separate *R* genes may interact with each other to form a link between pathogen perception and the

similar coiled-coil domains participate in interactions between proteins with a variety of other functions (Lupas et al., 1991; Hamm and Gilchrist, 1996). It is therefore possible that the leucine zipper domains in some R gene products play a role in the interaction of the R gene products with other proteins to regulate signal transduction upon recognition of a pathogen-encoded elicitor.

An N-terminal domain with similarity to the cytoplasmic signalling domain of the Drosophila melanogaster Toll protein and mammalian interleukin-1 receptors (IL-1R) occurs in proteins encoded by the tobacco N resistance gene (Whitham et al., 1994) and the flax L6 resistance gene (Lawrence et al., 1995). Toll is a receptor that is involved in the activation of a transcription factor that plays a role in the establishment of polarity during embryonic development in Drosophila melanogaster (Morisato and Anderson, 1995). IL-1R is an interleukin-1 (a cytokine) responsive receptor that activates the transcription factor NF-κB (Kuno and Matsushima, 1994). A number of interesting similarities, in addition to the sequence similarities, exist in the pathways controlled by the N, L6, Toll and IL-R proteins. For instance, NF-κB leads to elevated production of activated oxygen and is involved in host antimicrobial responses (Kuno and Matsushima, 1994); it is known that the production of reactive oxygen species is one of the hallmarks of avr-R gene-for-gene defence responses (Dangl et al., 1996; Hammond-Kosack and Jones, 1996; discussed further in this review). It has been shown that the activity of NF-κB is modulated by derivatives of salicylic acid such as aspirin (Kopp and Ghosh, 1994), which is interesting

triggering of host cell signal transduction that eventually leads to defence responses. For example, *Pto* encodes a protein kinase and *Prf* encodes a NBS-LRR and both of them are required for resistance against *Pseudomonas syringeae tomato* (expressing *avrPto*). Similarity between the domains of these two proteins, as a combination, with Xa21 suggests that Pto and Prf may interact to perceive the elicitor encoded by the pathogen (this would be a function of Prf) and activate a signal transduction pathway (via the phosphorylation of other proteins) that results in a defence response (as a result of the protein kinase activity of Pto). It follows that proteins encoded by *R* genes function in pathogen perception and triggering of a signal transduction pathway that results in a defence response aimed at conferring resistance to the pathogen.

Triggers and signalling mechanisms in plant defence reactions

There is evidence that pathogen recognition and wounding lead to efflux of K^+ from the cytoplasm, through K^+ ion channels in the cell membrane (Blatt *et al.*, 1999; Seo *et al.*, 1995, Seo *et al.*, 1999). This may be mediated by Avr-R interaction-activated phosphorylation of the K^+ channels. Ca^{2+} influxes that are observed during Avr-R interactions (Levine *et al.*, 1996) occur due to the opening of Ca^{2+} channels and this may compensate for the loss of K^+ (Atkinson *et al.*, 1990). The Ca^{2+} is hypothesised to bind to EF hands (which are helix-turn-helix motifs that bind Ca^{2+}) in transmembrane NADPH oxidases and facilitate phosphorylation of the NADPH oxidase by Ca^{2+}-responsive/dependant protein kinases, with calcium-dependent protein

kinase (CDPK) as the most recent example (Romeis et $al.$, 2000). Such phosphorylation can activate the NADPH oxidases which then convert O_2 to O_2^- (superoxide), resulting in the rapid accumulation of reactive oxygen species (ROS) (Levine et $al.$, 1994; Romeis et $al.$, 2000; Wojtaszek, 1997) namely superoxide, hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). In response to oxidative stress caused by the reactive oxygen species, endogenous plant superoxide dismutases convert the superoxide to H_2O_2 (Apostol et $al.$, 1989; Levine et $al.$, 1994). This rapid, transient production of high levels of reactive oxygen species in response to external stimuli (biotic or abiotic stresses) is known as the oxidative burst.

The major reactive oxygen species in the oxidative burst is H_2O_2. The accumulation of reactive oxygen species in stressed plants is rapid. In suspension-culture cells, the increase in the levels of reactive oxygen species starts within 2 minutes after the application of the stress stimulus, reaches its maximum within 10 minutes, after which the level of the reactive oxygen species starts decreasing and returns to basal levels within 60 minutes (Apostol et $al.$, 1989; Bradley et $al.$, 1992; Bowler et $al.$, 1989; Legendre et $al.$, 1993; Levine et $al.$, 1994). However, the accumulation of reactive oxygen species in whole plants occurs considerably later (at least 2 hours) after the application of stress stimuli to the plant.

The oxidative burst has been shown to occur at the cell surface (Apostol et $al.$, 1989; Levine et $al.$, 1994) and requires the presence of NADPH. The oxidative burst plays a central role in the activation of programmed cell death and the expression of defence genes (Lamb and Dixon, 1997).

H₂O₂ and programmed cell death

Exogenous application of H_2O_2 to plant cells has been found to lead to programmed cell death, as shown by Evans Blue staining (Levine *et al.*, 1994; Levine *et al.*, 1996; Solomon *et al.*, 1999). It has been observed that a threshold level of approximately 8 mM of exogenously applied H_2O_2 is required for the induction of programmed cell death. Below this threshold, cellular protectant genes against oxidative stress (glutathion- S-transferase at 2 mM H_2O_2 and glutathione peroxidase as examples) are expressed and programmed cell death does not occurs (Levine *et al.*, 1994). As described in the previous section, recognition of a pathogen or wounding of the plant cell leads to H_2O_2 accumulation. H_2O_2 accumulation has also been shown by Evans Blue and TUNNEL staining to lead to programmed cell death (Levine *et al.*, 1994; Levine *et al.*, 1996; Romeis *et al.*, 2000; Seo *et al.*, 1999; Solomon *et al.*, 1999).

From these results, it follows that H_2O_2 is a selective signal that can induce either cellular protectant genes or programmed cell death, depending on the concentration of H_2O_2 generated on the cell surface. H_2O_2 is diffusible across the cell membrane and can thus function in cell-to-cell communication. It has also been observed that host plant cells collapse during incompatible interactions in a manner similar to that seen in apoptosis (Dietrich *et al.*, 1994; Greenberg, 1996; Greenberg and Ausubel, 1993; Greenberg *et al.*, 1994; Levine *et al.*, 1994; Levine *et al.*, 1996). The oxidative burst that results from reactive oxygen species leads only to transient accumulation of H_2O_2 that is rapid and sufficient to induce

programmed cell death. The programmed cell death itself occurs after a substantial lag and does not increase with increasing levels of H_2O_2 once it is initiated (Levine *et al.*, 1994).

Programmed cell death is suppressed by glutathione (Hockenbery *et al.*, 1993) and reactive oxygen species are detoxified by glutathione-S-transferase (Berhane *et al.*, 1994). There is evidence to suggest that ubiquitin may also function in the regulation of the programmed cell death that occurs during the hypersensitive response, as seen in transgenic plants dominant negative for a ubiquitin transgene (Becker *et al.*, 1993).

The requirement of different concentrations of H_2O_2 for the induction of cellular protectant genes and programmed cell death accounts for the dual roles of H_2O_2 as a localised signal for initiation of programmed cell death and as a diffusible signal that induces cellular protectant genes that function in preventing undesired oxidant-triggered programmed cell death. Such a regulatory mechanism ensures strict spatial limitation of wounding-/pathogen-induced cell death and allows the surrounding cells to express defence genes even though they are adjacent to dying cells.

H_2O_2 has also been noted to mediate oxidative cross-linking of cell wall structural proteins and may have a direct role in the killing of invading pathogens. The cross-linking of cell wall proteins may serve to contain the pathogen within the infected cell and thus prevent spread of the pathogen into neighbouring cells (Bradely *et al.*, 1992; Brisson *et al.*, 1994). The rapid dehydration of tissues undergoing programmed cell death during the hypersensitive response may also act to deprive the pathogen of nutrients

from the plant tissue thereby inhibiting pathogen growth. In addition to these roles, H_2O_2 plays a role in the activation of genes that promote programmed cell death and genes that lead to other defence responses to environmental, mechanical or pathogen elicitation.

Signalling molecules programmed cell death in plants

It has been observed that salicylic acid, ethylene, jasmonic acid and abscisic acid play a major role as signalling molecules in plant responses to pathogen, mechanical and environmental stress (Busk and Pages, 1998; Creelman *et al.*, 1992; Delaney *et al.*, 1994; Jonak *et al.*, 1996; Malamy and Klessig, 1992; Kumar and Klessig, 2000; Seo *et al.*, 1995; Seo *et al.*, 1999). There is evidence that these signals can lead to programmed cell death in plants, however, the molecular mechanisms by which they operate is not yet elucidated. It is possible that when the stimuli are perceived by the plant cells, phosphorylation of receptors for the stimuli leads to potassium and chloride ion effluxes, activation of specific proteases and Ca^{2+} and H^+ influxes, which then mediate the accumulation of the signalling molecules that mediate programmed cell death (Blatt *et al.*, 1999; Felix and Boller, 1995; Hahlbrock *et al.*, 1995; Legendre *et al.*, 1993; Romeis *et al.*, 2000).

It has also been demonstrated that the accumulation of H_2O_2 precedes salicylic acid accumulation (Hammond-Kosack *et al.*, 1996). This indicates that high levels of H_2O_2 also act as a signal for salicylic acid accumulation. H_2O_2 accumulation was shown to activate benzoic acid 2-hydroxylase. Benzoic acid 2-hydroxylase is an enzyme that catalyses the hydroxylation of

benzoic acid to form salicylic acid, the benzoic acid being synthesized from the decarboxylation of trans-cinnamic acid that is derived from the de-amination of phenylalanine (Leon *et al.*, 1995; Yalpani and Raskin, 1993). The H_2O_2-activated enzyme-mediated formation of salicylic acid can thus account for the observed salicylic acid accumulation in response to biotic and abiotic stimuli.

Accumulation of these signalling molecules is known to induce a number of protein kinases in the presence of Ca^{2+} (Àdàm *et al.*, 1997; Jonak *et al.*, 1996; Kumar and Klessig, 2000; Levine *et al.*, 1996; Romeis *et al.*, 1999; Seo *et al.*, 1995; Seo *et al* 1999). Examples of such protein kinases are the salicylic acid-induced protein kinase (SIPK) (Zhang and Klessig, 1997; Zhang and Klessig, 1998) and wounding-induced protein kinase (WIPK) (Seo *et al.*, 1995; Seo *et al* 1999; Zhang and Klessig, 1998).

Protein kinases may cooperate with the H_2O_2 that is generated in response to the stress stimulus or work independently to activate the expression of defence genes. It is interesting to note that salicylic acid blocks the activity of a tobacco catalase (Chen *et al.*, 1993) and that exogenously applied salicylic acid potentiates programmed cell death that occurs in the hypersensitive response during an incompatible plant-pathogen interaction (Levine *et al.*, 1994, Chen *et al.*, 1995). Salicylic acid has also been suggested to modulate local programmed cell death at the site of pathogen entry (Chen and Chen 2000, 1995; Vernooij *et al.*, 1994). Blocking of catalase activity by salicylic acid may enhance or prolong the accumulation of H_2O_2 and hence allow the accumulation of the H_2O_2 to the threshold level

that is required for the initiation of the programmed cell death. This provides a system of communication between the programmed cell death and defence gene activation. Typically, protein kinases activated in the presence of the signalling molecules possibly phosphorylate other proteins, which in turn activate the expression of transcription factors that can up-regulate the expression of defence genes (for example, salicylic acid accumulation leads to the expression of pathogenesis-related proteins and antimicrobial peptides which have inhibitory activity against pathogens).

Proteases and their inhibitors in plant programmed cell death

In animal cells, activation of cysteine aspartate proteases known as caspases triggers a series of signal transduction events that promote programmed cell death. Caspase-like proteolytic activity has been detected in tobacco during the hypersensitive response of tobacco to viral infection (del Pozo and Lam, 1998). Introduction of caspase-specific inhibitors into these plants abolishes the hypersensitive response normally induced by avirulent bacteria. This suggests a possible role of caspase-like plant proteases in the programmed cell death of plants during the hypersensitive response.

