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THE ISOLATION AND CHARACTERIZATION OF A
SENESCENCE ASSOCIATED NODULIN cDNA

by

Christina Chi-Yat Chan

Thesis submitted to
the School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the M.Sc.
degree in Biology

Ottawa-Carleton Institute of Biology



Christina C.Y. Chan, Ottawa, Canada, 1995



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ABSTRACT

The goal of this thesis is to study cellular senescence with the use of the determinate nodule of soybean as the model system. Cellular senescence involves orchestrated processes that culminate in disintegration and death. The study of the molecular mechanisms of cellular senescence may aid our understanding of the systems necessary for cell maintenance under healthy and senescent conditions. The determinate nodule is an appropriate model system for the study of senescence due to the homogeneity of infected cells of various developmental stages within the organ.

In this model system consisting of *Glycine max* (L.) Merrill, Maple Arrow, infected with *Bradyrhizobium japonicum* strain 61A76, we showed that nodule senescence occurs late in nodulation, at the time of flowering and pod filling. We also demonstrated that nodule senescence can be induced with the addition of 20mM ammonium nitrate. The detection of a senescence associated process that is present in both the natural and senescent systems will aid in defining a universal mechanism.

The strategy to study the molecular mechanisms of nodule senescence involves the isolation of senescence-associated nodulin (SAN) cDNAs by the differential screening of a cDNA library representing mRNA from senescent nodules. This library constructed in the λ gt11 vector is screened with two bulk probes made from the PCR amplification of the cDNA

inserts of libraries in the λ gt10 vector: one representing mRNA of healthy nodules that are actively fixing nitrogen and the other of senescent nodules. In this thesis, one SAN cDNA, clone 8-4 was characterized through a Northern analysis to demonstrate the up-regulation of the corresponding gene with senescence. In the natural senescent system, SAN 8-4 expression was demonstrated to increase with nodule senescence. In the induced system, the up-regulation of SAN 8-4 was not as dramatic as seen with the natural system. Southern analysis suggests that this gene belongs to a multigene family. A search through nucleotide and protein sequence banks demonstrates that SAN 8-4 may be a novel F3H related protein.

Through this correlative study, it is proposed that this novel F3H related protein may be involved in the biosynthesis of a flavonoid produced in the soybean nodule for signalling processes associated with nodule senescence.

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CHAPTER ONE:

INTRODUCTION

1.1 What is Cellular Senescence?

In multicellular organisms, a biological mechanism has evolved whereby specific cells, tissues, and organs are programmed to degenerate. It is based on a series of biochemical and physiological events that comprise the final phase of development. Cellular senescence is the ordered series of events leading to the activation and expression of genes giving rise to new RNA and enzymes, culminating in degeneration and finally, death of the cell (Nooden, 1988). It has been postulated that cellular senescence is not caused by effects contributed by translational control or by the deterioration of the ribosomal system (Speirs and Brady, 1981), but rather by an internal program.

Cellular senescence is a fundamental process in plant development which operates at many stages and at many levels during the life cycle. Knowledge of the biological processes involved may provide clues to the maintenance of cellular viability under healthy and senescent conditions.

Thus far, the physiology of senescence has been studied in plants and in animals although the concept of senescence for each system was derived independently. Little is known at the molecular level except that the process starts with

the synthesis of new mRNAs giving rise to new proteins (Nooden, 1988).

This section will deal with the recent advances in the area of cellular senescence in plant systems. The emphasis will be placed on the molecular aspects of this phenomenon and the implications of this research to evolutionary biology and cellular biochemistry.

1.1.1 The Concept

The phenomenon of plant senescence was first described in *Science* (Leopold, 1961), where the concept of a "driving force" causing senescence was introduced. Only now, is the emphasis being placed on discovering factors which cause cellular senescence. Research has been focused on the mechanisms involved in the senescence syndrome. The idea that such processes are controlled by endogenous factors is gaining support.

Cellular senescence occurs at many stages and at many levels during the development of cells, tissues and organs. In fact, cellular senescence is thought to be the fate of differentiated cells (Woolhouse, 1967). If senescence and differentiation are aspects of the same process, groups of genes which are repressed during development may become active during senescence, disrupting the normal function of the cell which leads to death.

Cellular senescence, also known by many other names

believed to have evolved in organisms for the purpose of nutrient reallocation to healthy, more metabolically demanding parts of the organism (Peoples and Dalling, 1988). The viability of the organism is dependent on the normal function of its cells. Thus, a proposed role for this phenomenon is the elimination of damaged or of abnormally functioning cells that may harm the entire organism (Umansky, 1981).

Senescence is one of three phenomena leading to cell death: 1) senescence or programmed cell death is endogenously controlled with the induction of gene activity; 2) necrosis is due to massive trauma such as, wounding or damage caused by pathogenic attack; and, 3) aging or chronic degeneration is caused by the accumulation of sub-lethal damage with time (Nooden, 1988).

The common characteristic of all three processes is the eventual loss of the cell's ability to maintain homeostasis. The cell's viability is dependent on the integrity of the plasma membrane. It is essential for keeping the cell's entropy below that of its environment. This requires a constant input of energy and once it is disrupted the cell dies (Penning de Vries, 1975).

Characteristic only to cellular senescence is the activation of genes giving rise to new mRNA and proteins.

activation of genes giving rise to new mRNA and proteins. Advances in molecular techniques, allow us to address the questions concerning the genetic program that are central to the processes of cellular senescence.

1.1.2 Basic Components

The challenge in deciphering the central processes of cellular senescence is in defining its basic components. Carefully orchestrated processes involved in the dismantling of some but not all cellular structures have been identified. These include hormonal effects, loss of membrane integrity, degradation of macromolecules such as DNA, RNA and proteins, and in some cases, the degeneration of plastids, such as the chloroplast. Eventually, senescence would involve the metabolism of the entire cell; thus, it is important to identify the processes central to cellular senescence and to look at the universality of these processes amongst living systems.

Studies have indicated that senescence, a pervasive event in cell, tissue and organ development of multicellular organisms is initiated by specific gene activity and synthesis of new mRNA and proteins (Thomas and Stoddart, 1980; Nooden, 1988). It seems that senescence is a ubiquitous process, though, it is not clear whether the central processes in all senescing cells are the same. Genetic control and the synthesis of mRNA and proteins

leading to the senescence syndrome will be discussed in the following section.

1.1.2.1 Genetic Program

Studies have shown that cellular senescence is a well orchestrated sequence of events. This implies the involvement of an active genetic program (Brady, 1988). Genetic mutations that limit the progress of senescence support this theory and will be discussed in a later section. Research is now focused on defining specific gene activity which seems to be central to cellular senescence. It is generally accepted that senescence, like other developmental processes is regulated by differential gene expression including the activation of new genes (Stoddart and Thomas, 1982; Nooden, 1988). Although cellular senescence has been studied in many plant systems, the isolation and characterization of specific genes that are induced at senescence is still at its infancy. These systems will be discussed in greater detail in the proceeding section.

1.1.2.2 Protein and Nucleic Acid Synthesis

Cellular senescence begins with changes in gene expression and protein synthesis. The phenomenon most relevant to this thesis is the differential expression of genes giving rise to messenger RNAs specific to the

initiation of cellular senescence. The identification of mRNAs specific to developmental stages may provide clues to the function of these molecules. RNA is only one group of macromolecules vital to cellular senescence but the RNA species present and at what developmental stage they exist may reveal the regulation of this phenomenon. Is the control of cellular senescence at the level of transcription, translation, RNA or protein turnover? The RNA species that appear or disappear immediately before senescence are of most interest since the product of these templates are prime candidates as regulators or catalysts in the process.

In examining several developmental stages in leaf tissue, *in vitro* translation of existing polysomes revealed that ribulose-bis phosphate carboxylase (Rubisco) protein content diminished with age (Speirs and Brady, 1981). The polypeptides which declined with senescence were the small and large subunits of Rubisco. *In vivo* labelling showed that in mature leaves, Rubisco had turned over as rapidly as the average protein. Into early senescence of rice leaves, the net content of Rubisco was shown to decline but the turnover persisted (Makino et al., 1984). Northern analyses determined that the decline of this protein directly correlates with the decline of the corresponding mRNA. The changing pattern of protein synthesis during development directly correlates to the mRNA population. This study

disproves the causal effects contributed by translational control or by the deterioration of ribosomal system. In the same study, and in another study of natural leaf senescence, new species of mRNA were found (Malik, 1987). Thus the senescence syndrome is not brought on randomly by the cell's inability to maintain homeostasis, but is an active, temporal process requiring energy and an endogenous program.

1.1.3 Importance in Plant Developmental Processes

Senescence operates at many stages and at many levels during the life cycle of an organism. In plants, senescence may play an active role during cell differentiation of conducting cells in xylem tissue or the development of lobing pattern in leaves, fruit ripening and the breakdown of specialized cells in the embryo and the female gametophyte, or during the orderly breakdown of the chloroplast in senescent leaves (Nooden, 1988). Then, there is post-reproductive senescence of the whole organism known as monocarpic senescence, which occurs, for example in, soybeans.

The most widely accepted theory for the evolution of senescence is the necessity of this process in the redistribution of nutrients. In nature, there may be a selective advantage for those organisms with the ability to reclaim scarce, energetically expensive nutrients (Nooden,

1988). These macromolecules include sugars, phosphates and amino acids. There is movement of amino acids from stamens and petals to the developing ovary, from the pod wall to seeds, from foliage to growing fruits, and from old leaves to the new. It is not surprising then that the phloem is the last tissue to senesce in the whole plant since this organ is necessary for the transport of macromolecules between organs.

To the whole plant, senescence may have important adaptive functions to cope with stress and environmental changes. Stress of various types can induce or hasten symptoms of senescence. Environmental stresses such as severe chilling, heat, drought, etc. trigger senescence-like changes. Senescence can be induced artificially by dark induction or excision of leaves (Becker and Apel, 1993; Stoddart and Thomas, 1980) and by the addition of fixed nitrogen to nodules (Sutton, 1983). These stresses and environmental changes may not have any bearing on natural senescence; that is, induction of different genes may be involved in various senescence related processes depending on the source of stress. Differences in gene expression between natural and artificially induced leaf senescence has demonstrated that only a minor fraction of the mRNA changes observed during dark incubation of detached leaves is connected with leaf senescence, whereas stress-related transcripts appear to predominate quantitatively (Becker and

Apel, 1993).

1.2 Model Systems for the Study of Cellular Senescence

Biochemical and physiological pathways in healthy cells can be better understood by studying mechanisms in senescing systems and the disassembly of their components. At the level of the organism as a whole, such a study can aid in the understanding of the organism's focus of its resources at particular sites and mobilizing centres.

To understand cellular senescence, its induction and control in a tissue of interest must be addressed. In order to study a cellular process, molecular biologists are concerned with the isolation of senescence markers; that is, we are focusing our resources on finding particular cellular components that play a major role in the induction of senescence. Subsequently, such markers can be used to indicate the timing of development in the test model.

The following section will provide an overview of the research being conducted in the area of cellular senescence. Presently, various models are being used to study this phenomenon. The evidence presented will support the principle of evolutionary conservation of a well-orchestrated pathway for senescence amongst the species.

1.2.1 Animal Models

Active cell death (ACD) is the fashionable term for cellular senescence in animal models. ACD in animals can be

demonstrated in the cells found in the tadpole tail induced to die by thyroid hormone secreted by the thyroid gland at metamorphosis (reviewed in Raff, 1992), regression of prostate tissue in rats after castration (Leger et al., 1987) and many others. Although the evidence for ACD is indirect, an increase in specific mRNA has been shown to precede cell death in several systems (Montpetit et al., 1986). The contribution of the protein products of these mRNAs to ACD has yet to be demonstrated.

Cell death can sometimes be suppressed by the inhibition of RNA or protein synthesis in the cells that should die. This would suggest that ACD is controlled at the transcriptional and/or translational levels during development. The most direct evidence that cell death in animals is activated by an endogenous program can be found in *Caenorhabditis elegans* (Ellis and Horvitz, 1986). Mutations in either genes, *ced-3* and *ced-4* cause cells that would normally die, to survive instead. Another *C. elegans* gene, *ced-9*, when abnormally activated through a mutation, postpones or eliminates ACD (Hengartner et al., 1992). If its function is inactivated by mutation, many cells that normally survive now die and the animal dies early in development. Such studies are conducted in *C. elegans* as a model to examine mechanisms that specify cell fate.