A study on soybean suspension cultures (Solomon et al., 1999) treated with 5 mM H_2O_2 (which is within the 5-8 mM H_2O_2 concentration known to induce programmed cell death) showed cytosolic induction of protease activity 30 minutes after H_2O_2 treatment, which decreased 4 hours after the H_2O_2 treatment. The cultures subsequently exhibited programmed cell death. This shows that H_2O_2 acts as a signal for the activation of proteases,

which ultimately leads to programmed cell death. Cells pre-treated with cyclohexamide (an inhibitor of protein synthesis in plants) prior (but not post) H_2O_2 treatment retained the H_2O_2-induced protease activity, showing that the activity was not due to the activation of pre-existing proteases but rather due to the expression of protease genes in response to the H_2O_2 signal. Protein extracts prepared from cells not treated with H_2O_2 showed no change in proteolytic activity when the extracts were treated with 8 mM H_2O_2. This in turn suggests that the protease activity observed in H_2O_2 treated cells is not a result of direct activation of proteases by H_2O_2 (by, for example, H_2O_2-dependent oxidation of sulfhydryls in the proteases), but rather that H_2O_2 acted as a signal for the induction of the expression of active proteases via signal transduction pathways in intact cells.

Cells labelled with ^{35}S-cysteine (which would be incorporated only into proteins containing cysteine) and ^{35}S-methionine (which would be incorporated into all proteins) were treated with 5 mM H_2O_2. SDS-PAGE and autoradiography showed that there was degradation of specific proteins (rather than non-specific degradation) 1 hour after H_2O_2 treatment; during this time *de novo* protein synthesis has occurred. The band corresponding to the newly synthesized protein was not detected 4 hours after the H_2O_2 treatment (Solomon *et al.*, 1999). This indicates that H_2O_2 rapidly induces the expression of specific proteins (cysteine proteases) whose synthesis is transient and its activity is inhibited only several hours after H_2O_2 treatment. When cells were treated with protein synthesis inhibitors before the H_2O_2 application, programmed cell death was inhibited; however, programmed

cell death was not inhibited in cells that were treated with the protein synthesis inhibitors after H_2O_2 application. This proves that H_2O_2 acts as a signal for the synthesis of proteins required for programmed cell death (Levine *et al.*, 1994; Solomon *et al.*, 1999).

Measurement of protease activity against fluorogenic peptides showed that there is higher protease activity in fractions from H_2O_2 treated cells than in untreated cells (Solomon *et al.*, 1999). When the proteolytic fractions were treated with protease inhibitors that successfully inhibited programmed cell death, the fractions lost their proteolytic activity. This confirms that the proteases that are H_2O_2-inducible as indeed those that promote programmed cell death induced by H_2O_2.

Expression of the endogenous plant cysteine protease-specific inhibitor, cystatin, under the CaMV 35S promoter inhibited the observed H_2O_2-induced programmed cell death; CaVM 35S promoter driven expression of other types of protease inhibitors did not inhibit H_2O_2-programmed cell death. This is direct evidence that H_2O_2-mediated programmed cell death is due to the activation of cysteine proteases in response to the H_2O_2 signal and not due to non-specific activation of other types of proteases (Solomon *et al.*, 1999).

A response similar to the one above was observed when the CaMV 35S promoter-protease inhibitor transformants were treated with an avirulent strain of *Pseudomonas syringae*, where hypersensitive response-associated programmed cell death was blocked in a similar manner. This unifies the role of H_2O_2 as a signal for the induction of programmed cell death using

multiple pathways in response to stress (Draper, 1997; Greenberg *et al.*, 1994; Jabs *et al.*, 1996; Levine *et al.*, 1994; McDowell and Dangl, 2000).

Protein extracts from the CaMV 35S-cystatin transformants after H_2O_2 treatment showed no proteolytic activity, proving that the highly expressed cystatin in these transformants inhibited cysteine protease activity. Treatment of the same extracts with 4 M urea and 0.5 % SDS resulted in the restoration of protease activity. This is due to the urea/SDS-mediated dissociation of the protease inhibitor (cystatin) from the cysteine proteases and shows that it is necessary for the cysteine protease inhibitors to bind to their target cysteine proteases in order to exert the inhibition.

Another set of experiments showed that accumulation of salicylic acid represses cystatin expression (Solomon *et al.*, 1999). This suggests that salicylic acid can potentiate H_2O_2-induced programmed cell death by relieving cysteine protease inhibition via the repression of cysteine protease inhibitor gene expression. These experiments are evidence that cysteine proteases play a role in the regulation of programmed cell death in plants.

The role of the Bcl-2 family in plant programmed cell death

The Bcl-2 family of proteins is characterised by having at least one of four conserved Bcl-2 homology domains. These proteins are important regulators of programmed cell death in animals. They act to promote or inhibit programmed cell death, depending on the type of the members of the Bcl-2 family interacting at the particular instance. Bax is a member of the Bcl-2

family which heterodimerises with Bcl-2 and acts to promote programmed cell death (Oltavi *et al.*, 1993).

Expression of murine Bax in tobacco has been demonstrated to lead to programmed cell death similar to the one seen in the hypersensitive response to tobacco mosaic virus infection (Lancomme and Santa Cruz, 1999). Bax functional deletion mutants (lacking apoptosis-promoting function) over-expressed in tobacco plants prevented programmed cell death. The ability of the murine Bax to induce programmed cell death in tobacco suggests the presence of biologically active Bcl-2 homologues in plants. Sanchez *et al.* (2000) infected *Arabidopsis thaliana* plants with the avirulent strain of *Pseudomonas syringae* to induce programmed cell death that occurs during the hypersensitive response. *A. thaliana* genes whose expression was up-regulated by the pathogen were isolated using cDNA-AFLP display. One of the up-regulated genes had significant sequence similarity to a human Bax Inhibitor-1 (*BI-1*) gene (Sanchez *et al.*, 2000). This *A. thaliana* Bax Inhibitor-1 homologue was termed *AtBI-1*. The gene was also found to be induced by wounding. The fact that this gene is up-regulated by both pathogen challenge and wounding suggests that it is involved in both biotic and abiotic stress responses.

Yeast cells expressing a mouse Bax gene under the regulation of the galactose inducible *GAL10* promoter die when grown in galactose containing media. Death is due to the expression of the mouse *Bax* gene known to promote programmed cell death in mice. When *AtBI-1* was expressed in yeast cells that express the cloned galactose inducible mouse

Bax gene, the yeast cells did not die even in galactose-containing media. This suggests that *AtBI-1* plays a role as an inhibitor of Bax-induced lethality (Sanchez *et al.*, 2000).

Analysis of transcript levels of *AtBI-1* in the jasmonate-insensitive *coi1* mutant in *A. thaliana* plants (Feys *et* al., 1994) showed that *AtBI-1* transcript accumulation was delayed in these jasmonic acid-insensitive mutants compared to wild type plants. *AtBI-1* transcript levels were found to be low in *ndr1* (Century *et al.*, 1995) and *eds1* (Aarts *et al.*, 1998) *A. thaliana* plants. The *eds1* mutants are sensitive to *Peronospora parasitica* and the *ndr1* mutants are not resistant to *P. syringae* and *Peronospora parasitica*, to which wild type plants are normally resistant. *Coi1* plants have been shown to be more susceptible to fungal infection than wild type plants and the wild type *COI1* gene modulates responses to wounding. Both the *ndr1* and the *eds1* plants are compromised in elicitation of race-specific disease resistance mediated by *avr-R* gene interactions. These results suggest a general role of *AtBI-1* in pathogen responses and in abiotic stress responses. It therefore seems that *AtBI-1* plays a role as an inhibitor of programmed cell death in plants by suppressing programmed cell death promoting Bax proteins in plants. Interplay between these members of the Bcl-2 family in plants seems to be involved in the regulation of programmed cell death. It is interesting to note that there seems to be some common role players in programmed cell death in both plants and animals. Further genes of the Bcl-2 family are yet to be identified and their role in plants await extensive characterisation.

Domain With No Name (DWNN) - Homologues in plants

Promoter-trap mutant Chinese hamster ovary (CHO) cells expressing

receptors for cytotoxic T-lymphocytes were generated by retroviral

insertional mutagenesis (George, 1995). These mutant CHO cells were

resistant to cytotoxic T-cell lymphocyte mediated killing (George, 1995).

Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

performed on the cytotoxic T-lymphocyte resistant CHO cells revealed that

one of the cDNA products of the RACE-PCR has similarity to a human

expressed sequence tag (EST) named 21c4 (George, 1995). Treatment of

these cytotoxic T-cell lymphocyte resistant CHO cells with staurosporine, a

chemical inducer of programmed cell death in wild type CHO cells showed

that these CHO cells were also resistant to staurosporine (A. Pretorius,

UWC, personal communication). The resistance of these CHO cells to

programmed cell death induced by staurosporine was thought to suggest that

a 21c4-like gene may have a role in programmed cell death in animals.

Sequencing of the entire 21c4 EST revealed that the EST has sequence

similarity to novel candidate genes from a variety of organisms, including a

putative gene from chromosome 4 of *A. thaliana* (A. Pretorius and D.J.G.

Rees, UWC, personal communication). The putative gene has been named

DWNN (Domain With No Name). Since the gene thought to be knocked out

in the CHO cells has similarity to DWNN and these CHO cells appear to be

resistant to staurosporine, it is hypothesised that DWNN may have a role in

the regulation (promotion) of programmed cell death. The reasoning is that

if a DWNN homologue is knocked out and this mutation causes resistance

to programmed cell death induced by staurosporine, then wild type DWNN

may promote programmed cell death. The putative DWNN gene of

Arabidopsis thaliana (Accession number Z97343) is predicted to have the

structure shown in Figure 1.2 below:
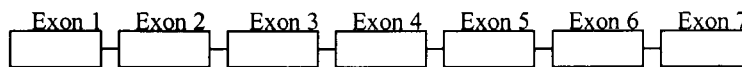
Figure 1.2. Predicted structure of the *DWNN* gene. The lines between the

exons signify introns.

In total, the putative DWNN gene is 1878 base pairs long and the predicted

cDNA (from joining the seven exons) is 825 base pairs long.

DWNN - Is there a role in PCD?

Based on the speculated role of DWNN in animals, it will be tested if the

expression of DWNN from *Arabidopsis thaliana* promotes programmed cell

death in plants or is coincidental with processes associated with

programmed cell death. The specific aim of this study is to partially

characterise the *DWNN* gene in chromosome 4 of *Arabidopsis thaliana*. To

achieve this aim, the experimental strategy below was followed:

1. Analysis of the sequence of the *DWNN* gene and identification of motifs

   in the hypothetical protein (derived from conceptual translation) encoded

   by the gene.

2. Expression studies using the following strategies:

a) Treatment of plants with stimuli known to induce programmed cell death in plants to find out if these stimuli lead to the up-regulation of *DWNN* expression.

b) *β*-glucuronidase (GUS) activity assays (Jefferson *et al.*, 1987) on transgenic plants in which the promoter of the *DWNN* gene has been fused to the GUS gene to study transcription al activity in response to stimuli that induce programmed cell death in plants.

c) Sense/Antisense over-expression of the *DWNN* gene using the CaMV 35S promoter to find out if enhanced/inhibited expression of *DWNN* results in a phenotype indicative of altered susceptibility to programmed cell death in the plants.

d) Expression of *DWNN* in the tapetum of *Arabidopsis thaliana* using the tapetum specific A9 promoter (Paul *et al.*, 1992) to determine a putative role of the *DWNN* in anther development.

e) Analysis of the phenotypic characteristics of *Arabidopis thaliana* plants in which the DWNN gene has been knocked out by transposon mutagenesis.

In summary, the proposed experimental program will help to identify the biological role and partially characterise modes of action of a novel protein with a putative role in programmed cell death in stress responses and development.

2. MATERIALS AND METHODS

Identification of the *DWNN* gene

The predicted protein of the 21c4 cDNA (A. Pretorius, University of the Western Cape, personal communication) was used to conduct BLAST searches (Altschul *et al*, 1997) to identify proteins with homology to the 21c4 protein. One of the proteins showing sequence homology to the 21c4 protein was a predicted protein encoded by a putative gene (now referred to as the *DWNN*) on chromosome 4 of *Arabidopsis thaliana*.

Sequence analysis

The DNA sequence of the putative *DWNN* gene was used to query BLAST (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al*, 1997) to identify nucleic acid sequences with similarity to the *DWNN* gene (genomic and mRNA using blastn against the nr database of GenBank, and EST using blastn against dbest (the EST database of GenBank).

The protein sequence (from a conceptual translation) of the *DWNN* gene in chromosome 4 of *Arabidopsis thaliana* was also used as a query to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al*, 1997) for protein sequences (blastp against the nr database of GenPept) with similarity to putative DWNN proteins.

A search for EST (expressed sequence tag) sequences with similarity to putative *DWNN* genes was also done by BLAST (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al*, 1997) using tblastn

with the DWNN protein sequence as query against dbest (GenBank's EST database).