The following section describes a study that was used as a precedent for this thesis.

1.2.1.1 Testosterone Repressed Prostate Message-2

A novel mRNA produced during the cellular regression induced in rat prostate following androgen removal has been isolated and characterized (Leger et al., 1987). Testosterone repressed prostate message-2 (TRPM-2) is the most widely used marker for active cell death (Tenniswood et al., 1992). The gene or the protein product has been isolated from a wide variety of cells and has been given many names. These include, rat Sertoli cells as SGP-2 (Griswold et al., 1986), or from ram rete testes fluid as clusterin (Blaschuk et al., 1983) or from human serum as apolipoprotein J (de Silva et al., 1990) and many others.

Not only is this gene expressed during the regression of rat ventral prostate after castration, but it is also expressed in regressing mammary gland after weaning (Guenette et al., 1992). A homolog of the gene is found in *Manduca sexta* (tobacco hornworm) and its expression is found in regressing intersegmental muscles after cessation of ecdysterone production (Schwartz et al., 1990).

A function for this protein has been proposed (Tenniswood et al., 1992). In some systems, TRPM-2 may protect senescent cells from the immune system. Because ACD requires extensive membrane remodelling, the process must occur without the onset of the immune response. TRPM-2 may protect senescent cells and neighbouring cells from complement activity which may induce inflammation and

compromise physiological function.

TRPM-2 is constitutively expressed in certain prostate cancer cell lines. If TRPM-2 protects surviving cells from immune surveillance during membrane remodelling, hormone therapy for certain cancers must be designed to eliminate the presence of incomplete programmed cell death. For instance, such cancerous cells could spread and not be recognized by the immune system (Tenniswood et al., 1992). In this case, the study of TRPM-2 and cell death processes may have a ready application in cancer treatment.

The induction of TRPM-2 in a number of systems undergoing ACD, suggests the universal importance in the role of this protein in senescence processes.

1.2.2 Human Fibroblast Tissue Culture Model

The fibroblast is a spindle shaped cell capable of sustained proliferation in culture (Norwood and Smith, 1985). Unlike immortalized cell lines, normal human fibroblasts in culture undergo replicative senescence in which the number of population doublings is limited. Because fibroblasts possess a finite growth potential, this system is commonly used for the study of cellular senescence *in vitro*.

Senescent cells express a dominant inhibitor of cell proliferation. Fusions of normal human fibroblast cells with various immortal cell lines generated cells with a

limited proliferative capacity (Pereira-Smith et al., 1990); thus, the limited-division phenotype is a dominant characteristic. A protein in senescent cells is capable of suppressing DNA synthesis in the younger dividing nucleus (Lumpkin et al., 1986). If replicative senescence is genetically controlled by growth inhibitors, it may be possible to identify genes that are up-regulated during senescence. Besides serving as markers, these genes may serve as targets for regulation or may themselves be regulatory.

Recently, a novel marker of cellular senescence has been isolated from human fibroblast in culture (Wistrom and Villeponteau, 1992). The expression of *SAG* (senescence associated gene) is up-regulated in senescent cells and is unaffected by the cell cycle. It is expressed in various cell lines during senescence and the sequence of this gene is evolutionarily conserved. Its expression is repressed in immortal cell lines which implies that there is a correlation between this gene's expression and the fibroblast cell's ability to senesce. The possible functions of *SAG* are unknown, but the C-terminal region of the *SAG* protein contains a potential DNA-binding domain with sequence homology to the basic domains found in the muscle-determining factor MyoD1. The sequence data and the up-regulation of *SAG* during senescence suggest that *SAG* may be a regulatory protein.

Although cellular senescence involves the fluctuation in the amounts of various mRNA species, this study suggests that up-regulated genes are of greater relevance to cellular senescence. The senescent phenotype is possibly a dominant trait (Pereira-Smith et al., 1990). The isolation of SAG is additional evidence that senescence is under dominant genetic control. Like SAG, the expression of senescence associated genes isolated in other organisms may also be used as markers for cellular senescence.

1.2.3 Plant Models

Studies of cellular senescence in plant and animal models developed independently. Most studies on plant senescence have focused on particular organs such as leaf, flower, and fruit, rather than processes. This may be due to the difficulty in obtaining tissue at synchronous developmental stages. Induction of the developmental event by artificial means is one method commonly used to circumvent the problem. This will be discussed in the following section.

Molecular techniques are now being used to discover the genetic control of cellular senescence in plant organs. The following are a few examples of plant models used to investigate cellular senescence.

1.2.3.1 Leaf Senescence

Leaf senescence is the sequence of events leading to cellular disassembly resulting in eventual death of the organ (Stoddart and Thomas, 1980). Released nutrients and other macromolecules are reallocated to metabolically active sinks such as growing leaves and developing seeds. This recycling process is physiologically advantageous, which would lead us to believe that senescence is an endogenously controlled developmental process rather than a passive degeneration.

The role of the chloroplast genome in foliar senescence has long been disputed. An initial process associated with leaf senescence is the decline in chlorophyll and other chloroplast components (Nooden, 1988). There is considerable evidence that the degeneration of the chloroplast is mediated by the nuclear genome (Yoshida, 1961; Nooden, 1988).

Sid, a nuclear gene in *Festuca* and other related temperate grasses has been implicated in the control of chloroplast disassembly during foliar senescence (Thomas, 1987). A mutation in this gene disables the breakdown of intrinsic pigment proteolipid complexes of thylakoid membranes resulting in retention of greenness in mesophyll tissue (Hilditch et al., 1989). The *Festuca* non-yellowing mutant, which carries a mutation of the *Sid* gene, shows all the biochemical changes seen at the end of reproductive

life: a decline in total protein, total RNA, glutamate pyruvate transaminase activity, with the exception of loss of total chlorophyll and the light harvesting chlorophyll a/b protein (Thomas and Stoddart, 1980). This is definitive evidence that chloroplast degeneration is not a causal event of senescence but a symptom of the phenomenon.

Changes in the levels of enzyme activity (Lauriere, 1983) and in translatable mRNA (Thomas, 1990; Thomas et al., 1992) during leaf senescence have been demonstrated in several experimental systems. The characterization of genes specifically induced in senescent leaves is still in its infancy.

Only a few gene transcripts have been shown to increase during leaf senescence, some of which are related to fruit ripening in tomatoes (Davies and Grierson, 1989). By exploiting the phenomenon that genes are differentially expressed during senescence, differential screening methods can be used to assist the isolation of mRNAs that correspond to senescence associated genes. Care must be taken when choosing an experimental system for the study of leaf senescence. Traditionally, leaves were induced to senesce by excision or incubation in darkness. Artificial induction of senescence would commit plant tissues synchronously thus a defined starting point for the process can be controlled. Recently, a difference in gene expression in induced and in naturally senescent barley leaves was found (Becker and

Apel, 1993). Stress related cDNAs that were up-regulated in excised or dark-induced senescent leaves but not in naturally senescent leaves were identified. Although artificially induced senescence is convenient and results obtained from this experimental system are informative in explaining catabolic processes, the validity of the data with respect to natural senescence can be questioned.

Recently, several senescence associated cDNAs were isolated from naturally senescing leaves of *Brassica napus* (Buchanan-Wollaston, 1994). Through Northern analysis, different times and levels of expression of these cDNAs were demonstrated. It was speculated that several modes of regulation may function in the expression of the proteins involved in the senescence process. There were three groups of senescence associated cDNAs classified by times of up-regulation. Group one genes were strongly activated at the start of senescence. These genes may be activated at the transcriptional level by some senescence-specific signal. Group two genes showed an increase in mRNA levels as senescence proceeded. Expression of these genes may not be specifically induced, but their mRNAs may not be degraded at the same rate as the bulk of the mRNA population. The resulting enzymes may have important functions in the senescence process. The third group of cDNAs were expressed early in leaf development and had levels of mRNA that fell considerably in mature green leaves and then increased again

during leaf senescence. It was proposed that these genes were induced in cells that were undergoing active metabolic functions and showed high level of respiratory activity. In both young leaf tissue and in senescing tissue, metabolic activity is high due to increased levels of protein synthesis (Thomas et al., 1992). In mature leaves, protein synthesis is at a very low level and metabolic activity is confined to the synthesis of fixed carbon by photosynthesis.

Further analysis of LSC54, a cDNA induced at the start of senescence showed that the translated sequence showed significant homology to metallothionein-like proteins. Although increased expression of plant metallothionein-like genes during leaf senescence has not previously been reported, there is some evidence that metallothioneins may act in mammalian systems to protect DNA from oxidative damage caused by free radicals (Chubatsu and Meneghini, 1993). Perhaps the presence of LSC54 protein may protect the nuclear DNA from damage, thus allowing expression of senescence-specific genes which participate in the process.

1.2.3.2 Studies on Nodule Senescence

The processes of cellular senescence have also been examined in the root nodule. The soybean root nodule is a product of nitrogen-fixing symbiosis involving combined interactions of a species of soil bacteria known as *Bradyrhizobium* or *Rhizobium* and the host plant, soybean

(Postgate, 1982).

The contribution of both plant and bacterium in the development of the root nodule has been studied extensively. Nodule formation is induced by signals produced by both organisms. Several specific plant genes known as nodulins involved in the symbiosis have already been characterized. These function in nodule development and in nitrogen fixation (Verma and Delauney, 1988).

Plant nodulin genes are classified into three groups depending on the stage of development in which they function, and during the process of nitrogen fixation. Early nodulins are involved in the formation of the nodule. Late nodulins are involved in nitrogen fixation. We could postulate that very late or senescence associated nodulins are involved in nodule disassembly during the reproductive stage of development.

Like all other stages of nodule ontogeny, senescence may also be controlled by differential gene expression. Senescence has been demonstrated in other plant organs and in the tissues of other organisms to be an active process requiring the synthesis of new RNAs and proteins (Nooden, 1988). The study of senescence processes in the soybean nodule may offer insight into the universality of this phenomenon.

1.3 Nodule Senescence

1.3.1 General Physiology

Soybean root nodules senesce naturally with whole plant senescence at the end of the growing season (Vance et al., 1986). It is postulated that the mobilization of assimilated carbon from the nodules to the seeds and pods suppresses nodule growth. Nodule senescence is characterized by a decline in nitrogen fixation rates and the oxidation of leghemoglobin (Vance et al., 1986). In other systems the onset of senescence is dependent upon protein synthesis (Smart et al., 1991; Bursch et al., 1990; Nooden, 1988). The active nature of the process leads to an eventual decrease in the steady state levels of mRNAs. The process culminates in cellular disintegration and death. It has not been proven that the onset of nodule senescence is dependent on protein synthesis.

1.3.2 Developmental Senescence

The soybean root nodule is a determinate nodule (Vance et al, 1986). About 50% of the cells are infected with the endosymbiont. In a single nodule, the infected cells constitute one developmental zone; thus, a homogeneous population of cells is available for biochemical study. In many other plant systems, a limitation when studying biochemical processes is the difficulty in obtaining tissue at synchronous developmental stages for analyses.

Senescence of the soybean root nodule occurs naturally during flowering and pod filling (Gottlob-McHugh, 1990). The result is a decreased capacity to reduce atmospheric nitrogen, thus potentially limiting the supply of amino acids and nitrogen to the plant. This is coupled with the degeneration of the nodule leading to loss of soluble protein and eventual abscission of the nodule from the root (Nash and Schulman, 1976). Similar to other senescent organs, disassembly and mobilization of nutrients to more energy requiring plant parts seem to be occurring during nodule senescence. Mobilization of sugars and other macromolecules with stored energy to the reproductive organs may improve the survival of the species (Nooden, 1988).

Senescent, determinate nodules are characterized by a green or dark brown colour, disintegration of nodule tissue, and the return of the endosymbiont to the free living form. Biochemically, the process is typified by a decline in nitrogen fixation rates, a net loss of soluble proteins, and by the oxidation of leghemoglobin (Vance, et al, 1986).

Little is known of the genes activated leading to and during nodule senescence. As yet no senescence associated genes have been cloned from soybean nodules undergoing natural or induced senescence.

1.3.3 Induced Senescence

Nodules also senesce under various environmental conditions. These include detopping, shading, and the addition of combined nitrogen. Nodule function is reduced when nitrate or ammonium are applied to active nitrogen-fixing legumes.

More energy is utilized in symbiotic nitrogen fixation than in the assimilation of soil nitrate. Addition of fixed nitrogen will cause premature senescence in various nodules. (Sutton, 1983; Becana and Sprent, 1987; Alcantar-Gonzales et al., 1990).

This system may be an excellent alternative to naturally senescent nodules when examining biochemical processes since it can provide material under a controlled environment. When considering the option of analyzing the differential gene expression during natural and induced senescent systems, one should be wary of variations in processes at the molecular level. In the study of natural and artificially induced leaf senescence in *Hordeum vulgare*, a difference in gene expression was found between the two systems. This phenomenon may also apply with senescent nodules.

In a recent review (Becana and Sprent, 1987), four theories pertaining to the inhibitory effects of nitrate on nitrogenase activity and subsequent senescence of the nodule were proposed:

1. inhibition by carbohydrate deprivation;
2. inhibition of N₂ fixation activity by the products of nitrate metabolism;
3. inhibition of N₂ fixation activity by decreased oxygen diffusion into nodules;
4. effect of respiratory nitrate reductase activity.

The intricate nature of combined nitrogen metabolism in nodules would suggest that gene expression during premature and natural nodule senescence may differ. Although informative results about catabolic processes in senescing nodules may be obtained with this method of inducing premature nodule senescence, the validity of such data with respect to natural senescence may be limited. The differences in gene expression between these systems may be exploited in explaining the processes involved in each.

1.3.4 Senescence Related Hydrolytic Enzymes

The most pervasive feature of plant senescence is the activity of hydrolytic enzymes. Most of these enzymes partake in degradative processes: proteases, nucleases and others. These enzymes aid in the release and redistribution of nutrients to more metabolically active cells. Increased activity of hydrolytic enzymes has been correlated with the processes of nodule senescence. Two thiol proteases have been purified from senescent nodules of French bean and

appear to be active only during the late stage of nodule development (Vance, 1986; Peoples and Dalling, 1988). They were shown to digest, *in vitro*, the peptidoglycan of the bacteroid cell wall (Pladys and Rigaud, 1988). In senescent soybean nodules, serine protease activity was found (Malik et al., 1981; Pfeiffer et al., 1983) but no role in senescence was proposed.

1.3.5 Senescence Associated Nodulin in Winged Bean

Several proteases have been found and their activities were postulated to be senescence related. Little is known of senescence associated nodulins (SANs). To this date, only one senescence associated nodulin has been cloned (Manen et al., 1991). This protease inhibitor is expressed in senescent Winged bean nodules. The authors speculated that this trypsin inhibitor disrupts the host-microsymbiont relationship which causes the plant to reject the invading bacteria. Little is known about the regulation and expression of this particular nodulin gene or any other senescence associated nodulin genes.

1.4 The Soybean Nodule: An Ideal Model for the Study of Cellular Senescence

The soybean determinate nodule is an ideal model system made up of infected cells at the same developmental stage, thus, presenting a homogeneous cell population for biochemical studies.

Biochemical and physiological pathways in healthy cells may be better understood by studying mechanisms in senescing systems and the disassembly of their components. Senescence involves the loss of self-maintenance in a cell. Information from a study of the biochemical processes in nodule senescence may provide clues as to how nodule cells maintain themselves under nonsenescing conditions.

Also, this information may be of agricultural importance. In knowing the mechanisms of nodule senescence, perhaps manipulations may be performed to delay the senescence of root nodules at the stage of seed formation. Such applications may some day be used to improve the nitrogen value of legume crops.

1.5 Thesis Objectives

Cellular senescence involves orchestrated processes that culminate in disintegration and death. The study of the molecular mechanisms of cellular senescence may aid our understanding of the systems necessary for cell maintenance under healthy and senescent conditions. As in the gene encoding TRPM-2, it is clear that certain messages involved in the senescence process are highly conserved in species as diverse as rats and insects. Studies conducted to understand the senescence process, its induction and control in the soybean nodule will offer evidence to support the principle of evolutionary conservation of a well-

orchestrated pathway for senescence amongst various species.

The objectives of this research have been:

1. to isolate a SAN cDNA clone by differential screening based on the principle of increased representation of SAN sequences in the cDNA library of senescent nodule cells.

2. to study the expression of the SAN gene by Northern analysis.

3. attempt to identify the senescence related function of the SAN by sequence analysis.

4. to investigate the expression of the SAN cDNA in senescent nodule cells induced by flowering and by addition of fixed nitrogen.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth and Collection of Plant Tissues

Soybean seeds (*Glycine max* (L.) Merrill cv. Maple Arrow) were obtained from H. Volding, Agriculture Canada, Ottawa. Seeds were surface sterilized in a 20% solution of commercial bleach for 10 minutes. The seeds were then rinsed with distilled water and dispersed on sterile trays covered with moistened Whatman 3MM paper. The seeds were germinated in the dark at 25°C for 3 days. Seeds that did not germinate, or seedlings that appeared abnormal were discarded. Healthy seedlings, 4-6 cm long were planted in 4 inch pots containing vermiculite (day 0). Each seedling was inoculated with 3 ml of a late log phase culture of *Bradyrhizobium japonicum* strain 61A76. The plants were grown at 25°C with a 16 hour light/8 hour dark photoperiod. The plants were watered with a nitrogen-free nutrient solution (Cutting and Schulman, 1969). All plant tissues were hand-picked, immediately frozen in liquid nitrogen and stored at -80°C until further use.

Premature senescence was induced with addition of 20mM ammonium nitrate at day 21 after planting. Nodules from the induced system were harvested at time 0 hour (day 21), 12 hours, 24 hours and 48 hours after induction.

Healthy nodules, actively fixing nitrogen were hand-picked from 24 day old plants for the construction of the control cDNA library. Senescing nodules used for cDNA library construction were hand-picked from 40 day old plants.

2.2 Nitrogenase Assay

Nitrogenase activity was measured by the acetylene reduction assay (Hardy et al., 1968). Root systems were removed from the plants at the base of the stem and placed in 30 ml serum stoppered bottles. 3 ml of acetylene was injected. 3 ml samples were removed from the bottle at 0, 30 and 60 minutes and injected into 3 ml vacutainers. Ethylene production was measured by injecting 0.5 ml of the vacutainer sample into a Carle Instruments GC9700 Basic Gas Chromatograph (Agriculture Canada, Ottawa). Known amounts of ethylene were run as calibration standards. Nodules were then removed, dried, and weighed and nitrogenase activity was calculated as nmoles ethylene produced/minute/gram of dry weight of nodule. The data presented are the mean of three different plants.

2.3 Bacterial Strains, Plasmids and Phage

Bradyrhizobium japonicum strain 61A76 from Nitragin Corporation, Wisconsin, was used to nodulate soybeans.

Echerichia coli XL1-BLUE (Stratagene) was used as a host for the growth and purification of the pGEM-7Zf plasmid. *E. coli* strain DH5 α mcrrF' (BRL) was used as the host for M13 vectors.

For the cDNA libraries, *E. coli* strain Y1090 (Amersham) was used as the host for λ gt11 (Huynh et al., 1985). *E. coli* strain NM514 (selective host) and NM538 (non-selective host) (Watson and Jackson, 1985) were used as the hosts for λ gt10 (Huynh et al., 1985).

M13 mp18 or mp19 phage (Yanisch-Perron et al., 1985) and pGEM-7Zf plasmid (Promega) were used as sequencing vectors.

2.4 Isolation of DNA

2.4.1 Isolation of Plant DNA

High molecular weight soybean DNA was isolated from sprouts. Soybean tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. 10 ml of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 1% SDS, pH 8.0) were added per gram of powdered tissue and the slurry was poured into an 50 ml Oakridge centrifuge tube or 250 ml centrifuge bottle. The slurry was extracted with an equal volume of phenol (buffered in 50 mM Tris, 10 mM EDTA, pH 7.0) and centrifuged for 10 minutes at 8,000 rpm at 20°C. This was followed by two phenol-chloroform (prepared as in Sambrook et al., 1989) extractions. Total nucleic acids were precipitated overnight at -20°C following the addition of

0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The precipitate was then collected by centrifugation at 8,000 rpm for 20 minutes at 4°C. The pellet was dissolved in TE and treated with RNase (50 µg/ml) for 30 minutes at room temperature. Following extraction with an equal volume of phenol-chloroform, the nucleic acid was precipitated with sodium acetate and ethanol as before. The precipitate was collected by centrifugation at 8,000 rpm for 20 minutes and washed 2X with 70% ethanol. The pellet was dissolved in 25 ml of TE and 25 grams of CsCl and 1 ml of ethidium bromide (10mg/ml) were added. DNA was purified by CsCl equilibrium gradient centrifugation at 44,000 rpm with a 50 VTi rotor (Beckman), for 16 hours at 15°C. Following centrifugation, the ethidium bromide was removed by butanol extraction (Sambrook et al., 1989). Three volumes of TE were added to the aqueous phase, and following the addition of 2.5 volumes of ethanol, the DNA was precipitated overnight at -20°C.

2.4.2 Preparation of Plasmid DNA

Plasmid DNA was prepared from the 'Magic Miniprep Method' (Promega), the 'Easy prep' Boiling Method (Berghammer and Auer, 1993) or the QIAGEN Miniprep Plasmid Kit.

2.4.3 Isolation of DNA Fragments

DNA restriction fragments were isolated from agarose gels by using the SEPHAGLAS kit as recommended by Pharmacia.

2.4.4 Isolation of Lambda DNA

Lambda phage was grown, titred and purified as described in Sambrook et al., (1989). Briefly, the plate lysate technique involves the elution of phage grown on L-agar plates with SM buffer (5.8g/L sodium chloride, 2g/L $MgSO_4 \cdot 7H_2O$, 100mg/L gelatin, 50mM Tris-HCl, pH7.5) at 4°C with shaking. The lysate can be stored over 1/100 volume of chloroform. The lysate is then treated with DNase and RNase before precipitation in PEG8000 on ice. The pellet was resuspended in SM buffer and extracted twice with chloroform. SDS (0.1%) and proteinase K (0.1 mg/mL) were added to the phage sample and incubated at 50°C for 1 hour. A series of organic extractions was performed before precipitation in 0.3M sodium acetate, pH5.5 and 2.5 volumes of 95% ethanol.

2.5 RNA Isolation Procedures

2.5.1 Isolation of Total RNA

Total RNA from soybean roots or nodules was isolated using the guanidinium isothiocyanate method of Chirgwin et al. (1979) and Glisin et al. (1974). The typical yield was 200 µg total RNA per gram of nodules. Briefly, the

isolation procedure involves the homogenization of frozen plant material with a mortar and pestle using a small piece of dry ice. The ground powder and at least 3 volumes (or up to 10 volumes, enough to give a final volume of 10 mL) of ice cold homogenization buffer (4M guanidine isothiocyanate (ultrapure from BRL or Schwartz-Mann), 25 mM sodium citrate (pH 7.0), 0.1M mercaptoethanol were added to an ice cold polytron vessel for homogenization (small size).

After homogenizing the sample for 1 minute with the polytron (max. speed), 0.5 mL of 20% sarcosyl per 10 mL of total sample volume was added and homogenization was repeated. The supernatant (\approx 9.0 mL) was collected after centrifugation and loaded onto a 2 mL CsCl cushion (5.7 M cesium chloride, 10 mM EDTA) in a Beckman open top polyallomer tube (autoclavable). This was centrifuged at 30 600 rpm for 16 hours at 18°C in an SW41.Ti rotor. The RNA pellet (clear lens-like pellet) was resuspended in 500 μ L of GHCl buffer (7.5M guanidine hydrochloride (ultrapure from BRL, Schwartz-Mann or ICN), 0.025 volume of 1M sodium citrate, 5mM dithiothreitol). The RNA was precipitated in 0.025 volume of 1M acetic acid and 0.5 volume of EtOH.

The pellet was dissolved in 250 μ L of water that was treated with diethylpyrocarbonate (DEPC) and precipitated in 0.1 vol 3M sodium acetate (pH 5.0) and 2.5 vol EtOH. The RNA may be stored in EtOH at -20°C indefinitely.