In addition, the DWNN protein sequence was used as a query on BEAUTY/CRSeqAnnot (http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html, Worley *et al.*, 1995) to search for proteins with similarity to the DWNN protein and the motifs contained in the DWNN protein. A protein motif search was also done using Block Searcher (http://blocks.fhcrc.org/blocks/blocks_search.html; Henikoff and Henikoff, 1994) and ProfileScan (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) with the DWNN protein as the query.

Plant DNA extraction

1 g of fresh whole plant *Arabidopsis thaliana* was ground into a fine powder in liquid nitrogen. Genomic DNA was extracted from the ground plant tissue using a mixture of equal volumes of the DNA Grinding Buffer (0.2M Tris-HCl, pH 8.0; 0.25M NaCl; 0.25M EDTA and 1% SDS) and Tris-saturated phenol. This mixture was incubated at 50°C for 20 minutes; then the aqueous phase was separated from the organic phase by centrifugation at 10 000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to a new tube and retreated twice with Tris-saturated phenol and once with chloroform. The DNA was precipitated from the aqueous phase three times with absolute ethanol and 3M NaOAc (pH 5.5), with one wash of 70% ethanol after every precipitation followed by resuspension in sterile

distilled water. The DNA was quantified using a spectrophotometer and its purity was determined using the A_{260}/A_{280} absorbance ratio.

Plant RNA extraction

1 g of fresh *Arabidopsis thaliana* whole plants at the flowering stage, either treated or untreated with 8 mM H_2O_2, was ground to a fine powder in liquid nitrogen. Total RNA was extracted from the ground tissue using a mixture of equal volumes of the RNA Grinding Buffer (0.2M Tris-HCl, pH 9.0; 0.4M LiCl; 0.25M EDTA and 1% SDS) and H_2O-saturated phenol. The extract was incubated at 60°C for 20 minutes and then the aqueous phase was separated from the organic phase by centrifugation at 10 000 rpm for 20 minutes at room temperature. The aqueous phase was transferred to a new tube and treated twice with H_2O-saturated phenol and subsequently once with chloroform. The RNA was precipitated from the aqueous phase overnight on ice twice with 8M LiCl, with one wash of 2M LiCl after every precipitation followed by resuspension in DEPC-treated sterile distilled water. The RNA was then precipitated two times with absolute ethanol and 3M NaOAc (pH 5.5), with one wash of 70% ethanol after every precipitation followed by resuspension in DEPC-treated sterile distilled water. The RNA was clarified by heating at 60°C for 5 minutes followed by centrifugation at room temperature for 5 minutes. The RNA supernatant was transferred to a clean tube. The RNA was quantified using a spectrophotometer and its purity was determined using the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios.

Isolation of the *DWNN* sequence

The DWNN gene was isolated by polymerase chain reaction (PCR) from *A. thaliana* genomic DNA using primers designed for the putative *DWNN* gene in chromosome 4 of *A. thaliana*. The primers used were designed to contain appropriate restriction enzyme recognition sites for use in subsequent cloning of the isolated gene. Conditions of the PCR were: 30 cycles (denaturation at 94°C for 1 minute, annealing at 60°C for 45 seconds and extension at 72°C for 2 minutes) followed by a final extension at 72°C for 10 minutes.

Isolation of the *DWNN* promoter

The putative promoter of *DWNN* was isolated by PCR from *A. thaliana* genomic DNA using primers specific for a 1 kb genomic DNA fragment immediately upstream of the coding sequence of the *DWNN* on chromosome 4 of *A. thaliana*. The primers used were designed so that they have the relevant restriction enzyme recognition sites for cloning purposes. The conditions of the PCR were: 30 cycles (denaturation at 94°C for 1 minute, annealing at 50°C for 45 seconds and extension at 72°C for 2 minutes) followed by a final extension at 72°C for 10 minutes.

Isolation of *DWNN* cDNA

RT-PCR was performed using total RNA from *A. thaliana* plants either treated or untreated with 8 mM H_2O_2. The RT-PCR was performed according to the manual supplied with Promega's (Promega Corporation,

Madison, USA) Introductory Access RT-PCR Kit, except that the annealing temperature used was 50°C and 45 cycles of the PCR steps were done. 5' and 3' *DWNN*-specific primers against the predicted first and last exon were used for the RT-PCR. An oligo-dT primer (Sigma-Aldrich South Africa, Virna Valley, South Africa) was also used, together with the 5' *DWNN*-specific primer designed for the first exon. This was done in a separate RT-PCR according to Promega's Introductory Access RT-PCR Kit manual; 2 μl from each of the RT-PCR reactions were used in a "second-round" PCR to re-amplify the products, separately, with the *DWNN*-specific primer pair and the oligo-dT/5' *DWNN*-specific primer pair in a Roche LightCycler (Roche Diagnostics South Africa, Randburg, South Africa) using the LightCycler-DNA Master CYBR Green I kit as recommended by the supplier.

Cloning and sequencing

All the plasmids resulting from the cloning experiments mentioned in this section were introduced into *Escherischia coli* XL-1 Blue (Stratagene, La Jolla California, USA) by electroporation (GenePulser II RF Module; BioRad Laboratories, California, USA) and propagated in this bacterial strain. All plasmids were prepared using the alkaline lysis large-scale plasmid DNA preparation method (Birnboim and Doly, 1979; Maniatis *et al.*, 1982). Successful cloning was confirmed by colony PCR (Güssow and Clackson, 1989).

The PCR-isolated *DWNN* gene was cloned into the plasmid vector pBluescript SK (+/-) (Stratagene, La Jolla, California, USA) to make the construct named pdwnn1. Subsequently, the tapetum-specific A9 promoter (Paul *et al.*, 1992) was excised from the plasmid pWP127 (Paul *et al.*, 1992) and cloned upstream of *DWNN* in pdwnn1 to yield the construct named pTap1. The A9 promoter-*DWNN* fusion was excised from pTap1 and cloned into the pBin19 (Bevan, 1984) based plant transformation vector pCIRCE-NOS (constructed and kindly donated by Stuart Casson, University of Durham, United Kingdom) to make the construct named pHA1 for expression of the *DWNN* in the tapetum. Constructs pdwnn1 and pTap1 were sequenced to confirm that the cloned gene was *DWNN*.

DWNN was cloned immediately downstream of the double CaMV35S promoter in sense orientation in the plant expression vector pJIT60 to make pSens1. The CaMV35S promoter-sense *DWNN* fusion was excised from pSens1 and cloned into the pBin19 based plant transformation vector pCIRCE (kindly donated by Dr. Mike Bevan, John Innes Centre, Norwich, United Kingdom) to make the construct named pUN1 for over-expression of *DWNN*. Construct pSens1 was sequenced to confirm that the cloned gene was DWNN.

DWNN was cloned immediately downstream of the double CaMV35S promoter in antisense orientation in the plant expression vector pJIT60 (kindly donated by Dr. R. R. Croy, University of Durham, United Kingdom) to make the construct named pAnt1. The CaMV35S promoter-antisense *DWNN* fusion was excised from pAnt1 and cloned into the plant

transformation vector pCIRCE to make the construct named pUM1.
Construct pAnt1 was sequenced to confirm that the cloned gene was
DWNN.

The promoter of the *DWNN* gene was cloned upstream of the *β*-
glucuronidase (GUS) encoding gene, *uidA* [*gusA* (Jefferson *et al.*, 1987)], in
the plasmid vector pGUS-1 (kindly donated by Dr. J. F. Topping, University
of Durham, United Kingdom) to make the construct named pProm-GUS1.
The *DWNN* promoter-GUS gene fusion was excised from pProm-GUS1 and
cloned into the plant transformation vector pCIRCE to make the construct
named pExp1 for expression of the GUS gene. Construct pProm-GUS1 was
sequenced to confirm that the cloned fragment was the putative promoter of
DWNN.

The sequencing of the constructs was done using the ABI Prism™
BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with
AmpliTaq FS (PE Applied Biosystems, California, USA) as described in the
supplier's manual for sequencing reactions on the GeneAmp® PCR System
9700 and the ABI Prism® 310 Genetic Analyzer or the ABI Prism® 377
Genetic Analyzer (PE Applied Biosystems, California, USA) as described
in the supplier's manual for the sequence determination.

Plant transformation

Introduction of plasmids into *Agrobacterium tumefaciens*

The constructs pExp1, pHA1, pUM1 and pUN1 were mobilised from
Escherischia coli XL-1 Blue (Stratagene, La Jolla California, USA) into *A.*

tumefaciens LBA4404 (Ooms *et al.*, 1982) for tobacco transformation and *A. tumefaciens* C58 for *A. thaliana* transformation (Koncz and Schell, 1986) by triparental mating (Hoekma *et al.*, 1983; Bevan, 1984), using *Escherischia coli* HB101 (kindly supplied by Dr. J. F. Topping, University of Durham, United Kingdom). Selection of *A. tumefaciens* harbouring the constructs was done using the antibiotics kanamycin and rifampicin (both from Sigma-Aldrich South Africa, Virna Valley, South Africa) at 100 mg/L for both antibiotics.

Transformation of tobacco (*Nicotiana tabaccum* Petit Havana SR1) and *Arabidopsis thaliana* Columbia

Tobacco leaf explants were transformed with *A. tumefaciens* LBA4404 (Ooms *et al.*, 1982) harbouring the constructs pExp1, pHA1, pUM1 and pUN1 and the transformed plants were selected using kanamycin as described in Horsch *et al.* (1985). *A. thaliana* plants were transformed with *A. tumefaciens* C58 (Koncz and Schell, 1986) harbouring the constructs pExp1, pHA1, pUM1 and pUN1 using the floral dip method (Clough and Bent, 1998). Seeds from the transformed *Arabidopsis thaliana* plants were selected using kanamycin according to the protocol of Clough and Bent (1998).

Identification of a *DWNN Ds* transposon insertion knockout mutant

The database of the Arabidopsis Information Resource (TAIR, at http://www.arabidopsis.org), was searched for the availability of a seed line of knockout mutants of the *DWNN* gene from publicly available mutants in the Nottingham Arabidopsis Stock Center (NASC, at http://nasc.nott.ac.uk), using the locus/gene name "dl4735w" assigned by GenBank for the *DWNN* gene.

Assaying of GUS activity from pExp1 transformed plants

Roots and leaves were cut from *A thaliana* and tobacco plants transformed with pExp1 (has the *DWNN* promoter fused upstream of the *uidA* gene) and placed in Murashige and Skoog (from Sigma-Aldrich South Africa (Pty) Ltd, Virna Valley, South Africa) medium supplemented with 30 g sucrose per litre of medium (pH 5.8). Leaves were wounded by scraping the leaf tissue with the tip of a surgical blade. The plant tissue was left on the medium for 15 minutes. GUS activity was assayed in the roots and leaves using X-GlcA (from Sigma-Aldrich South Africa (Pty) Ltd, Virna Valley, South Africa) as the substrate for the GUS enzyme as described in Jefferson *et al.* (1987). The reactions were allowed to occur over 24 hours, followed by chlorophyll clearing with 70% ethanol overnight for leaf and stem tissue. Blue staining resulting from GUS activity was visualised using the Leitz light microscope (Wetzlar, Germany), photographed with the Pulnix LCD camera (USA) and recorded using the HL – image 97++ image software.