2.5.2 Isolation of Poly (A)⁺ RNA

Poly A⁺ RNA was isolated using the paramagnetic particle system in the PolyATtract kit (Promega). Briefly, 500µg of total RNA in a volume of 500µL was annealed to a biotinylated-oligo dT probe and subsequently annealed to Streptavidin-paramagnetic particles (SA-PMPs). The mRNA-probe-SA-PMP hybrid was washed four times with 0.1X SSC (0.3mL each wash) to remove unbound RNA. After the final wash, the hybrid SA-PMPs were captured and the poly A⁺ RNA was eluted from the SA-PMP pellet in sterile RNase-free water. Approximately, 10 µg of poly A⁺ RNA is isolated per 500 µg of total RNA.

2.6 Labelling of Probes

2.6.1 Preparation of Radioactively Labelled Fragments

Isolated restriction fragments were labeled by the random primer method (Feinberg and Vogelstein, 1983) using kits purchased from Amersham. The radioactive label was [α -³²P]-dCTP (3000 Ci/mmol). Unincorporated nucleotides were removed by passing the labeled fragments through Sephadex G-50 by the spun-column procedure as indicated in Sambrook et al., (1989). Prior to use, the required amount of labelled fragment was heated to 100°C for 2 minutes, fast cooled on ice and added to the hybridization solution. This step is omitted when preparing radioactive λ HindIII markers (as a fill-in reaction).

2.7 Construction of cDNA Libraries in λ gt10 and λ gt11 Vectors

The cDNA libraries in λ gt10 and λ gt11 vectors were constructed using the Riboclone cDNA synthesis system by Promega. The reverse transcriptase and its buffer supplied by the Promega kit was substituted with the Moloney murine leukemia virus RNase H⁻ reverse transcriptase and buffer (BRL). The cDNA inserts were cloned into the phage vectors using the Amersham cDNA cloning system - λ gt10 or λ gt11 vectors.

2.7.1 First Strand Synthesis

The poly A⁺ RNA was annealed to the primer/XbaI adaptor maintaining a 2:1 ratio of RNA to primer/adaptor (w/w). The first strand cDNA was synthesized according to the suggested protocol in the Riboclone cDNA synthesis system (Promega). Moloney murine leukemia virus RNase H⁻ reverse transcriptase and its buffer (BRL) were used in place of the supplied enzyme. A tracer reaction to follow the synthesis was performed in parallel by labelling with $\alpha^{32}\text{P}$ -dCTP.

2.7.2 Second Strand Synthesis

The primary unlabelled first strand reaction was combined with recommended amounts of the 10X second strand reaction buffer, dNTP mix (10mM), $\alpha^{32}\text{P}$ -dCTP, DNA pol I and RNase H. A 5X dilution of the first strand reaction was

maintained. The yield for the cDNA synthesis was determined by the trichloroacetic acid (TCA) method as recommended by Promega.

2.7.3 Size Fractionation of Double Stranded cDNA

The newly synthesized cDNA was passed through a size fractionation column of Sepharose 4B CL-200 beads at room temperature. The column was made in a 1 mL syringe with the plunger removed. The size fractionated cDNA was eluted in TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0).

Twenty fractions were collected. Samples were counted for radioactivity. 1 μ L samples of each radioactive sample were run on an agarose gel to produce a profile of the fractions. The gel was dried and exposed to Kodak XAR film.

Those fractions containing cDNA \geq 500 bp were pooled and precipitated in 0.3M sodium acetate and 2.5 volumes of 95% EtOH at -20°C.

2.7.4 Cloning of Double Stranded cDNA into λ gt 11 and λ gt 10 Vectors

The ligation of the EcoRI adaptor to the cDNA and the subsequent cloning of the adapted cDNAs were performed according to the protocol recommended in the Amersham cDNA cloning system for λ gt 11 and λ gt 10.

2.8 Screening of cDNA Libraries in λ gt10 and λ gt11 Vectors

The cDNA libraries were plated onto L-agar plates and plaque lifts were taken onto Biodyne nylon membranes (ICN). The lifts were denatured (0.5M sodium hydroxide, 1.5M sodium chloride) for 5 minutes, neutralized (1.5M sodium chloride, 0.5M Tris-HCl, pH 7.0) for 5 minutes and soaked in 2XSSC for 5 minutes, before U.V. linking the DNA onto the membrane for 5 minutes.

2.8.1 Screening with Leghemoglobin and Clone 15-9-A

The integrity of the newly synthesized cDNA libraries were tested by screening with two nodulin clones, leghemoglobin (Lb) and clone 15-9-A. The 15-9-A clone was isolated by Gottlob-McHugh and Johnson, 1991). This gene belongs to the nodulin 20 family. The Lb probe was a multiprime labelled 550 bp Lb insert containing the 3' untranslated region of Lb and most of the coding region. The 15-9-A probe was a multiprime labelled Bgl I and Bgl II fragment (Gottlob-McHugh, 1990). The probes were hybridized to the filters in a 1×10^6 cpm/ml hybridization solution (Meinkoth and Wahl, 1984) in the standard formamide method. 50% formamide, 5X SSC pH 7.0, 250 μ g/ml herring sperm DNA, 50mM sodium phosphate, 5X Denhardt's, 0.1% SDS for 16 hours at 42°C. The blots were washed 3 times for 10 minutes each time in 2X SSC, 0.1% SDS at room temperature. If background counts were greater than 250 cpm, an additional wash in 0.1X

SSC, 0.1% SDS at 50°C was performed. The filters were exposed on KODAK XAR-5 film overnight at -80°C with a Picker Spectra Blue intensifying screen.

2.8.2 Screening by Differential Hybridization

The cDNA library made from mRNA of 40D nodules was screened by a PCR based differential cross hybridization method for libraries cloned into phage-lambda-based vectors (Wong et al., 1989).

2.8.2.1 Probes for Differential Screening

The probes for the library screening were made from the PCR amplification of the purified phage DNA of the 24D and 40D libraries in λ gt10. Phage DNA was extracted from elutions of 300 000 recombinants. 30 ng of phage DNA was used for PCR amplification with λ gt10 amplimers. Amplification was performed in a total volume of 100 μ L spiked with 10 μ L of [α -³²P]-dCTP (3000 Ci/mmol). Labelled product can be run on an agarose gel to check extension, and a more accurate estimation of the amount of PCR product can be made in preparation for the following multiprime labelling. The unincorporated nucleotides were removed from the amplified product by passing through a Sephadex G50 column. 200 ng of PCR product was used for multiprime labelling. Specific activity of the probe must be greater than 1×10^8 cpm/ μ g DNA. Concentration of probes used to

hybridize to duplicate lifts of the 40D library was 1×10^6 cpm/ml.

2.8.2.2 Screening of Duplicate Lifts

Two methods for hybridization of probes to lifts were used; both are effective methods. The standard formamide method is described in section 2.11. Seventeen positive clones were selected based on the differential intensities of the hybridizing 24D and 40D probes as determined by autoradiography.

2.9 Agarose Gel Electrophoresis

2.9.1 Gel Electrophoresis of DNA

Restriction fragments of DNA were separated by electrophoresis through horizontal agarose slab gels. The concentration of the gels varied from 0.8-1.25% depending on the size range of the fragments to be resolved. The electrophoresis buffer contained 45 mM Tris-borate, 1 mM EDTA (Sambrook et al., 1989). Lambda HindIII digests or pBR322 TaqI digests were generally used as size markers.

2.9.2 Gel Electrophoresis of RNA

RNA was separated by electrophoresis through 1.5% agarose gels containing formaldehyde (Lehrach et al., 1977). RNA samples for electrophoresis were prepared as described in Sambrook et al. (1989) with 10 μ g/mL final concentration

of ethidium bromide in the sample. The gels contained 20 mM MOPS (pH 7.0) and 2.2 M formaldehyde. The running buffer contained 20 mM MOPS (pH 7.0). *E. Coli* 23S, 16S, and soybean 25S, 18S rRNAs were used as size standards. Since the samples were run with ethidium bromide, a picture of the gel could be taken immediately after completion of the run.

2.10 Transfer of Nucleic Acids to BIOTRANS (BIODYNE)

2.10.1 Transfer of DNA to BIOTRANS (Biodyne)

Restricted DNA was separated electrophoretically in agarose gels. Following electrophoresis, the gels were stained with ethidium bromide and photographed. Gels containing plant genomic DNA restriction digests were then depurinated with 0.25 M HCl for 5 minutes to facilitate transfer of large molecular weight fragments. Gels containing restriction digests of phage and plasmid DNA were not depurinated. The DNA in the gels was denatured by gentle agitation in several volumes of 1.5 M NaCl, 0.5 M NaOH for 30 minutes. This step was repeated for an additional 30 minutes. The denaturation buffer was then replaced by 2 changes of neutralization buffer containing 3 M sodium acetate (pH 5.2). The DNA was transferred to BIOTRANS membranes by the method according to Southern (1975). Following transfer, the DNA was immobilized onto the membrane by U.V. crosslinking for 5 minutes (Church and Gilbert, 1984).

2.10.2 Transfer of RNA to BIOTRANS (Biodyne)

RNA in 10 $\mu\text{g}/\text{mL}$ ethidium bromide was electrophoretically separated in formaldehyde gels. Following electrophoresis, the gel was photographed and transferred overnight to BIOTRANS membrane as described in Thomas (1983) using 20X SSC.

2.11 Hybridization of Radiolabeled Probes to DNA and RNA Immobilized on BIOTRANS Filters

Filters containing immobilized DNA or RNA were prehybridized in 4 ml of hybridization solution per 100 cm^2 of membrane in sealable bags for a minimum of one hour. The standard formamide hybridization solution contains 50% formamide, 5X SSC pH 7.0, 250 $\mu\text{g}/\text{ml}$ herring sperm DNA, 50mM sodium phosphate, 5X Denhardt's, 0.1% SDS. Hybridization was performed in 2 mL of hybridization solution per 100 cm_2 for 16 hours at 42°C. The blots were washed 4 times for 10 minutes each time in 2X SSC, 0.1% SDS at room temperature. High stringency wash conditions were sometimes necessary. These blots were washed with 0.1 X SSC and 0.1% SDS at 65-68°C. The filters were exposed overnight at -80°C with a Picker Spectra Blue intensifying screen. Blots were then wrapped in plastic and exposed to Kodak XAR-5 film at -80°C using a Picker Spectra Blue intensifying screen.

2.12 Standard Conditions for PCR Amplification of Clones in λ gt10 or λ gt11 Vectors

Individual plugs of single plaques may be selected and amplified by first eluting the DNA in 50 μ L of SM buffer. 5 μ L of the eluate was used for the reaction. The source of DNA may also be from whole libraries. The VENT polymerase, its buffer and $MgSO_4$ was supplied from New England BIOLABS and the conditions used under their recommendations: 1 X VENT PCR buffer, 6mM $MgSO_4$, 200 μ M each dNTP, 30 pmoles of each of the appropriate amplimer pairs, 5 μ L plug eluate in SM in a total volume of 25 μ L. 30 ng of phage DNA from a library was amplified in a total volume of 100 μ L containing 10 μ L of [α - ^{32}P]-dCTP (3000 Ci/mmol).

The sequence of the λ gt11 amplimers are as follows.

Forward amplimer sequence:

5'-GGT GGC GAC GAC TCC TGG AGC CCG-3' 24MER

Reverse amplimer sequence:

5'-TGA CAC CAG ACC AAC TGG TAA TGG-3' 24MER

With λ gt11 amplimers, the annealing temperature was 60°C and successful extension of inserts of ~900 bp was at a temperature of 72°C for 1.5 minutes.

The sequence of the λ gt10 amplimers are as follows.

Forward amplimer sequence:

5'-CTT ATG AGT ATT TCT TCC AGG GTA-3' 24MER

Reverse amplimer sequence:

5'-CTT TTG AGC AAG TTC AGC CTG GTT-3' 24MER

With λ gt10 amplimers, the annealing temperature was 54°C and extension conditions of 72°C for 2.5 minutes.

2.13 Preparation of Templates for DNA Sequencing

Restriction fragments for sequencing were subcloned into either M13mp18/mp19 vectors (Yannish-Perron et al., 1980), or pGEM-7Zf plasmid vector (Promega). Fragments for subcloning were determined by PCR amplification of the cDNA inserts. All ligations into M13 phage vectors were transformed into *E. coli* strain DH5 α merF' by the method of Hanahan (1985). Recombinant phage was grown and isolated as described in the M13 cloning and sequencing handbook provided by Amersham. Single-stranded template for sequencing was prepared as described in the Amersham handbook.

Plasmid clones in pGEM7Zf (Promega) were placed into *E. coli* strain XL1-Blue (Stratagene) by electroporation under the conditions recommended by BIORAD. Plasmids were isolated with the QIAGEN Miniprep Plasmid Kit.

2.14 DNA Sequencing

The pGEM7Zf subclones were sequenced using the dideoxy chain termination method (Sanger et al., 1977). Sequencing of plasmids was as described in the USB Sequenase Kit.

Cycle sequencing of M13 recombinant phage DNA was as described in the New England BIOLABS sequencing handbook using VENT Polymerase. Most sequencing reactions were performed using [α -³⁵S]-dATP (800-1000 Ci/mmol) as the radioactive label.

A PCR based method was used for sequencing with internal primers designed to resolve ambiguities in clone SAN cDNA 8-4. The AmpliTaq DNA Polymerase was used according to the procedure for Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Sequence information was manipulated using PCGENE and SeqEd software.

Internal primers designed to walk SAN cDNA 8-4 are as follows.

G1F1 sequence: 5'-GGT GCC AGT GCA ATA CC-3' 17MER

G1R1 sequence: 5'-TTC CCT CTG TGG ACA AG-3' 17MER

2.15 Protein Alignments

The nucleotide sequence of SAN cDNA 8-4 was searched in GENBANK, EMBL, and the conceptual protein sequences were searched in all 6 reading frames in SWISSPROT and PIR with the BLAST algorithm (Altschul et al., 1990). The protein

alignments were performed in MACAW and in CLUSTAL. Significance of the protein alignments was determined by using a simple method which utilizes the High Score value presented by the BLAST search and the normalized lengths of the sequences (Doolittle, 1986, pp. 14). Motifs were found using the PROSITE program.

CHAPTER THREE:

RESULTS

Seventeen SAN cDNA clones were isolated from a λ gt11 library representing mRNA from senescent nodule tissue by the screening of 100 000 clones. This was facilitated by differential hybridization with two bulk probes constructed from two libraries cloned into λ gt10: one library representing mRNA from senescing soybean nodules and the control library representing mRNA from young soybean nodules that were actively fixing nitrogen. This procedure utilized the PCR amplification of inserts in the λ gt10 libraries to be used as probes to screen the λ gt11 library for the identification of sequences which were common in both. Thus, sequences specific to the 'senescent' libraries and not in the 'healthy' library were putative SAN cDNA clones.

The use of PCR for the differential screening of libraries cloned into phage-lambda-based vectors is a powerful tool which exploits the amplification of the cDNAs in each λ gt10 library to enhance the detection sensitivity of up-regulated genes in a stressed system (Wong et al., 1989).

This strategy was made possible by the nature of the vectors used to generate the two libraries. Although both λ gt10 and λ gt11 have regions of common sequence, the genes in which the cloning sites are situated are different. The

cloning site of λ gt10 is an EcoRI site in the cI repressor gene. The cloning site of λ gt11 is an EcoRI site in the lacZ gene. With the use of primers which flank the insert site, the PCR reaction amplifies the insert DNA and a small portion of the λ gt10 cI repressor gene. Under standard hybridization conditions (see Materials and Methods), the amplified portion of the λ gt10 vector does not cross-hybridize with the λ gt11 vector.

Before the screening experiment was conducted, time points at which to harvest soybean nodules for the isolation of mRNA to be used for the construction of the cDNA libraries were determined. The acetylene reduction assay was used to determine the relative rates of nitrogen fixation. This information aided the monitoring of nodule development. Total RNA was extracted from nodules collected at various times during nodule development. In a previous study, it had been documented that the soybean nodule was actively fixing nitrogen at day 24 (i.e. 24 days after inoculation), and the nodule was beginning to senesce at day 34, 7 days after flowering (Gottlob-McHugh, 1990). In the present study, senescence was predicted to occur later, at day 40. The delay in the flowering may have been due to variability in the age of the seeds, conditions for germination, or growth conditions in the incubator between studies.

To illustrate the correlation between the predicted time points and the developmental stages, Northern blot analyses were performed to show the decline of nodulin messages with nodule senescence as monitored in the decline in the relative rate of nitrogen fixation. On the assumption that successful Northern analyses would demonstrate the quality of the total RNA, cDNA libraries could then be constructed from the mRNA purified from these total RNA preparations.

The quality of RNA fit for cDNA library construction was characterized by:

1. spectrophotometric means giving an O.D._{260/280} of 1.9 to 2.2
2. the visual analysis of clean, tight rRNA bands on an ethidium bromide stained denaturing gel containing 10 µg total RNA in each track
3. the existence of clean, tight signals of the hybridizing message corresponding to the probe of interest in a Northern analysis.

3.1 The Investigation of the Senescence Phenomenon through Northern Analyses and Acetylene Reduction Assay

3.1.1 The Decline in the Level of the Leghemoglobin and 15-9-A mRNA with Natural Senescence

During natural senescence, the decline of specific nodulins in soybean nodules (Gottlob-McHugh, 1990) and the net decline of RNA in other systems (Nooden, 1988) had been

documented (see Introduction). The progression of nodule development, specifically during nodule senescence, was monitored by plotting the relative rate of nitrogen fixation (as calculated from acetylene reduction assays) against time.

Figure 3.1 is an attempt to fit the decline in the level of leghemoglobin (Lb) and 15-9-A mRNAs with the progression of natural senescence as monitored by the relative rate of nitrogen fixation. The graph taken from the results of Gottlob-McHugh, 1990, shows the rate of nitrogen fixation during the time of nodule development. In the latter stages of development, a gradual decline in the rate is seen with natural senescence. From day 24 to day 36, the rate of nitrogen fixation diminishes 0.2 nmole/min/g dry wt. of nodule. In 12 days, the rate of nitrogen fixation diminishes to 80% of that at day 24.

The decline in signals corresponding to the hybridizing mRNAs of known nodulins was demonstrated through Northern blot analysis. With approximately equal amounts (10 μ g) of total RNA from 24 day old (24D) and 40 day old (40D) nodules, leghemoglobin (Lb) and 15-9-A clones were used as probes to demonstrate the decline in the expression of the corresponding late nodulins with nodule senescence (Figure 3.1). All signals were scanned to estimate the relative band intensities. The relative values were standardized

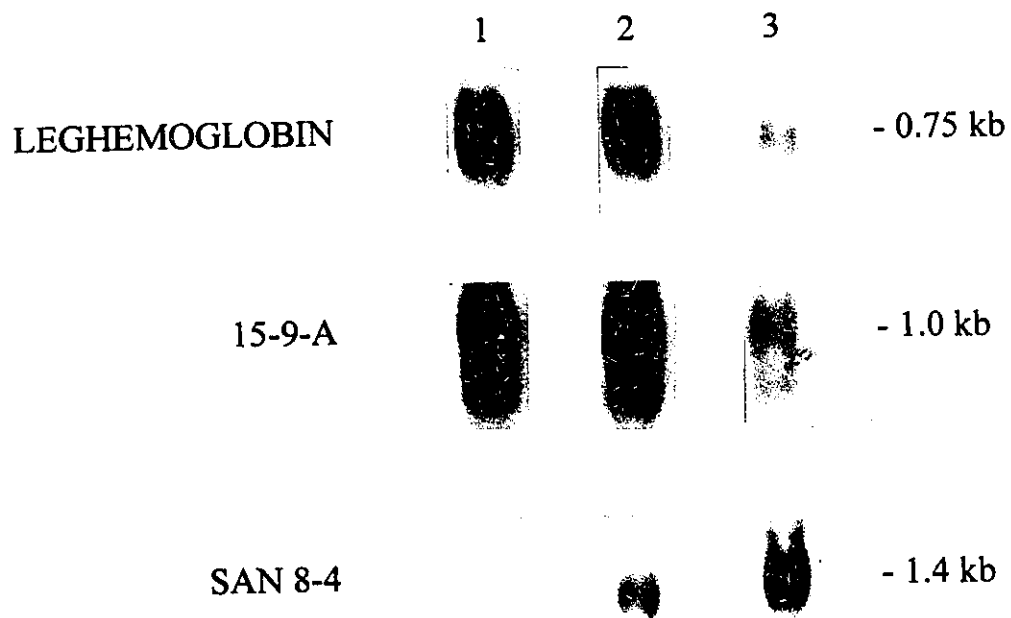
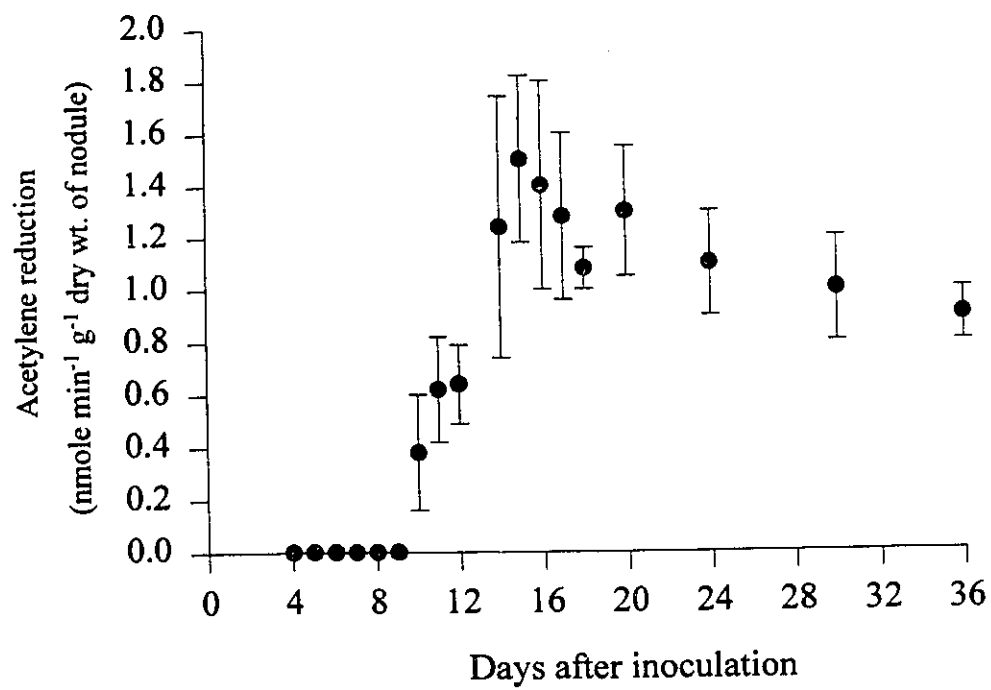
**FIGURE 3.1 NORTHERN BLOT ANALYSIS DEMONSTRATING THE
DIFFERENTIAL EXPRESSION OF THE LEGHEMOGLOBIN,
15-9-A AND SAN 8-4 cDNA CLONES WITH NATURAL
SENESCENCE**

Natural senescence was monitored by the acetylene reduction assay during the development of the nodule. The graph was taken from a previous study conducted by S.G. McHugh, 1990. The error bars indicate the standard deviation. Three sets of soybean root systems at each time point were assayed.

To demonstrate the differential expression of nodulins along this time course, 10 μ g of total RNA from the nodules at various time points after inoculation was used for northern analysis versus probes from clones of leghemoglobin, 15-9-A and SAN 8-4. The analysis was performed under standard hybridization conditions. The time points consisted of:

1. day 22
2. day 24
3. day 40

after inoculation. The sizes of the hybridizing messages are indicated.



with the scanned 25S rRNA bands on a photograph of the denaturing gel.

At 40D, the band intensity of the hybridizing message to the Lb probe was 17% of the intensity found at 24D. Similarly, at 40D, the band intensity of the hybridizing message to the 15-9-A probe declined to 13% of that at 24D (raw data not shown).

3.1.2 **The Decline in the Relative Rate of Nitrogen Fixation and in the Levels of Leghemoglobin and 15-9-A mRNAs with Induced Nodule Senescence**

The addition of fixed nitrogen induces premature senescence of nodule tissue (Sutton, 1983). The system was induced with the addition of 20mM ammonium nitrate. This procedure of induction was suggested by Chen and Phillips, 1977. Similar to natural senescence, the induced system was monitored by the decline in the relative rate of nitrogen fixation and in the levels of late nodulin mRNAs, such as Lb and 15-9-A. These experiments are described in Figure 3.2.