3. RESULTS

Identification of *DWNN* homologues in *Arabidopsis thaliana*

A BLAST search using the predicted protein sequence of the 21c4 cDNA from

mutant CHO cells resistant to staurosporine-induced apoptosis showed that the

21c4 cDNA-derived protein sequence has homology to a predicted putative

gene of *Arabidopsis thaliana* (Accession number 2245073). The gene is named

DWNN and is found on chromosome 4.

```
1     ATGGCAATAT ATTACAAGTT TAAGAGTGCG AGGGATTATG ATACCATCTC CATGGATGGT
61    CCTTTTATAA CAGTTGGTTT ACTCAAAGAA AAAATTTACG AAACTAAGCA TTTGGGAAGT
121   GGTAAAGACC TTGACATTGT CATCTCTAAT GCCCAAACTA ATGAAGGTTT GTCTTCCTCC
181   TTCTTATCCT ATTCACCTAG AAGATATTGT GCAATGTGTT TTGTTGTTAT CTAGGTTGGA
241   TTCTGGTCTG ATTGTGATCA GTATTCACTT ATCAAAATAT ATGGTTATTA CTTGCTTTGT
301   CAAAATTTCC ATGGTTTATG GCTTTCTTCC GAAGTAATCA CATTTAGCAC TGTTAATCTG
361   GCCATGGATT GTTCAGTATC TAACTGCTTC AGTAAAATGG ATGTTATGTA CCTGGTACTT
421   CCTTGTTAGT TGAATAAAAA AAATAAAAGT GAAGTTCTTG CGTACTGTAT TAGCTTATAT
481   GTTAAATGTG ATTACTTCTG AGACTACTGT TCTTGATTGG TTGTGGTTGT GAATCATTAG
541   AAAGAAACCC TTAACTACGA TTCTCTGTTG GTATAGTTGC AATATGTGGT GGCCTTCATA
601   CTTATTTTTT TTTTTGCAAT TTCAGAATAT CTGGATGAAG CAATGTTAAT ACCAAAAAAT
661   ACTTCTGTGC TAATTCGCCG GGTCCCTGGA CGCCCTCGCA TCAGAATTAT TACCAGAGAA
721   GAGTAATTTC TCTATAACTT TAATACCTAA TTTCTCAACT GAGTTGTTCA ATCTTTGTGT
781   TCTTTCGCTC TAAGCTGCGC ATTTCATTAT TAGTTGTGTG AAATTATACC AGATTCCCAT
841   GTGTCATGAT GTAAATGACT ACATTTTGCA GGCCGAGAGT CGAGGATAAA GTGGAAAACG
901   TTCAAGCTGA TATGAACAAT GTTATTACTG CTGATGCATC TCCCGTGAGT TGGCTTTCTC
961   AGTTTTTATT GGCATGGAAT CCTTCTACAC AAATATTGAT TTACAACTTA ATCTACCTTA
1021  TAGGTTGAAG ATGAATTCGA TGAGTTTGGG AATGATTTGT ATTCAATTCC TGATGCCCCA
1081  GCAGTCCATT CTAATAACCT ATGTCATGAT TCTGCACCAG CTGATGATGA AGAAACCAAG
1141  CTAAAGGCAT TAATTGACAC TCCAGCACTA GACTGGCATC AGTAAGTAAT CTTCTTGAAC
1201  TGATGGACAT TAGCATGCTA AATGAGATCT TGACATACAT TTTGCATAAC ATTCGTATTT
1261  TTCTTTCATG TTACAGACAA GGTGCAGATA GTTTTGGCCC GGGTAGAGGT TATGGGAGGG
1321  GTATGGCTGG AAGGATGGGT GGCCGCGGTT TTGGTAATTA TATTCCTGTA TATGATTGAC
1381  TTATATGCTT CAGTCTCCCC AAGTATTTAG CTTTGAGACT CGGATGTTCT GTTAATTATT
1441  ATTGTCCAAA TATTTTGTTT GCTCTTTCAT TGAAATGTAA GATATAGAGA CTGATTGTAT
1501  TTGTTTGAGT AGGAATGGAG AGGACAACAC CGCCACCAGG CTATGTTTGC CATCGGTGCA
1561  ATGTTTCTGG TATGACTGAC TTAGAGAATC TTTATATATT TTTAATTTGG TTAAGTAATG
1621  TCTCGAGTGT GTACGGTTAT TACCGAGAAA TGAATGTTCT GAACATCATT TTCTGTCTCA
1681  GGACATTTTA TTCAACACTG CTCCACAAAC GGTAATCCTA ACTTTGATGT TAAGAGAGTT
1741  AAACCACCTA CTGGTATCCC CAAGTCGATG CTGATGGCAA CCCCAAATGG CTCTTACTCC
1801  TTGCCAAGTG GCGCAGTAGC AGTTTTAAAA CCAAACGAGT ACGTTGTCAC TAAATTAAC̲A̲
1861  TGGCGAGTTG TTTGTTAG
```

Figure 3.1. Sequence of the *DWNN* gene.

Position 1 to 1878 in Figure 3.1 correspond to position 81131 to 83008, respectively, on the chromosome 4 contig (Accession no. Z97343). Exons are in black font and introns are in blue font. Primers for isolating the gene by PCR and its cDNA by RT-PCR were designed against the underlined sequences in Figure 3.1 and are within exon 1 (forward primers, bases 1 to 26) and exon 7 (reverse primers, reverse complement of bases 1851 to 1878). The conceptually translated protein sequence (Accession no. CAB10521) from the predicted cDNA (Accession number 2245073, from joining the seven exons) is shown below.

```
1    MAIYYKFKSA RDYDTISMDG PFITVGLLKE KIYETKHLGS GKDLDIVISN AQTNEEYLDE
61   AMLIPKNTSV LIRRVPGRPR IRIITREEPR VEDKVENVQA DMNNVITADA SPVEDEFDEF
121  GNDLYSIPDA PAVHSNNLCH DSAPADDEET KLKALIDTPA LDWHQQGADS FGPGRGYGRG
181  MAGRMGGRGF GMERTTPPPG YVCHRCNVSG HFIQHCSTNG NPNFDVKRVK PPTGIPKSML
241  MATPNGSYSL PSGAVAVLKP NEYVVTKLTW RVVC
```

Figure 3.2. Sequence of the predicted *DWNN* protein.

Sequence analysis

There were no significant similarities between expressed sequence tags (ESTs) and the *DWNN* gene or its protein, as indicated by the high E-values (> 0.01) in Figure 3.3.

```
Sequences producing significant alignments:                     (bits)  E-Value

emb|AJ397671.1|AJ397671   AJ397671 dkfz426 Gallus gallus cDNA...   42    0.96
gb|BF058621.1|BF058621    7k24b10.x1 NCI_CGAP_Ov18 Homo sapien...  40    3.8
```

Figure 3.3. First 2 hits of the BLAST search results for EST sequences with similarity to the *DWNN* gene.

The predicted DWNN protein from this *DWNN* gene showed a high degree of similarity to a number of novel proteins from different species and phyla, as shown in Figure 3.4.

```
Sequences producing significant alignments:              Score(bits)  E-Value

pir||D71443   hypothetical protein - Arabidopsis thaliana >gi...    461   e-129
dbj|BAB11612.1|   (AB018117) DNA-binding protein-like [Arabid...    334   7e-91
pir||T40809   conserved hypothetical zinc-finger protein - fi...    102   6e-21
ref|NP_012864.1|   Ykl059cp >gi|549683|sp|P35728|YKF9_YEAST H...     96   3e-19
gb|AAD34765.1|   (AF132177) unknown [Drosophila melanogaster]...     76   3e-13
pir||T21861   hypothetical protein F36F2.3 - Caenorhabditis e...     46   3e-04
```

Figure 3.4. BLAST search result of the six hits with the highest degree of similarity to DWNN.

Further *in silico* analyses were performed to identify conserved protein motifs within DWNN. The motifs are shown in Figure 3.5.

```
1     MAIYYKFKSA RDYDTISMDG PFITVGLLKE KIYETKHLGS GKDLDIVISN AQTNEEYLDE
61    AMLIPKNTSV LIRRVPGRPR IRIITREEPR VEDKVENVQA DMNNVITADA SPVEDEFDEF
121   GNDLYSIPDA PAVHSNNLCH DSAPADDEET KLKALIDTPA LDWHQQGADS FGPGPGYGRG
181   MAGRMGGRGF GMERTTPPPG YVCHRCNVSG HFIQHCSTNG NPNFDVKRVK PPTGIPKSML
241   MATPNGSYSL PSGAVAVLKP NEYVVTKLTW RVVC
```


Key to colour codes:

■ Protein Kinase C phosphorylation site (positions: 9 - 11, 40 - 42, 269 - 271)

■ N-glycosylation site (positions: 67 - 70, 207 - 210, 245 - 248)

■ Glycine rich region (positions: 167 – 191)

□ N-myristoylation site (underlined in the protein sequence, positions: 191 – 196, 234 – 239)

■ CCHC Zinc finger domain (the CCHC is in bold font in the protein sequence, positions: 201 – 218)

Figure 3.5. Structure of the DWNN protein as predicted from the motif analysis.

Sequence alignments of the proteins showing homology to the DWNN protein

were done using Clustal X and are shown in Figure 3.6.

```
Arabidopsis              ---MA YYFKSARDYDT SMX -PFITVGLKEK YETKHL S KLLB  50
DNA-binding_Arabidopsis  ---MA  FKSARDYDT AM  -PFIS GILDK FETKH  T KL
Schizosaccharomyces      -MSGV   F GQKDPSR T   TIGMS F V  IMQKK N L F
Saccharomyces            -MSST  KFKSQRNTSRILF  -TGLTVF KK IQENKL D TDFQI
Candida                  -MSSVV  KFLHQKNKSVIHF  -TSISVF KF ILQNQ S QDFN
Drosophila               ---MSVH    TLNFDT T   -LHIS G    VQQKR KII
Caenorhabditis           MHTIVLFNA LRMFAYDR AL L-AVFNLK VDSGNSSSSERSSPP--TR

Arabidopsis              VISNAQT-NEEY I EAML     VL IK V GR-PRIRIITREEPRVEDK
DNA-binding_Arabidopsis  VVSNAQT-    EAM        I    GR-PRITVITTQEPRIQNK
Schizosaccharomyces      LLYNANS- EE   TFI  K  VIV K  AQ-KSGKGTAA---R----
Saccharomyces            KIYNPDT-EE Y  AFV K  VI VK S AI-KSFSVHSRLKGN----
Candida                  RLYHSEQPDQ Y EL QDV   SY  AK S AF-VKLGKYNN-ALR----
Drosophila               QITNAQS-KE K  GF      TLIIS I IAHPTKKGWEPPAAENAFS
Caenorhabditis           IMSQAQNINDE SGSSGYGSPTR PLASPSNDIFSSVLPLQVQ---R----

Arabidopsis              VENVQADMNNVITADASPVEDEF-DEFGNDLY--SIPDAPAVHSNNLCHD
DNA-binding_Arabidopsis  VEDVQAETTNFPVADPSAPEDEY-DEFGTDLY--SIPDTQDAQHIIPRPH
Schizosaccharomyces      ----------YVSGAPKTTGARS-DSVKRPVP--MLQKKAPITSGESNIN
Saccharomyces            ----------V--GAAALGNATR-YVTGRPR---VLQKRQHTATTTANVS
Candida                  ----------YITGKPRINRKAITSTVGHNSNSNPLVSAQLQQQQQQQLD
Drosophila               AAPAKQDNFNMDLSKMQGTEEDKIQAMMMQSTVDYDPKTYHRIKGQSQVG
Caenorhabditis           -------------AFSAQSPEEITRHFGVSPSPQAVKPVPEVQQQREQAQ

Arabidopsis              SAPADD---EETKLKALIDTPALDWHQQGADSFGPG--RGYGRGMAGRMG
DNA-binding_Arabidopsis  LATADDKVDEESKIQALIDTPALDWQQQGQDTFGAG--RGYGRGMPGRMN
Schizosaccharomyces      KSPSSS---EDAAIQQMFQVSSDQWRETQDKMASAT--PIYKPNQRRIAA
Saccharomyces            GTTEEE------RIASMFATQENQWEQTQEEMSAAT--PVFFKSQTNKNS
Candida                  ENATEE-----DRIKLMFQNQSNAWEQTQEDLAHHK--MVFNKTASSTAN
Drosophila               EVPASYRCNKCKKSGHWIKNCPFVGGKDQQEVKRNTGIPRSFRDKPDAAE
Caenorhabditis           VSQTSA------QGLAAVDGFSMAEIAGFAELLSSP--KKVGEGMKIEGN

Arabidopsis              GR-----GFGMERTT       HR N----------------------
DNA-binding_Arabidopsis  GR-----GFGMERKT  E    HR N----------------------
Schizosaccharomyces      S---------VPDKP   I YR G----------------------
Saccharomyces            AQ--------ENEGP   M YR G----------------------
Candida                  K---------QDDH    I YR G----------------------
Drosophila               NESADFVLPAVQNQEI EDLI GI RDIFVDAVMIPCCGSSFCDDCVRTS
Caenorhabditis           L----------EYSTP PRYQ HI Y----------------------

Arabidopsis              ---VSG FIQH S- G  FDVK--------RVKPP      SMLMATP
DNA-binding_Arabidopsis  ---IPG FIQH P- G  YDVK--------RVKPP      SMLMATP
Schizosaccharomyces      ---QKG WIQA P- AD YDGKP-------RVKRT GI KSFLKNVE
Saccharomyces            ---GRD WIKN P- SD FEGK--------RIRRT GI KFLKSIE
Candida                  ---KKD WIKN P- N  FEGK--------KIMRT      SYLKTIS
Drosophila               LLESEDSECPD KEK CS GSLIPNRFLRNSVNAFKNE YN SAAKPAA
Caenorhabditis           --ATGH YISD PQRFNT YDELTP-------YQGRKKCYGEFTCQLCK
```

Figure 3.6. Alignment of DWNN homologs. Conserved sequences are in red; CCHC zinc fingers are represented in magenta.