The shape of the graph differs from that found in the natural system. In the induced system, there is an asymptotic decline in the relative rate of nitrogen fixation versus time of induction of nodule senescence. The relative rate of nitrogen fixation decreased to nearly 50% of the maximum level of activity in only the first 12 hours of

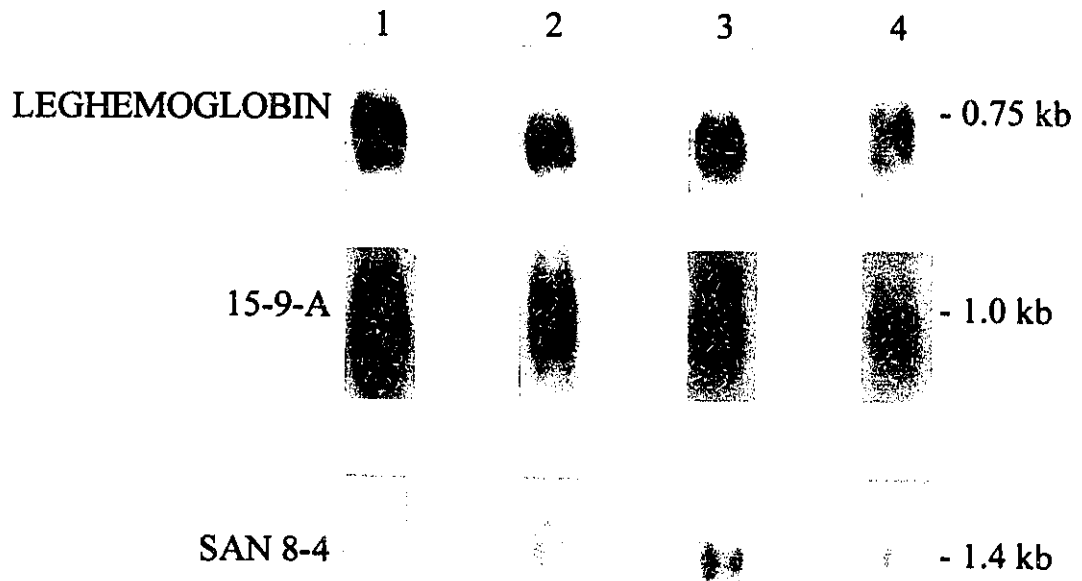
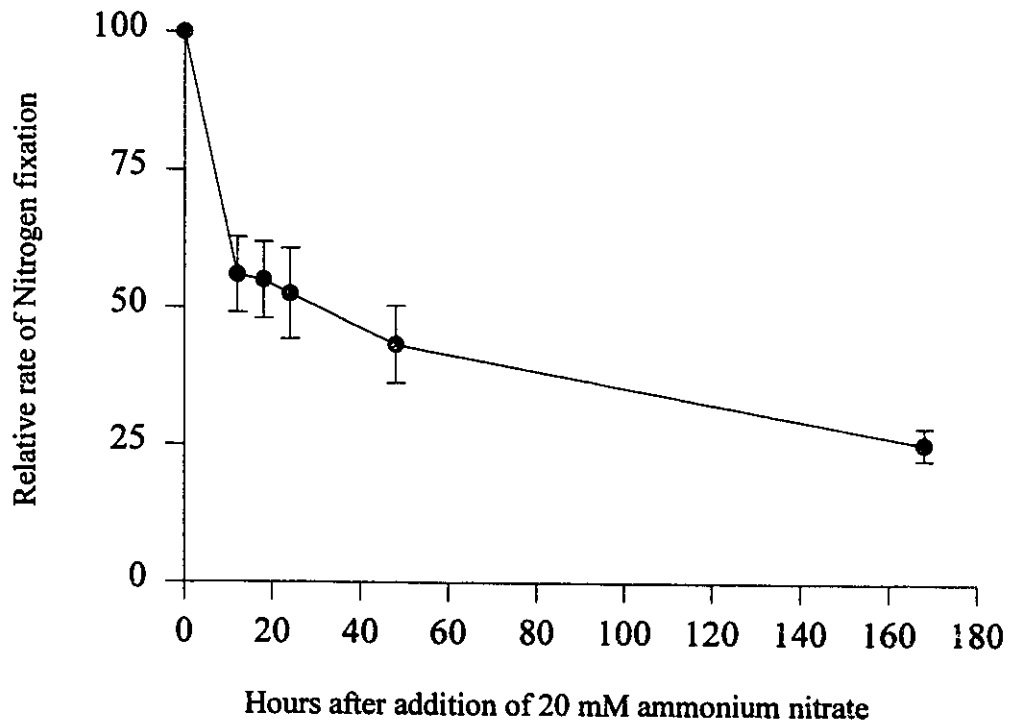
**FIGURE 3.2 NORTHERN BLOT ANALYSIS DEMONSTRATING THE
DIFFERENTIAL EXPRESSION OF THE LEGHEMOGLOBIN,
15-9-A AND SAN 8-4 mRNAs WITH INDUCED
SENESCENCE BY THE ADDITION OF FIXED NITROGEN**

Induced nodule senescence was brought on by the addition of 20mM ammonium nitrate. The relative rate of nitrogen fixation was monitored by the acetylene reduction assay. The error bars represent standard deviations. Five sets of soybean root systems at each time point were assayed.

10 μ g of total RNA from the nodules at various time points after induction was used for northern analysis versus probes from clones of leghemoglobin, 15-9-A and SAN 8-4. The analysis was performed under standard hybridization conditions. The time points consisted of:

1. 0 hour
2. 12 hours
3. 24 hours
4. 48 hours

after induction. The sizes of the hybridizing messages are indicated.



induction. The decrease is most pronounced in the first 12 hours.

As induced senescence progressed, there was a decline in the hybridizing message of Lb and 15-9-A. Typically, in the first 12 hours of induction, the band intensity of the hybridizing message of Lb diminished to 71% of that at time 0, and likewise with the 15-9-A probe, 72% of that at time 0. By 48 hours of induction, the band intensities corresponding to the Lb and 15-9-A probes were 43% and 34% respectively, of that at time 0.

3.1.3 Natural Senescence Versus Induced Senescence in the Soybean Nodule

The decline in the rate of nitrogen fixation was accelerated in the induced system when compared to the natural system. This was depicted in the shapes of the graphs of Figure 3.1 and 3.2. In the natural system, the decline in the rate was gradual, producing a gentle negative slope when plotted against time. The decline for the induced system was asymptotic. It reached 50% of the maximum relative rate of nitrogen fixation in the first 12 hours of induction.

There was a trend in the decline in late nodulin mRNAs, as shown for Lb and 15-9-A in both the natural and induced systems. Evidence suggested that the decline in the quantity of late nodulin mRNA was accelerated in the induced

system but a quantitative study must be performed to prove this hypothesis. This will be discussed in Section 4.1.

The mechanisms of senescence in the two systems may be explained by a fundamental difference in the rates at which the phenomenon occurred. A model will be presented in Section 4.1. In doing so, the observed patterns of expression of the gene corresponding to the isolated SAN cDNA may be explained in both systems.

3.2 Preliminary Screening of Soybean cDNA Libraries for Nodule-Specific Clones

cDNA enriched for an average size greater than 500 base pairs was used to construct the cDNA libraries. This length promoted the success in isolating a full length cDNA. In achieving full length clones, the identification of putative SAN cDNAs was facilitated.

Upon construction of the cDNA libraries (the 24 day cDNA in λ gt10 vector, and the 40 day cDNA in λ gt10 and λ gt11 vectors), their quality, defined by the number of true recombinants was tested. By screening with homologous probes made from known nodulins, the fraction of positive clones would represent the proportion of their mRNAs at each developmental stage. These numbers were compared to the theoretical values predicted in the literature; thus, the quality of the libraries was judged on the accuracy of the positives to the theoretical amounts.

1000 recombinants from each library were screened with the leghemoglobin and the 15-9-A clones under standard hybridization conditions (see Materials and Methods).

At 24 days, the Lb message makes up 12-15% of the poly A⁺ mRNA (Fuller et al., 1983) and the 15-9-A message, approximately 4% (Gottlob-McHugh, 1990). The results of screening experiment showed that the 24 day library contained 1.3% detectable Lb and 1.3% detectable 15-9-A cDNA clones. The lower percentage of Lb and 15-9-A messages represented in this library compared to the cited values may be due to:

- 1) the enrichment of the newly synthesized cDNA for lengths greater than 500 bp and thus smaller pieces resulting from incomplete extensions during the synthesis of cDNA would be eliminated,
- 2) the cloning of ribosomal RNA.

The 40 day library in λ gt10 contained 0.3% Lb cDNA and 0.1% 15-9-A cDNA. The relative amounts of mRNAs represented by the corresponding cDNA clones decreased from the 24 day library. The decline in the level of Lb and 15-9-A mRNAs was demonstrated in Figure 3.1. The relative number of clones corresponding to each late nodulin is expected to diminish from the 24 day library to the 40 day library, perhaps due to the senescence phenomenon.

The screening of the 40 day library in λ gt11 presented no detectable clones corresponding to Lb or 15-9-A cDNA. The difference in the 40 day libraries in λ gt10 and λ gt11 may be insignificant.

Because the above test gave inconclusive information on the quality of the libraries, DNA inserts from the detectable clones of Lb were PCR amplified and tested through a Southern analysis. The amplified products were found to be full length. And when hybridized to the Lb probe, gave a clean, sharp signal at approximately 750 bp corresponding to the full length hybridizing mRNA of Lb (Gottlob-McHugh, 1990). This showed that the synthesis of the cDNA was efficient to produce cDNA of at least 750 bp and these molecules were clonable. All three libraries were constructed in parallel, thus this evidence is the only positive control to the quality of the libraries.

3.3.2 Differential Screening for Putative SAN cDNAs

100,000 plaques from the 40 day library in λ gt11 were differentially screened for putative SAN cDNAs. Seventeen putative SAN clones were selected (refer to Materials and Methods for procedure). Both the standard formamide method of hybridization and the aqueous condition were tested on various membranes and no difference in sensitivity or the amount of background was noted.

3.4 Characterization of Putative SAN cDNAs by the PCR Method

The seventeen putative SAN cDNA clones were analyzed by attempting to PCR amplify the cDNA inserts with λ gt11 primers which flank the cloning site, and by running the PCR product on a sizing gel. The intention was to determine the approximate size of the cDNA inserts and to isolate the insert from the agarose gel for the purpose of radio-labelling. These probes would be used for Northern analyses. In demonstrating the increased expression of the putative SAN genes, further sequence and Southern analyses would be performed.

The seventeen putative SAN cDNA clones were plaque purified and only five clones were successfully amplified by the PCR method. Successful amplification was judged from the existence of one sharp band on an agarose gel. Only a few inserts were successfully amplified perhaps due to the cloning of very small inserts or to insufficient PCR conditions used for the amplification of all the inserts. In the span of this study only one putative SAN cDNA clone was analyzed extensively.

SAN cDNA clone 8-4 was chosen for further analysis because its PCR amplified insert was approximately 900 bp, the largest of the five clones that were successfully PCR amplified. More sequence information of the putative SAN cDNA would facilitate the characterization of the corresponding protein.

One other cDNA clone was characterized which showed decreased expression with senescence. Please see the Appendix for more information on the analysis of this putative late nodulin, clone 7-2.

3.5 Differential Expression of SAN cDNA 8-4

Northern analysis was used to demonstrate the increased expression of the putative SAN cDNA 8-4 with nodule senescence. Figure 3.1 shows the increased signal of the hybridizing message at 1.4 kilobase (kb). At day 22, there is no detectable signal. From day 24 to day 40, a low level of the hybridizing mRNA is detected with increased intensity during senescence. The analysis was repeated three times with new blots and new probes, giving the same results. Although a weak signal was seen at 48 hours, no over-exposures of the blots were performed.

Since the hybridizing mRNA is 1.4 kb and the PCR product of the SAN clone 8-4 is estimated to be approximately 900 bp, it is not full length. Sequence analysis will determine the actual length of the insert, as described in Section 3.6.

The Northern analysis detected the expression of SAN 8-4 in the nodules but not in root or stem tissue. The sensitivity of the Northern analysis may be insufficient to detect expression of SAN 8-4 in other tissues.

Differential expression of SAN 8-4 was also tested in

induced nodule senescence with the addition of 20mM ammonium nitrate (Figure 3.2). By Northern analysis, the hybridizing message at 1.4 kb was most evident at 24 hours of induction. The signal corresponding to the hybridizing message of SAN 8-4 was weaker than in the natural system.

Although a weaker signal for the hybridizing message of SAN 8-4 was detected in the induced system, perhaps the maximal expression of SAN 8-4 in induced nodule senescence may be observed if more time points were taken during the time course experiment.

3.6 Southern Blot Analysis of SAN 8-4

Four cultivars of soybean were tested to demonstrate the restriction fragment length polymorphism of SAN 8-4. DNA from soybean cv. Maple Arrow, cv. Maple Don, cv. Ac Bravor and cv. Harosoy were digested with BamHI, EcoRI or HindIII. The Southern blot was hybridized to the SAN 8-4 probe (performed by Dr. D.A. Johnson) and the band patterns analyzed.

Southern analysis showed that all four sources of DNA gave the same band patterns with each restriction digest (Figure 3.3). No polymorphism was detected with these restriction enzymes.

The SAN 8-4 probe strongly hybridized to DNA fragments of 12.5, 6.0, 4.2, 3.5 kb of EcoRI-digested genomic DNA. For the HindIII digests, SAN 8-4 strongly hybridized to

FIGURE 3.3 SOUTHERN BLOT ANALYSIS OF SAN cDNA 8-4

Southern transfer analysis was performed on four soybean cultivars: A. Maple Arrow

B. Harosoy

C. AC Bravor

D. Maple Donovan

10 μ g of total DNA was digested with four restriction enzymes: 1. BamHI

2. EcoRI

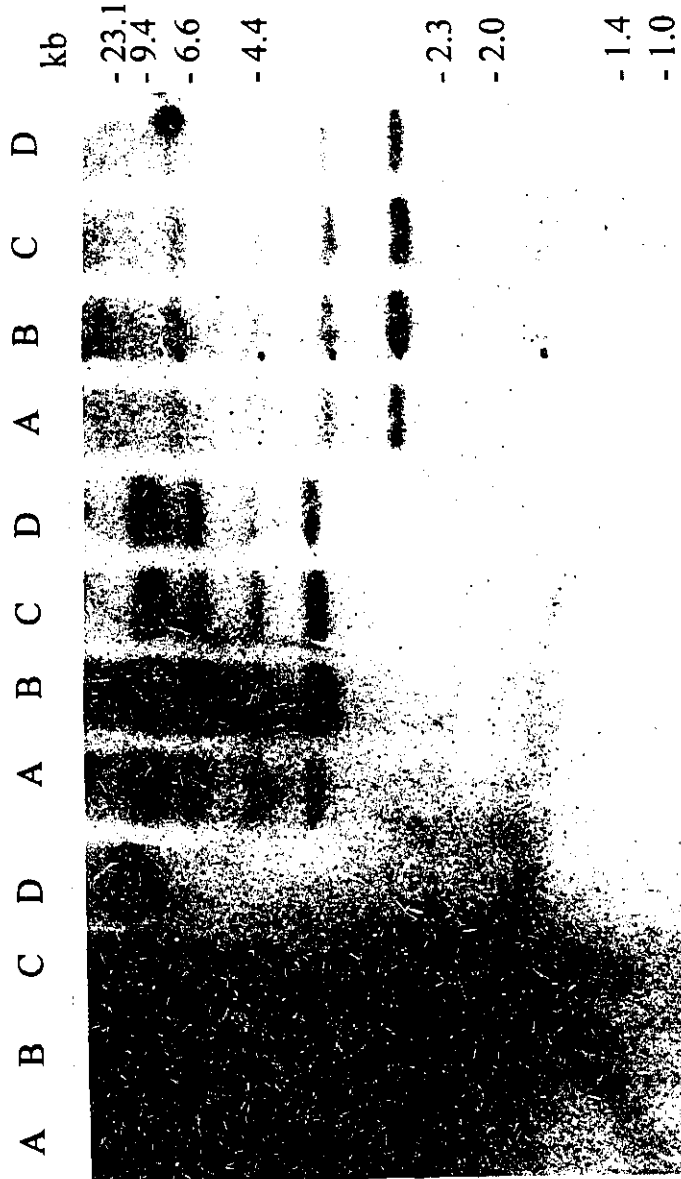
3. HindIII

An extended incubation period with maximum number of units for digestion was performed to ensure complete cutting of the DNA. 50 units of each enzyme was used in the first round of digestion with an additional 20 units and 3 hours in a sequential incubation. Hybridization was performed under standard conditions with a probe made from the PCR product of the SAN cDNA 8-4 clone. The final wash conditions are as follows: 10mM Tris-HCl, 1mM EDTA, 0.5% Sarcosyl (pH8.0) at 63°C.

1

2

3



fragments of 3.6 and 2.6 kb and weakly to fragments of 7.0, 4.3, 1.75 and 0.84 kb, respectively. These observations suggested that SAN 8-4 may belong to a small gene family. See Section 4.2.2 for a possible explanation. A single band at high molecular weight was observed when the SAN 8-4 probe was hybridized to the DNA fragments of the BamHI digest.

3.7 Sequence Analysis of SAN cDNA 8-4

Preliminary analysis involved the subcloning of PCR products into plasmid vectors. This analysis when combined with restriction analysis and Southern blotting with the original clone SAN cDNA 8-4 suggested that during the cloning process, one of the polylinkers (5' to the insert on the standard λ map) was missing. Therefore, our strategy to sequence both strands of the insert relied upon subcloning and sequencing of restriction fragments giving clones 8-4-12, 8-4-1/3, PS1G1 and PS1G17 and the use of synthesized oligonucleotide primers (see Figure 3.4). The size of SAN cDNA 8-4 was determined to be 935 bp. Figure 3.5 shows the final DNA sequence of SAN cDNA 8-4.

3.8 Analysis of the Conceptual Protein Sequence of SAN cDNA 8-4

The resulting DNA sequence was translated in all six reading frames and subsequently used to search protein banks using the BLAST algorithm (see Methods and Materials). The

FIGURE 3.4 SEQUENCING STRATEGY MAP OF SAN cDNA 8-4

The 935 bp sequence of SAN cDNA 8-4 was attained through the sequencing of various subclones: PS1G17, PS1G1, 8-4-1/3-3 and 8-4-12. G1F1 and G1R1 were internal primers designed to walk the sequence. Both strands of each subclone were sequenced.

λgt11 DNA SAM cDNA 8-4

PSIG17

PSIG1

8-4-1/3-3

8-4-12

↑ GIP1 ↓ GIP1

500 bp

FIGURE 3.5 THE cDNA SEQUENCE AND CONCEPTUAL PROTEIN SEQUENCE OF SAN 8-4

Shown is the cDNA sequence and the translation sequence of SAN cDNA 8-4. Starting at position 1, the number on the left indicate the nucleotide position and the bold number indicate the amino acid residue position. The ORF is in the +3 reading frame thus nucleotide one is unknown and is represented by a period. The stars represent stop codons. The segment containing 13 stop codons was not translated. The final three stars represent the stop codon followed by the 3' untranslated region. The underlined region is the putative polyadenylation site.

The bolded nonsense sequence correspond to a possible intron. ()₁ is the consensus sequence for a possible sulfation site Y. ()₂ is the consensus sequence for a possible phosphorylation site S.

SAN 8-4 Sequence

1 .TT GAG GAA TTT TTC ATC AAA GAT CAA ACT AGC TTT ATT CGA CTC AAC CAC
 1 E E F F I K D Q T S F I R L N H

 51 TAT CCT CCA TGC CCT TAC CCT GAC CTT GCT CTT GGC GTC GGT CGA CAC AAG
 17 Y P P C P Y P D L A L G V G R H K

 102 GAC CCC GGT GCC TTG ACC ATT CTT GCA CAG GAC GAA GTT GGA GGA CTT GAA
 34 D P G A L T I L A Q D E V G G L E

 153 GTG AGA CGT AAA CGG GAT CAA GAG TGG ATT AGA GTG AAA CCA ACC CCA AAT
 51 V R R K R D Q E W I R V K P T P N

 204 GCT TAT ATT ATC AAC ATT GGT GAT ACT GTT CAG GTG CCA GTG CAA TAC CAT
 68 A Y I I N I G D T V Q V P V Q Y H

 255 ATT GAA AAA TGA AAA TGT TAA ATT TCA CTT ACA CAC TAG TTG TAA CAC ACT
 85 I E K *

 306 CTA TCA TTG GTT TAA ATT AAT TGA AAA CTA TAA AAT CAT GAG AGA GAC ATT

 357 AAA TAA GAT GTG AGA ACA ACA ACT TTC TTG TAA TTT CCC ATA AAT TTA AAC

 408 CAA TGA TAG AGT GTG TTA GAG AGT GTG TTG CTA GCA TTT CTC TAA GGA AGA

 459 AAA ACT TTC ACT TGA GGA TTG GGG TTG CTT GTG CAG GTT TGG AGC AAT GAT
 88 * G L G L L V Q V (W S N D

 510 GCA TAT GAG AGT GTG GAT CAC AGA GTG GTG GTC AAC TCT GAG AAG GAA AGG
 100 A Y E S V D H)₁(R V V V N S E K E R

 561 GTT TCC ATT CCG TTC TTC TTC TTC CTT GCA CAC GAC ACC AAA GTG AAG CCT
 117 V)₂ S I P F F F F P A H D T K V K P

 612 TTG GAG GAG CTG ATA AAT GAA CAA AAC CCT TCA AAA TAT AGG CCA TAC AAT
 134 L E E L I N E Q N P S K Y R P Y N

 663 TGG GGC AAG TTT CTT GTC CAC AGA GGG AAC AGC AAT TTC AAG AAG CAA AAT
 151 W G K F L V H R G N S N F K K Q N

 714 GAG GAG AAC ATT CAA ATT CAT CAT TAC AAG ATA GCT TAA AGG GTA GTA TAT
 168 E E N I Q I H H Y K I A ***

 765 TAG CAG GAT TCC AGC GCC ATA TAG CTT ATG CAT ATA AAT TGG GTA TGA TAT

 816 CAT GAT ATG TAG CAG CGT TGA TCC AAG ACA ACG TTT CTG ATA TAA ATG ACT

 867 GAG TCA AAT TTA CTT CCT AGT GTG CCT TAA TGT ATG CAA GTA TCA CCC AAT

 918 AAA ACA ACT AAA TTA TTA

open reading frame (ORF) which showed significant similarity to a known protein was found in position +3.

Comparison of the newly determined sequence with sequence banks may be useful in deriving information on function or structure. The cDNA sequence was searched in Genbank and EMBL DNA databases and the amino acid sequences of all six reading frames for SAN cDNA 8-4 were searched in SWISSPROT and PIR protein databases according to the BLAST algorithm (Altschul et al., 1990). The last search was performed on March 23, 1995. The conceptual protein sequence in the +3 reading frame of SAN cDNA 8-4 showed most significant similarity to an enzyme known as flavanone 3-hydroxylase (F3H) in *Dianthus caryophyllus* (Stich et al., 1992) (Figure 3.6 A). The BLAST search found two blocks in the alignment that were significantly related between the conceptual protein sequences. The first block showed 50% similarity over a stretch of 69 amino acids and the second block showed 55% similarity over 29 amino acids. Between these two blocks is nonsense sequence which contain 13 stop codons. As described in Section 4.3, the nonsense sequence may represent a possible intron. If so, the removal this sequence improves the alignment by eliminating the gap. Figure 3.6 B illustrates the alignment of a small segment of DNA which was not detected by the BLAST search but was found upon the manual removal of the suspect nonsense sequence. Significance is dependent on the High Score value determined

FIGURE 3.6 **ALIGNMENT OF THE CONCEPTUAL PROTEIN SEQUENCES
OF SAN 8-4 AND F3H OF *Dianthus caryophyllus***

A. The amino acid sequences start at position 1. Because SAN cDNA 8-4 is not full length, its position 1 starts midway through the sequence of F3H of *Dianthus caryophyllus* (accession X72592). The dash lines '-' represent the gaps introduced into sequence X72592 when aligned with SAN cDNA 8-4. The upper case letters indicate the motifs present in both sequences which show high similarity. The '*' represent stop codons. The '~' represent the 3' untranslated regions. The bold nonsense sequence represents a possible position for an intron.