BEAUTY/CRSeqAnnot was used to search for motifs in the DWNN protein (http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html, Worley *et*

al., 1995) and to search for proteins with similarity to the DWNN protein. Block Searcher (http://blocks.fhcrc.org/blocks/blocks_search.html; Henikoff and Henikoff, 1994) and ProfileScan (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) were used for motif searching.

Isolation of the DWNN gene

The DWNN gene was isolated from *Arabidopsis thaliana* DNA by PCR using the primers designed against the first exon and the last exon of the *DWNN* gene, indicated with the underlined sequences in Figure 3.1. Essentially, forward primers were identical to bases 1 – 26 (primer sequence: 5'-ATGGCAATATATTACAAGTTTAAGAG- 3'), except that relevant restriction sites were added to the 5' end of the primer for cloning purposes. The reverse primers used were reverse complements of bases 1859 – 1878 (primer sequence: 5'-CTAACAAACAACTCGCCATG- 3'). Additional base pairs were added to form restriction sites at 5' end of the primer to facilitate for further sub-cloning. The PCR yielded a 1902 base pairs (bp) product (Figure 3.7). Of this product 1878 bp are derived from the *DWNN* gene template and the extra 24 bp result from the restriction sites and extra nucleotides added to the 5' end of the primers. The extra nucleotides allow for digestion with the relevant restriction enzymes.
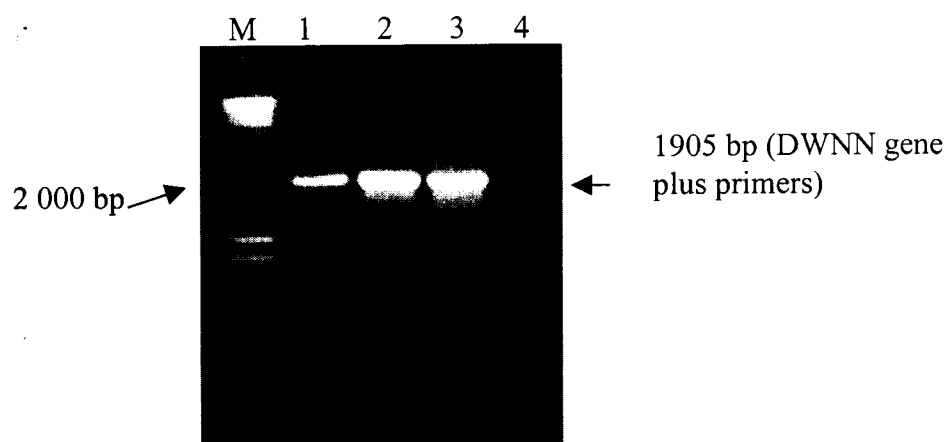
Figure 3.7. *DWNN* gene isolated from *A. thaliana* genomic DNA. Lane M is a DNA size marker (HyperLadder I: from Bioline, Humber Road, London, UK). Lanes 1–3 in represent the *DWNN* gene incorporating the relevant restriction enzyme sites at the 5' end of the PCR primers, lane 4 is the control without DNA in the PCR reaction.

Isolation of *DWNN* cDNA

To find out if the *DWNN* gene is expressed in *A. thaliana*, RT-PCR was performed using the Introductory Access RT-PCR Kit (Promega Corporation, Madison, USA) using total RNA from *A. thaliana* either untreated or treated with 8 mM H_2O_2. The rationale for treatment with 8 mM H_2O_2 was based on the fact that 8 mM H_2O_2 induces of the expression of a number of genes involved in programmed cell death in plants (see Introduction). The RT-PCR was performed using primers designed against the first exon and the last exon of the predicted putative DWNN gene, as indicated with the underlined sequences in Figure 3.1. Essentially, the forward primer was identical to bases

1–26 (primer sequence: 5'-ATGGCAATATATTACAAGTTTAAGAG- 3')
while reverse primer was the reverse complement of bases 1859 – 1878 (primer
sequence: 5'-CTAACAAACAACTCGCCATG- 3'). The concentration of the
total RNA used varied between independent reactions within a range of 100 ng
and 2 µg per reaction (with increments of approximately 100 ng in-between, to
cover the 100 ng–2 µg range) while the $MgSO_4$ concentration and annealing
temperature were kept constant at 1 mM and 50°C respectively. In a separate
optimisation experiment, the concentration of $MgSO_4$ was varied across a range
of 1–5 mM (with increments of 0.5 mM in-between the concentration) while
keeping the amount of total RNA at 1 µg per reaction and the annealing
temperature at 50°C. The final optimisation experiment was done in
independent reactions with 1 µg of total RNA at 1 mM $MgSO_4$ within the
temperature range of 48°C–58°C (with a difference of 1°C in-between each
temperature). Control RNA from the Introductory Access RT-PCR Kit yielded
the expected positive control RT-PCR product and this positive control reaction
was not affected when the control RNA was used in combination with 1 µg of
the *A. thaliana* total RNA used in the RT-PCR reactions thus confirming that
the template was not carrying inhibitory compounds. Sterile distilled water was
used in negative control reactions instead of total RNA. No *DWNN* transcript
was detected by this method. To date Northern blots were not attempted as the
RT-PCR strategies are thought to be the more sensitive method to detect low
transcript levels.

Isolation of the *DWNN* promoter

A fragment 1000 bp immediately upstream (-1 to -1000 relative to the first nucleotide of the *DWNN* gene) of the start of *DWNN* was selected as a region containing the putative promoter of the *DWNN* gene; this is shown in Figure 3.8.

```
1    CTCCCAACAC CTAACCCTCT GAAGAAACCT CATCACATCC GACTGCTTCT
51   TCCTCCATAG CTCCGACACG TATTTGTACG CACCTGTAAT CAAAAATCAC
101  ATTTCATCCT TAATTAACTT CTAGAATCTA ATGAACAATA CAGAAACGAC
151  ATCTGAGAGA GAGATAATAG TGAGAAACAT ACCCATTGCG ATTTTCCGGC
201  GTCGCAGTCA CTTCTTTCTT CGAATTAGGT TTGTGCTTTA AATAGATGGC
251  TCCATGGGAA TTAGGGTTTC CATCAGACAA AGAAAGCCCT TATTGGGCCT
301  TTTTCCGTTA GCTTTATGTA TTGGGCCTTC GCTACAACAT AAAACATCTC
351  TAATTGAACT GTATGGCCCA TTATAAATTT ATGTAAAAGG AGCCAAAACG
401  CAAGAGAATT AGGTGAAGAG TCTTTTGGAT TCGGTTACGA TAGTTCTCTA
451  TTCCTCTTCA AACCCTAGAG TTCGTGTTTC TGCGATATCC ATCGGTTTCT
501  CCTTCATATA TCCATCAAAC GCCAAAGATT CTCTAATTCT CTCTCGAGAT
551  CGTCTGCAAT TTCACCGTGA ATCATCAGGG ATACCGCGGT GGTTTCGATT
601  CTATTTCCTC GATTCGAAGC TTGGAGAGTT TCAGCTTCTG AATTGAAGCC
651  AGTGATTCAA TTGTTTGGAG ATATCTGAGC ATTGTCCCTG CGATGTATTC
701  GATTCAATCC AACACGGGCG TTCTCGTTTC GTTTCTGAGC TTTGTGGGAA
751  ATTCTCGTTG ATTTGATTTC GTCTTGAAGA GCGCGTGAGA GGTTTCTTCT
801  GTCGAATTTT GTTGAAGTCC AACCAGAGAA GTAAAGAATC ATATTTTGTA
851  ATTACTTTTT GTTTTTGTTT TGGAAATCAA CTTTCTGGCC TCCATTTTTT
901  TGGCCTGGGT TAAGGTTTTT TTCAAAGAGA GTAGATTCTG GTGTGTTTTA
951  ATAAATTAGT AATACAGATT GTATTTTTAT TCATTATCAG ATTAATTCCA
```

Figure 3.8. Putative *DWNN* promoter. The A of the ATG (encoding the first methionine) marks the start of the putative *DWNN* gene (position 1001).

The promoter was isolated by PCR using the underlined sequence (5' - CTCCCAACACCTAACCCTCTG- 3', base 1 – 21) as the forward primer and a reverse complement (5' -TGGAATTAATCTGATAATG- 3') of the underlined sequence (5' – CATTATCAGATTAATTCCA- 3', base 981 – 1000) as the reverse primer (Figure 3.8). Six nucleotides followed by a SalI site were added at the 5'

end of the forward primer and six nucleotides followed by a BamHI site were added at the 5' end of the reverse primer. Inclusion of these nucleotides and restriction enzyme sites will facilitate digestion of the PCR product with the restriction enzymes for cloning purposes. The PCR-isolated *DWNN* promoter is shown in Figure 3.9.
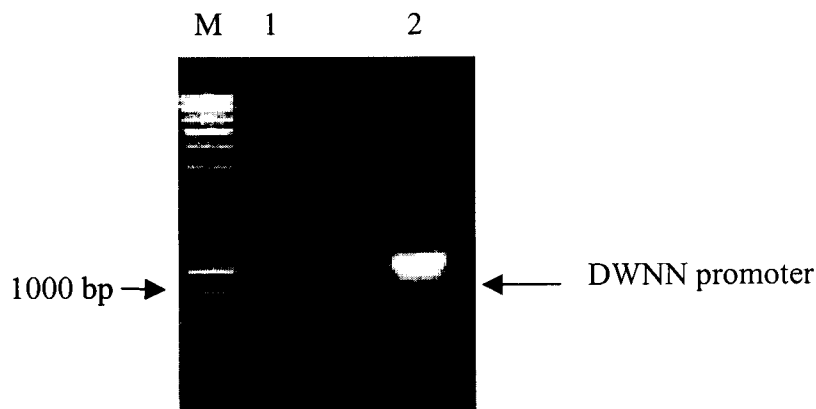

Figure 3.9. Promoter of the *DWNN* gene isolated from *A. thaliana* genomic DNA. Lane M is a DNA size marker (HyperLadder I: from Bioline, 16 The Edge Business Centre, Humber Road, London. UK). Lane 1 is a control in which water was added in the PCR instead of the *A. thaliana* genomic DNA. Lane 2 is the promoter of the *DWNN* gene with primers incorporating Sal1 (5'- end/forward primer) and BamHI (3'-end/reverse primer) sites. The Sal1 and BamH1 restriction sites were included to facilitate cloning of the PCR product.

Cloning and expression studies

The *DWNN* gene was sub-cloned for specific expression in the tapetum under the regulation of the A9 promoter and for constitutive overexpression in sense

and antisense orientation under the regulation of the double CaMV 35S promoter. An agarose gel (Figure 3.10) shows these predicted sizes of the constructs. The promoter of the *DWNN* gene was cloned upstream of the GUS gene into the pGUS-1 vector. This construct will allow analysis of the expression pattern of the *DWNN* gene (based on the activity of the *DWNN* promoter as indicated by GUS activity assays). Figure 3.11 below, panels A to D, shows the constructs used for the expression studies of *DWNN*.
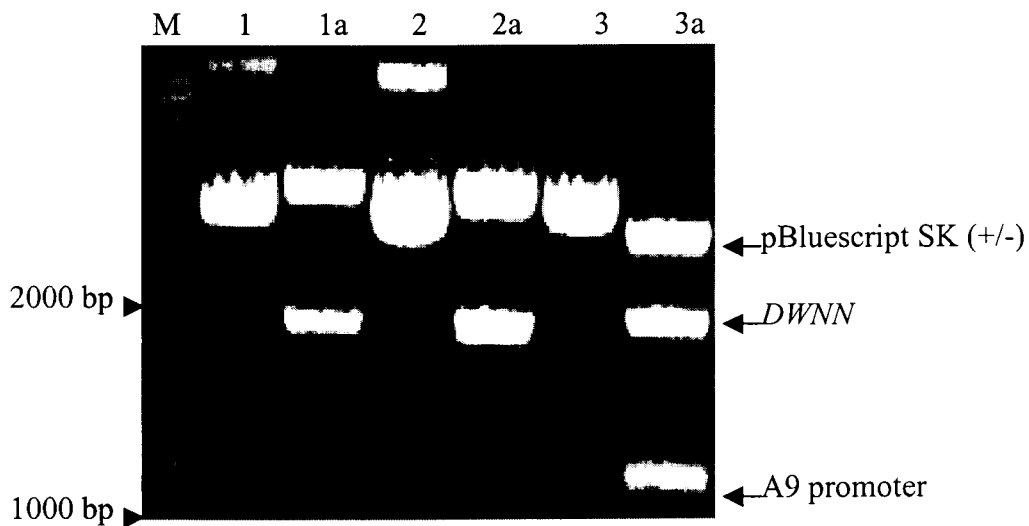
Figure 3.10. *DWNN* expression constructs. The constructs are pAnt1 in lane 1a, pSens1 in lane 2a and pTap1 in lane 3a. Lane M is a DNA size marker (HyperLadder I: from Bioline, London. UK).

In Figure 3.10, lane 1 is uncut pJIT60 (contains the double CaMV 35S promoter which drives overexpression of genes fused to its 3' end) with the *DWNN* gene in antisense orientation relative to the double CaMV 35S promoter, yielding the construct termed pAnt1; lane 1a is pAnt1 cut with

BamHI and HindIII, where the top band is pJIT60 and the bottom band is *DWNN*1; lane 2 is uncut pJIT60 with the *DWNN* gene in sense orientation relative to the double CaMV 35S promoter, giving the construct pSens1; lane 2a shows pSens1 cut with BamHI and HindIII, with pJIT60 as the top band and *DWNN* in sense orientation as the bottom band; lane 3 is uncut pBluescript SK (+/-)containing the A9 promoter and the *DWNN* gene and lane 3a is pBluescript SK (+/-) with the A9 promoter cloned as a SacI/XbaI fragment upstream of *DWNN* (which was cloned as a BamHI/SalI fragment), giving plasmid pTap1. For expression in plants; the A9 promoter-*DWNN* fragment from pTap1 was cloned into pCIRCE-NOS while the double CaMV 35S promoter-sense *DWNN* and the double CaMV 35S promoter-antisense *DWNN* fragments from pSens and pAnt1, respectively, were cloned into pCIRCE.

No phenotype was discernible for the anstisense and tapetum-specific expression experiments of the *DWNN* gene in the transformed plants, although germination and growth of the transformed plants on kanamycin-containing culture media (50 mg of kanamycin/L of culture medium) suggested that transgenic plants were obtained. The constructs used for the expression studies are detailed in Figure 3.11, panels A to D.

Panel A: pExp1 for GUS expression analysis

SalI   BamHI   EcoRI

Panel B: pUN1 for sense over-expression

KpnI   HindIII   BamHI

Panel C: pUM1 for antisense overexpression

KpnI   HindIII   BamHI

Panel D: pHA1 for tapetum-specific expression

SacI   XbaI   BamHI   SalI

Key:

■ : GUS-A gene

■ : DWNN promoter

▶ : Left Border

▷ : Nopaline synthase promoter

▶ : NPTII gene (Kan^R)

◗ : Nopaline synthase terminator

◀ : Right Border

▨ : Multiple cloning site

■ : CaMV 35S promoter
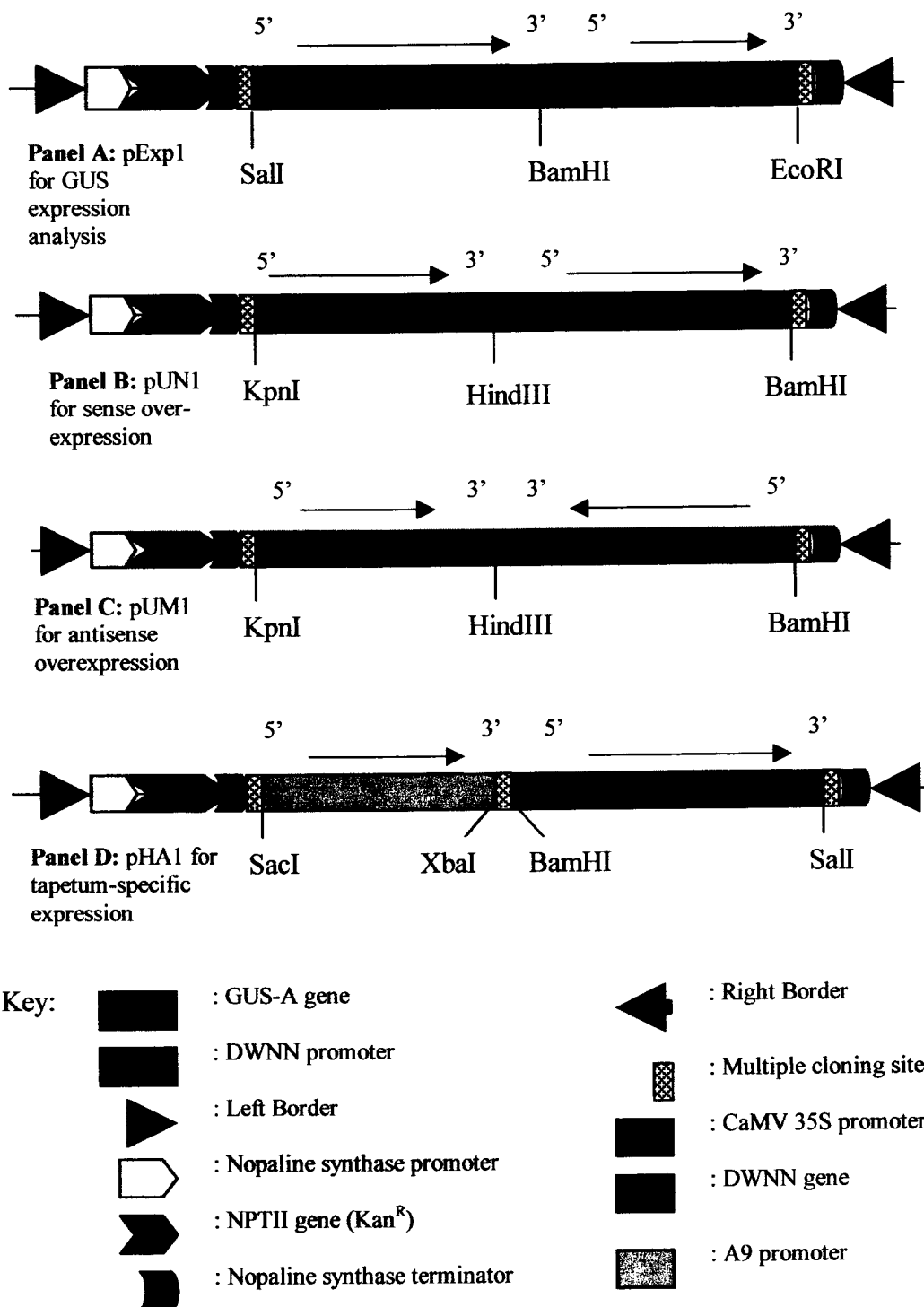
■ : DWNN gene

▦ : A9 promoter

Figure 3.11. Constructs for the partial characterisation of *DWNN*. The T-DNA (integrated into the plant genome) is between the left and right border.

Preliminary results from phenotypic analysis of tobacco plants generated from tobacco shoots either transformed (*DWNN* over-expressors) or untransformed (control plants) with pUN1 (Figure 3.11, panel B) show that *DWNN* over-expressors have severely stunted growth, defective development and appear bleached. Control plants look healthy, grow and develop well. These differences (described in Table 3.1) occur even though the growth conditions [25°C at a light intensity of approximately 200 μmol/m^2/s, in Murashige and Skoog medium (from Sigma-Aldrich, Virna Valley, South Africa) supplemented with 30 g of sucrose/L of medium at pH 5.8, with a light/dark cycle of 16 hours light and 8 hours dark] were the same for both the *DWNN* over-expressors and control plants. Both shoots and plants shown (Figure 3.12) were 6 weeks old when scored for the phenotypic observations.

Phenotype	Wild type	Over-expressor
Mean leaf diameter	15 mm, n=15	5 mm, n=15
Mean shoot length	60 mm, n=5	10 mm, n=5
Mean leaf length	30 mm, n=15	10 mm, n=15
Mean Root length	40 mm, n=15	No roots, n=15

Table 3.1. Comparison between DWNN over-expressors and control plants. The mean was calculated according to the formula mm/n, where mm represents sizes and n is the number of organs (leaf, shoot or root) counted.

It may be possible that over-expression of *DWNN* over a prolonged period of time confers a lethal phenotype in tobacco plants that express the *DWNN* gene under the regulation of the strong constitutive double CaMV 35S promoter.

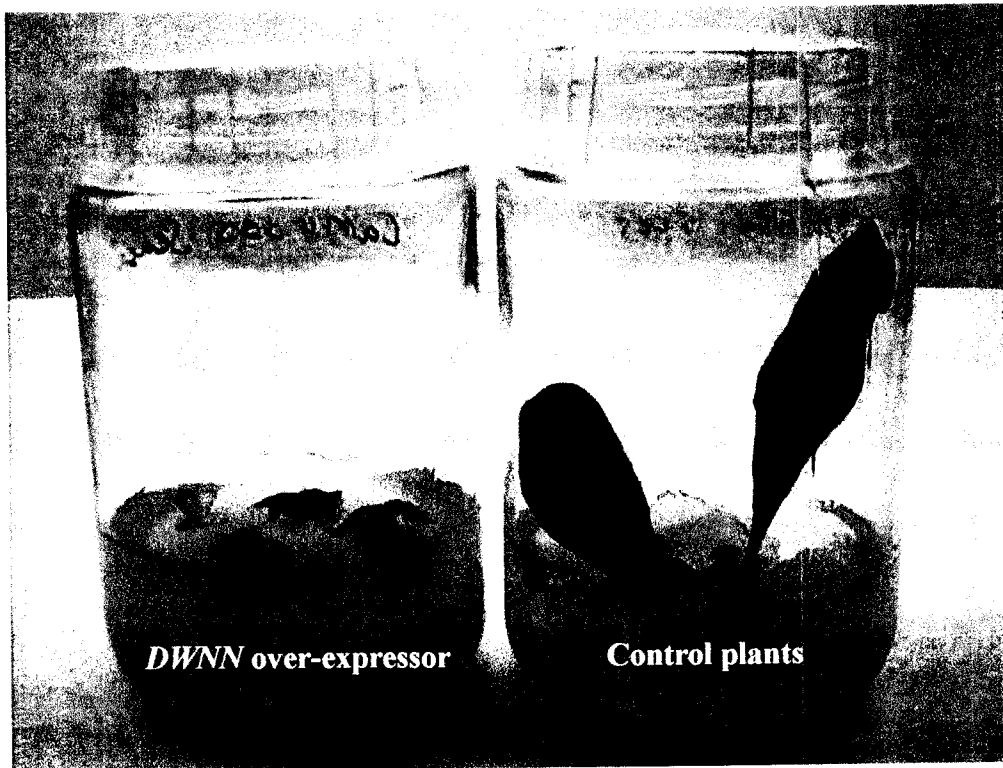Figure 3.12. Comparison of tobacco plants transformed with pUN1 (*DWNN* over-expressors) with plants that were untransformed (control plants).

DWNN over-expressors had not developed any visible root system. Furthermore, the leaves look significantly etiolated possibly pointing to a defect in pigment synthesis or processing. Growth and development of the control plants is as expected under the experimental conditions.

Identification of a *DWNN Ds* transposon insertion knockout mutant

A seed line of *A. thaliana* (SGT3743) in which the *DWNN* gene was knocked out by the insertion of the *DS* transposon was identified (Parinov *et al.*, 1999) and obtained from the Nottingham Arabidopsis Stock Centre. The seeds from this knockout mutant line were grown under standard tissue culture conditions and the phenotype of the resulting plants was compared to that of wild type plants of the same ecotype grown at the same time. As shown in Figure 3.13, the knockout mutant shows a slightly faster growth rate than the wild type plant. The knockout mutant plants are taller, the leaves are bigger/broader and their roots are longer as compared to the wild type plants. These differences (shown in Table 3.2) occur even though the ecotypes used were identical, the time of growth was the same and the growth conditions [25°C at a light intensity of approximately 200 $\mu mol/m^2/s$, in Murashige and Skoog medium supplemented with 30 g of sucrose/L of medium at pH 5.8, with a light/dark cycle of 16 hours light and 8 hours dark] were the same for both the knockout mutant and the wild type plants. To synchronise seed germination, seeds from both the wild type and knock-out plants were placed at the same time in the dark at 4°C for four days, then placed under the same growth conditions at the same time afterwards. The phenotypic characteristics of the plants were scored four weeks after the seed germinating.

Phenotype	Wild type	Knock-out
Mean leaf diameter	6 mm, n=25	10 mm, n=25
Mean shoot length	90 mm, n=25	115 mm, n=25
Mean leaf length	10 mm, n=25	17 mm, n=25
Mean Root length	20 mm, n=15	30 mm, n=15

Table 3.2. Comparison of wild type plants to knock-out plants after 4 weeks of growth. Both wild type and knock-out plants were grown under the same conditions [25°C at a light intensity of approximately 200 $\mu mol/m^2/s$, in Murashige and Skoog medium supplemented with 30 g of sucrose/L of medium at pH 5.8 (MS30), with a light/dark cycle of 16 hours light and 8 hours dark]. The mean was calculated according the formula mm/n, where mm represents size and n is the number of organs (leaf, shoot or root) counted.
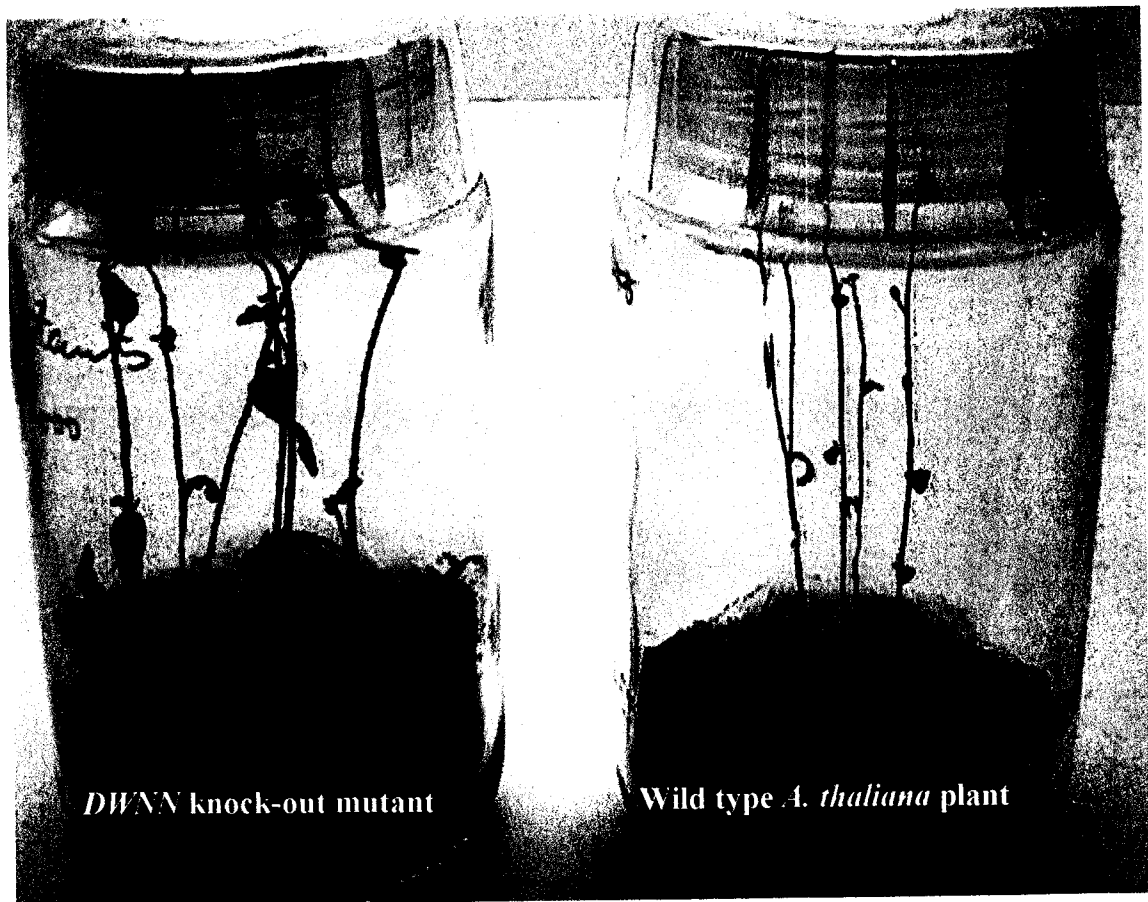
Figure 3.13. Comparison of the growth rate of *DS DWNN* knock-out mutants to

wild type plants. The plants were grown under the same conditions [25°C at a

light intensity of approximately 200 μmol/m^2/s, in Murashige and Skoog

medium supplemented with 30 g of sucrose/L of medium at pH 5.8 (MS30),

with a light/dark cycle of 16 hours light and 8 hours dark] for the same length

of time. The plants used were of the same ecotype and the ones shown here are

4 weeks old.

Roots and leaves were cut from *Arabidopsis thaliana* and tobacco plants transformed with pExp1 (see panel A of Figure 3.11) and left on MS30 [Murashige and Skoog medium from Sigma-Aldrich South Africa (Pty) Ltd, Virna Valley, South Africa, supplemented with 30 g of sucrose/L of medium at pH 5.8] for 15 minutes and then used for GUS activity assays. GUS activity (visualised by blue staining of plant tissue) was detected in the xylem tissue of roots and in leaves surrounding the wounded tissue but not in root hairs or any other tissue in the roots. GUS staining results for the roots and the leaves are shown in Figure 3.14 to 3.16 below.

Figure 3.14. Light microscopic visualisation of GUS activity in the xylem of an *Arabidopsis thaliana* root (magnification: 10x). The arrow points the diagnostic secondary lignification in the xylem; the single bracket delineates GUS staining in the stele.

GUS activity occurs only in the xylem in unstressed plants but not in other types of tissue. GUS expression under the regulation of the DWNN promoter in the xylem indicates that DWNN may be expressed during xylogenesis, most likely in xylem cells undergoing cell death.

Figure 3.15. Detail of GUS staining in the stele (magnification: 40x). The arrow points to the secondary lignification in the xylem.

Xylem tissue undergoes secondary lignification during xylogenesis and a majority of cells at this stage are undergoing cell death. The fact that DWNN promoter-driven GUS activity is observed in secondary lignification suggests that DWNN expression may occur in dying cells during this process. DWNN promoter-driven GUS expression occurs in wounded leaf tissue as shown in Figure 3.16.
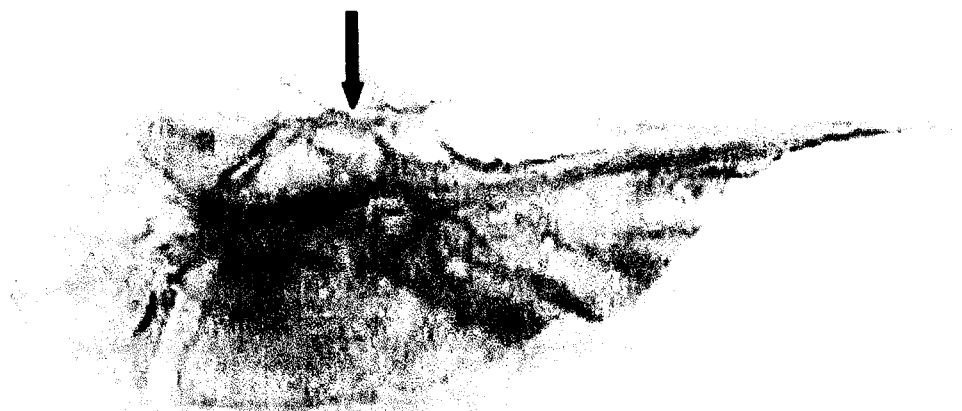
Figure 3.16. GUS activity in a whole leaf. GUS activity is enhanced in leaf tissue surrounding the wound. The arrow points to a mechanically induced lesion.

Wounding is a stress stimulus that triggers cell death and cell wall lignification in tissue surrounding the wounded area. The fact that the DWNN promoter drives GUS expression in leaf tissue only around the area of wounding suggests that DWNN is expressed in leaves only in response to stress, with wounding here as the demonstrated example of such a stress stimulus.

4. DISCUSSION

DWNN: Identification of a putative novel gene

This study has identified a putative novel gene encoding DWNN. The gene is located on chromosome 4 of *Arabidopsis thaliana*; sequence analyses predict a length c ⁏1878 base pair organised in seven exons and six introns. *DWNN* encodes a predicted protein of 274 amino acid residues. Analysis of the conceived DWNN protein sequence shows that it has significant sequence similarity to proteins encoded by homologous putative genes from a diverse range of species, including *Saccharomyces sereviceae*, *Schizosaccharomyces pombe*, *Caenorabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. In addition, another DWNN-like protein, referred to as putative DNA binding protein, is found on chromosome 5. The most conserved domain in all the DWNN-related proteins is a CCHC type zinc finger. This zinc finger domain occurs at positions 201 to 218 in the *A. thaliana* DWNN protein. Zinc fingers are diagnostic for nucleotide binding proteins. Specifically, the 18 residues CCHC zinc finger domain is often found associated with the nucleocapsid protein of retroviridae. It is required for viral genome packaging as well as the early infection process (Katz and Jentoft, 1989). It is also found in eukaryotic proteins involved in RNA binding or binding of single stranded DNA (Urbaneja *et al.*, 1999); CCHC zinc fingers are also found in the retroviral GAG nucleocapsid proteins (Katz and Jentoft, 1989). CCHC zinc finger domains

occur in human CNBP proteins involved in sterol-mediated repression (Ayala-Torres *et al.*, 1994; Rajavashisth *et al.*, 1989). It is known that CNBP proteins bind the 5' untranslated region of ribosomal protein mRNA, a region critical in translational regulation (Pellizzoni *et al.*, 1997; Pellizzoni *et al.*, 1998; Calcaterra *et al.*, 1999). The CCHC type of zinc finger is also present in the yeast BYR3 protein, a CNBP homologue in yeast, and thought to function in the mating pheromone recognition pathway and/or sexual differentiation pathways (Cullen *et al.*, 2000; Balciunas and Ronne, 1999; Kanoh *et al.*, 1995; Xu *et al.*, 1992). The *Caenorhabditis elegans* GLH1 protein, a putative ATP-binding RNA helicase also contains the CCHC type zinc finger motif. Furthermore, this type of zinc finger has also been identified in the *Leishmania major* hexamer-binding protein (HEXP), affinity isolated with a labelled DNA oligonucleotide from the 5'untranslated region of the GP63 gene (Weise *et al.*, 2000; Grandgenett *et al.*, 2000; Alvarez-Valin *et al.*, 2000). The consensus pattern of CCHC zinc finger proteins is C-X2-C-X4-H-X4-C. It can therefore be expected that DWNN-like proteins might be involved in nucleic acid or nucleotide binding. Such activities can potentially be associated with a number of different and possibly regulatory functions.

A role for DWNN in development, stress response and cell death

Analysis of GUS expression under the regulation of the *DWNN* promoter has shown that this promoter drives specific expression in cells associated with the stele thus including the xylem. This may signify that the DWNN gene is

expressed in association with programmed cell death that is part of the cellular differentiation from living meristem to dead but structurally functional mature xylem tissue. It is also interesting to note that there is a significantly stronger GUS signal in the tissue surrounding the experimentally induced leaf wounding. In contrast, unwounded leaf tissue has shown only background or no GUS staining at all. Mechanical wounding can trigger cell death and it is conceivable that dying cells around the wounded leaf tissue express *DWNN*. An alternative explanation for the expression pattern may be that the gene up-regulates the expression of a defence response that would include e.g. strengthening of cells in the affected tissue by the deposition of lignins. Secondary lignification again is common to both wound response and the development of mature xylem. The possibility that the GUS enzyme substrate used (X-GlcA) may have had better access to the conductive tissue than other tissues and therefore resulting in more GUS activity in the region of the conductive tissue has been ruled out since no GUS staining occurs in root hairs and epidermal cells which have direct access to the substrate. These results suggest that there is a direct or indirect correlation between *DWNN* expression and cell death as it occurs during tissue differentiation and stress responses. Further investigation to characterise the mode of action of DWNN in these processes is in progress.

The data obtained from the *DWNN* knockout mutants suggest that the absence *DWNN* enhances the growth rate of the knockout mutants. This results in taller plants and substantially larger leaf areas. The finding may indicate, that in the

absence of the functional wild type DWNN protein, accelerated cell proliferation possibly as a result of the suppression of developmentally regulated cell death. If this hypothesis proves correct, we expect the development of the xylem to be impaired or at least retarded in *DWNN* knockouts. A further reason for accelerated growth of the knockout mutant might be that cell cycle control is dependent on DWNN expression. We are currently planning experiments to further analyse the phenotype of the knockout mutant. In addition, we plan to transform the mutant with a functional copy of the gene to test if we can revert to the wild type phenotype.

The hypothesised role of *DWNN* is further strengthened by results obtained from plants transformed with a CaMV 35S promoter construct that drives over-expression of *DWNN*. The *DWNN* over-expressor phenotype shows severely stunted growth, abortive development and strongly impaired pigment synthesis. This suggests that over-expression of *DWNN* in these plants induces or promotes a cell death programme that is leading to extremely stunted growth and eventually results in a lethal phenotype. This phenotype is again consistent with a role for *DWNN* in cell death and/or cell cycle control.

The tapetum is one of the tissues where programmed cell death plays a key part in the developmental pathway. Tapetum tissue is the locus where pollen formation takes place; subsequent to this developmental process the tapetum undergoes programmed cell death. We have therefore used the A9 promoter (Paul *et al.*, 1992), a tapetum specific promoter, to drive *DWNN* expression. If *DWNN* promotes cell death, it would then be expected that expression of

DWNN in the tapetum will promote early tapetal cell death and possibly prevent the formation of viable and fertile pollen in plants expressing *DWNN* under the A9 promoter specifically in the tapetum. In the results reported here, no demonstrable phenotype in plants transformed to express *DWNN* can be detected. This indicates that the promoter acts tissue specifically as predicted and does not drive generalised expression. In addition, no phenotype was observed in the reproductive tissue to date and the first generation has set seeds. It remains to be tested whether or not the seeds are fertile. There is the possibility that for an effect, *DWNN* in the tapetum requires that the plants should be homozygous for the A9 promoter-*DWNN* construct. It will therefore be interesting to examine fertility in a homozygous A9 promoter-*DWNN* population. This requires back-crossing of plants transformed with this construct.

Transcript analyses and revised gene structure

To date, attempts to detect a *DWNN* transcript by RT-PCR in wild type *Arabidopsis thaliana* have not been successful. This was surprising for two reasons. Firstly, RT-PCR is a sensitive method that should detect even low amounts of transcripts. The transcript levels, especially in wounded tissue could not have been a restricting factor. Secondly, appropriate positive and negative controls have worked consistently (data not shown). Since the GUS assays have indicated that the 5' prime end of the gene is correctly annotated it was speculated that the 3' end of the (hypothetical) gene may have been predicted

wrongly by the annotating algorithm. Consequently, the presumed stop codon might in fact be located in an intron. This in turn would then suggest that one of the primers (designed to lie immediately up-stream of the putative stop codon) would in fact represent an intronic sequence rather than a transcript. The result is the absence of RT-PCR products.

This hypothesis is now being tested. The first step was a re-assessment of recent sequence data from the *Arabidopsis* and rice genome sequencing projects obtained during preparation of this thesis. It confirms that part of the 3' end of the fragment predicted to is within the last exon at the 3' end of *DWNN* (exon 7) may actually be within an intron. This is essentially based on the observation that a predicted putative gene found in chromosome 5 of *Arabidopsis thaliana* encodes a protein with a high degree of similarity to *DWNN* (approximately 70%). However, this gene is longer than *DWNN* discussed in this paper. At the time when sequence analysis of the DWNN protein (accession number CAB10521.1 and Genbank Identity number GI: 2245099) was done, genomic sequence data for chromosome 5 was incomplete and it appeared that the chromosome 5 gene had the same structure as the one predicted on chromosome 4. The additional sequence data now available for chromosome 5 shows that the protein (which is annotated as putative DNA binding protein, accession number BAB11612.1 and Genbank Identity number GI: 10178219) encoded by the putative chromosome 5 gene (homologous to the DWNN protein) has significant sequence similarity (approximately 67%) to a region further downstream of what was predicted to be the C terminal of the DWNN

protein described here. According to the annotation in Genbank at the time of preparation of this manuscript, the region downstream of the C terminal of the DWNN protein is a putative protein (which will be referred to as DNA binding protein 5-like, accession number CAB10522.1 and Genbank Identity number GI:2245100) encoded by a separate gene distinct from *DWNN*. The fact that the DNA binding protein encoded by the chromosome 5 gene has sequence similarity to both the entire predicted DWNN protein and the DNA binding protein suggests that *DWNN* and the "downstream sequence" constitute a single gene. If this proves the case, it implies that the 3' end fragment of the previously predicted last exon (exon 7) is indeed within an intron and thus spliced out and consequently absent from the mRNA.

This might also explain why there was no visible phenotype from plants transformed with the antisense construct and will be tested with modified constructs once the gene structure has been fully verified. However, plants transformed with the sense construct over-expressing the previously predicted DWNN protein do present a phenotype typical of the expected result from over-expression of *DWNN*. This could mean that the previously predicted DWNN protein is a fragment essential and sufficient to confer the observed phenotype. Further experiments will show if the extended sense construct will yield yet a different, (and possibly lethal) phenotype. Figure 4.1 gives a schematic comparison of the proteins referred to above to illustrate the possibility of *DWNN* being longer than predicted earlier.

——————————————[▭]—— Previously predicted DWNN as currently annotated in Genbank

Panel A

——————————————[▭]—— Previously predicted DNA binding protein before publication of
more sequence data for chromosome 5

Panel B

——————————————[▭]—[∷]————[⬚]———— Predicted DNA binding protein as
currently annotated in Genbank

Panel C

——————————————[▭]—[∷]————[⬚]———— Predicted new structure of DWNN

Panel D

Key: [▭]   CCHC Zinc Finger

[∷]   Ring Finger (C3HC4 Zinc Finger) domain
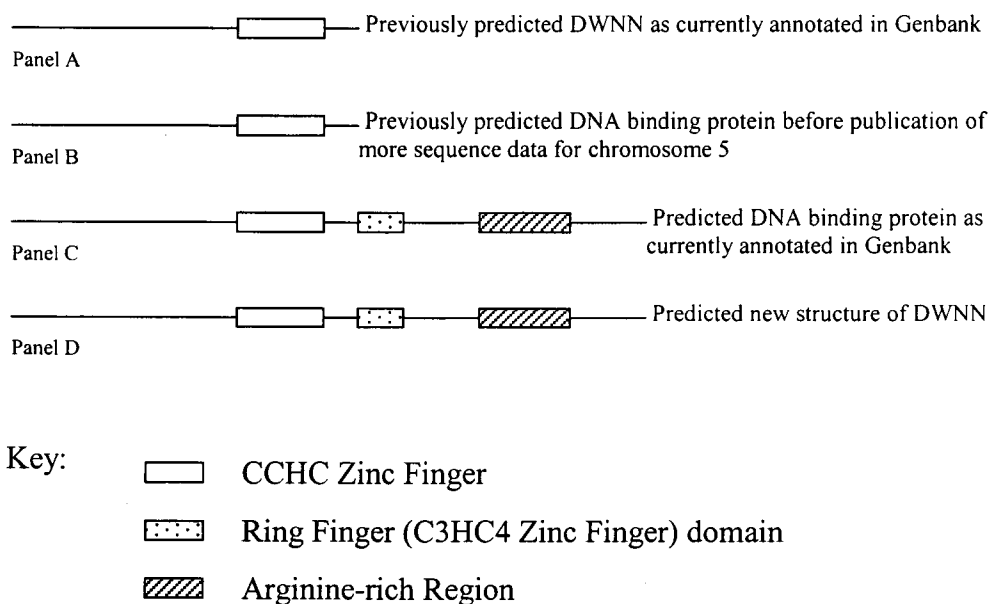
[⬚]   Arginine-rich Region

Figure 4.1. Illustration of the predicted structures of DWNN and the DNA binding protein. The annotated domains were predicted based on similarity to motifs found in the indicated domains as described in the section on sequence analysis.

The protein structure in Panel A of Figure 4.1 represent the 274 amino acids that make up the previously predicted DWNN protein, which was based on the annotation of the gene as it is presented in Genbank. This structure also represents the previously predicted protein structure formed by the amino acids in the DNA binding protein in chromosome 5 (Panel B) (similar to the previously predicted DWNN protein) before further sequence data was published for this chromosome. The further and additional sequence from chromosome 5 published recently showed that the DNA binding protein is longer than previously predicted and this is shown in Panel C.

Phylogenetic analyses

A phylogenetic tree was generated using the ClustalX protein alignment program (see Figure 4.1). The program predicts a relationship between the DWNN-like proteins from the different species containing members of this family of proteins. From the tree it can be concluded that DWNN-like proteins show a degree of homology that reflects conceived evolutionary relatedness of the species.
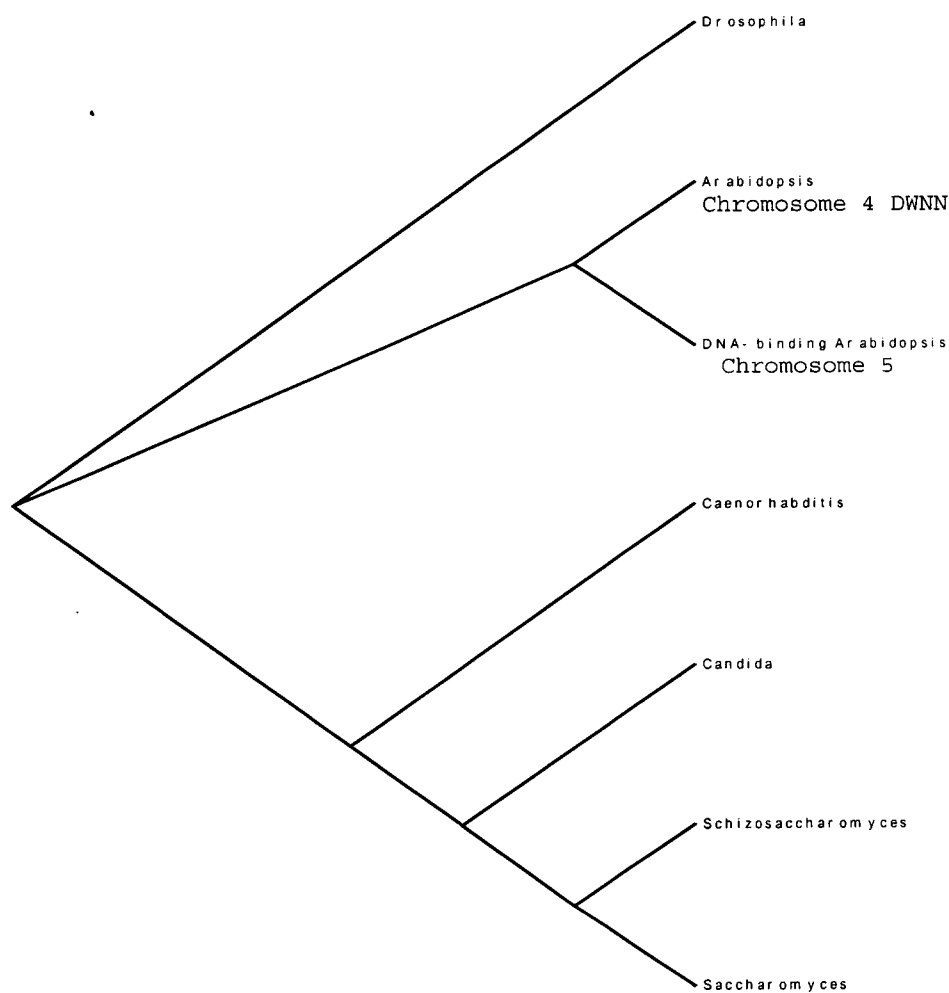

Figure 4.2. Phylogenetic tree of some of the DWNN-like proteins.

5. Summary and outlook

The data presented suggest the presence of a novel expressed gene in *Arabidopsis thaliana*. The gene shows an expression pattern that is compatible with a role in development as well as stress responses. A common link between these two types of responses is the changes in cellular protein turnover and programmed cell death. Further studies will characterise the molecular and developmental mechanisms involved in the DWNN-dependent processes.

Cell death during the hypersensitive response is important in the defence response of plants to pathogens. Further experiments are underway to study the effect of expression or inhibition of expression of *DWNN* in *A. thaliana* responses to stress and pathogen induced cell death. This will establish the link between *DWNN* and cell death. In these experiments, the role of *DWNN* in defence responses will be determined by a comparison of the defence responses against pathogens and downstream stress-related stimuli such as H_2O_2, between plants that express *DWNN* and those that do not (*DWNN* knockouts). Taken together, the results obtained so far suggest that *DWNN* plays a role in protein metabolism and specifically protein degradation. Interestingly, such a role is entirely compatible with modelled structural predictions of DWNN domains (Dr D. Pugh, University of the Western Cape; personal communication). These predictions indicate structural similarities of DWNN domains with ubiquitin, which is a major component in the regulation and protein turnover.

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