B. With the removal of the nonsense sequence, the following alignment results. The numbers flanking the block indicate the amino acid positions of each end residue. '#' indicates conserved amino acid residues. '+' indicates conservative amino acid substitutions.

by the PAM120 matrices and the number of amino acid residues in each sequence that is aligned (Doolittle, 1986, pp.13-14). According to this reference, the alignment was significant. The SAN cDNA 8-4 conceptual protein also aligned significantly with 7 other sequences all of which were F3H's from various plant species (Table 1).

Starting at nucleotide position 264, 13 stop codons were found over a span of 210 nucleotides (Figure 3.6). These were not due to sequence errors since this area was sequenced in both strands. The presence of stop codons in the middle of SAN cDNA 8-4 was alarming. This stretch of DNA, independent of the rest of the clone, was used to search Genbank and EMBL to attempt to identify its origin. No significant match was found. A possible explanation for the origin of this region will be discussed in Section 4.3.

F3H belongs to a class of enzymes known as dioxygenase that are 2-oxoglutarate dependent (E.C.# 1.14.11.9). The next most similar sequences were also dioxygenases. Although SAN 8-4 is not likely hyoscyamine 6-dioxygenase, gibberellin 20-oxidase, aminocyclopropane 1-carboxylate oxidase or flavonol synthase, blocks of similarity amongst all 15 sequences were found using the MACAW program (Schuler et al., 1991). Recently the same motifs were also reported in dioxygenases (Britsch et al., 1993). The aligned motifs and their relative positions within the individual sequences are highlighted in Figure 3.7.

**TABLE 1 PROTEIN SEQUENCES THAT SHOW SIGNIFICANT SEQUENCE
SIMILARITY TO THE CONCEPTUAL PROTEIN SEQUENCE OF
SAN cDNA 8-4**

The following is a list of various 2-ODDs and related enzyme which align significantly to the conceptual protein sequence of SAN cDNA 8-4. Their accession numbers in EMBL, SWISSPROT or PIR databases are indicated along with the organism and enzyme names. L_n is the normalized length of the protein sequence to that of SAN 8-4. The score of the alignment was found through the BLAST algorithm based on PAM 120. The level of significance of each sequence to SAN 8-4 was aided by a simple reference to Doolittle, 1986, pp. 13-14. The reference contains a plot of alignment score versus L_n . This graph is a rough guide to the significance in the comparison of amino acid sequences.

Table 1. Protein sequences that show significant sequence similarity to the conceptual protein sequence of SAN cDNA 8-4.

Accession number	Species	Protein	L _n	Score	Identity of SAN 8-4
X72592	<i>Dianthus caryophyllus</i>	Flavanone 3-Hydroxylase	204	210	probable
X72593	<i>Callistephus chinensis</i>	Flavanone 3-Hydroxylase	199	207	probable
X72594	<i>Matthiola incana</i>	Flavanone 3-Hydroxylase	199	201	probable
X69664	<i>Malus</i> sp.	Flavanone 3-Hydroxylase	203	198	probable
X75965	<i>Vitis vinifera</i>	Flavanone 3-Hydroxylase	203	198	probable
X60512	<i>Petunia hybrida</i>	Flavanone 3-Hydroxylase	206	196	probable
U04434	<i>Zea mays</i>	Flavanone 3-Hydroxylase	208	192	probable
X58138	<i>Hordeum vulgare</i>	Flavanone 3-Hydroxylase	211	192	probable
X81812	<i>Medicago sativa</i>	Flavanone 3-Hydroxylase	199	189	marginal
X78994	<i>Medicago sativa</i>	Flavanone 3-Hydroxylase	199	187	marginal
P24397	<i>Hyoscyamus niger</i>	Hyoscyamine 6-dioxygenase	192	141	improbable
X73314	<i>Cucurbita maxima</i>	Gibberellin 20-oxidase	215	139	improbable
X13437	<i>Lycopersicon esculentum</i>	Aminocyclopropane 1-carboxylate oxidase	203	128	improbable
S33510	<i>Petunia hybrida</i>	Flavonol synthase	194	127	improbable

**FIGURE 3.7 MOTIFS PRESENT IN SAN 8-4, 2-OXOGLUTARATE
DEPENDENT DIOXYGENASES AND RELATED PROTEINS**

Shown are three blocks of highly similar amino acid sequence motifs present in SAN 8-4, F3H, Hyoscyamine 6-dioxygenase, gibberellin 20-oxidase, ACC oxidase and flavonol synthase. The accession numbers for the sequences are indicated. (see Table 1 for more details) The numbers indicate the amino acid positions in each protein sequence. '#' indicates positions that are conserved amongst all the sequences. '|' indicates positions where the residue of SAN 8-4 is identical to one of the other sequences. '+' indicates positions of conservative amino acid substitutions

SAN 8-4	17	YPPCP
X72592	203	YPKCP
X72593	200	YPKCP
X72594	201	YPKCP
X69664	203	YPKCP
X75965	203	YPQCP
X60512	205	YPKCP
U00434	208	YPRCP
X58138	205	YPRCP
X81812	203	YPKCP
X78994	203	YPKCP
P24397	215	YPPCP
X73314	228	YPTCD
X13437	221	YPPCP
S33510	219	YPPCP

##|#|

SAN 8-4	32	HKDPGALTILAQDEVGGLE
X72592	218	HTDPGTITLLLQDQVGGLQ
X72593	215	HTDPGTITLLLQDQVGGLQ
X72594	216	HTDPGTITLLLQDQVGGLQ
X69664	218	HTDPGTITLLLQDQVGGLQ
X75965	218	HTDPGTITLLLQDQVGGLQ
X60512	220	HTDPGTITLLLQDQVGGLQ
U00434	223	HTDPGTITLLLQDLVGGLQ
X58138	220	HTDPGTITLLLQDLVGGLQ
X81812	218	HTDPGTITLLLQDQVGGLQ
X78994	218	HTDPGTITLLLQDQVGGLQ
P24397	231	HYDGNLITLL-QQDLPGLQ
P73314	243	HTDPTSVTILHQDPVSGGLQ
X13437	236	HTDIGFVTILLQDDMGGLQ
S33510	234	HTDMSYITILVPNEVQGLQ

#| |++#|#+| |||##

SAN 8-4	106	HRVVVNSEKERSIPFF
X72592	276	HQAVVNSECSRLSIATF
X72593	273	HQAVVNSSTSRLSIATF
X72594	274	HQAVVNSNSSRLSIATF
X69664	277	HQAVVNSNSSRLSIATF
X75965	276	HQAVVNSNHSRLSIATF
X60512	278	HQAVVNSNSSRLSIATF
U00434	281	HQAVVNSECSRLSIATF
X58138	278	HQAVVNGESSRLSIATF
X81812	276	HQAVVNSNHDRLSIATF
X78994	276	HQAVVNSNHDRLSIATF
P24397	288	HRVVTDPTRDRVSIATL
P73314	299	HRAVVNSMNARKSLAFF
X13437	292	HRAISNNVGSRMSITCF
S33510	290	HRTTVNKDKTRMSWPVF

#| || || || || |+##| || ||

3.9 Motifs of SAN cDNA 8-4 Conceptual Protein Sequence

Sequence motifs that had been found in members belonging to the class of enzymes known as dioxygenases (Britsch et al., 1993) also exist in SAN 8-4 (Figure 3.7). They include: two conserved histidines at positions 32 and 106 (SAN 8-4); an aspartate at position 34 (SAN 8-4); an R-X-S tripeptide 10 residues from histidine 106 (SAN 8-4); leucine 41 (SAN 8-4); glutamine 43 (SAN 8-4); and proline 18 (SAN 8-4).

In Figure 3.6, an alignment of SAN 8-4 and F3H of *Dianthus caryophyllus* shows the conserved regions in upper case letters. Histidines 78, 220 and 278 of the *Petunia* sequence (X60512) are highly conserved in dioxygenases and ACC. Only histidine 220 and 278 were found in SAN 8-4 (histidine 32 and 106) since the proposed cDNA of SAN 8-4 was not full length. Aspartate 222 (X60512) is also highly conserved. The conserved aspartate residue is at position 34 in SAN 8-4. An R-X-S tripeptide is present in the related proteins ten residues after the last conserved histidine (ie. position 116 of SAN 8-4). The importance of these motifs in iron binding will be discussed in Section 4.

Leucine 227 (SAN 8-4), glutamine 43 (SAN 8-4) and proline 18 (SAN 8-4) are also strictly conserved in the aligned sequences. In the above reference, it was proposed that these residues were important in the folding process of the polypeptide.

These blocks of similar sequences were also found when SAN 8-4 was aligned with dioxygenases and ACC. Amino acid residues in the motifs of SAN 8-4, dioxygenases and ACC (Figure 3.7) were categorized in the following groups. There are 41 positions for amino acid residues in the three blocks of sequences. Of these:

i. 35 residues are identical to at least 1 of the other aligned sequences at the same position;

ii. of these 35, 12 of the positions contain identical amino acids amongst all of the aligned sequences at the same position;

iii. 4 of the 41 positions in SAN 8-4 contain conservative amino acid substitutions (as characterized by the similar chemical properties of the amino acid side chains) with at least one of the other aligned sequences at the same position; and,

iv. 2 of the 41 amino acid residues of SAN 8-4 are not conserved or conservative with any of the other sequences at the same position.

Of the 6 unique residues in SAN 8-4, lysine 33 and glutamate 50 are the only positions that are not conserved or conservative amino acid substitutions. Conservative amino acid substitutions are found at leucine 38 (SAN 8-4) with all the aligned sequences at the same position. The following lists the amino acid positions in SAN 8-4 that are conservative substitutions only with the protein sequences

mentioned: alanine 37 for leucine 237 (Hyoscyamine 6-dioxygenase in *Hyoscyamus niger*); alanine 42 for leucine (all F3H sequences and ACC oxidase in *Lycopersicon esculentum*) and for valine 244 (flavonol synthase in *Petunia hybrida*); glutamate 115 for aspartate (F3H in *Medicago sativa* and Hyoscyamine 6-dioxygenase in *Hyoscyamus niger*).

Prosite Version 5.10 (November 1993) was also employed to look for sites and signatures in SAN cDNA 8-4 conceptual protein. This search identified two possible modification sites (Figure 3.5):

1. a tyrosine sulfation site at tyrosine 101 in the consensus sequence from residues 96-106; and,
2. a phosphorylation site at serine 112 in the consensus sequence from residues 106-117.

Although these motifs are not present in the other sequences of the alignment the proposed sites may still show the possible significance in the protein function of SAN 8-4 (see Section 4.3.1).

CHAPTER FOUR:

DISCUSSION

A putative SAN (senescence associated nodulin) cDNA was isolated from the differential screening of a λ gt11 library representing mRNA present in senescent soybean nodules and named SAN cDNA 8-4. The two bulk probes used in the screening were PCR amplified from two λ gt10 libraries: one from soybean nodules that were healthy and actively fixing nitrogen and the other from senescent nodules. Northern analysis demonstrated the increased expression of the gene corresponding to SAN cDNA 8-4 with nodule senescence. The hybridizing message was 1.4 kb. Southern analysis with the genomic DNA of four soybean cultivars of known genotype detected no polymorphism in the BamHI, EcoRI and HindIII sites in or flanking the SAN 8-4 gene. Evidence from Southern analysis suggest that SAN 8-4 belonged to a small gene family. The DNA sequence of the clone was translated into the 6 possible reading frames and the conceptual protein sequences were used to search SWISSPROT and PIR protein banks available through the BLAST algorithm. The conceptual protein sequence of SAN 8-4 showed significant similarity to flavanone 3-hydroxylase (F3H), a known 2-oxoglutarate dependant dioxygenase. Sequence motifs in SAN 8-4 and in F3H were also present in other members of the class of enzymes known as dioxygenases. The importance of

SAN 8-4 in nodule senescence will be discussed in the context of the possible identification of its gene product.

4.1 Characterization of SAN 8-4

4.1.1 Expression of SAN 8-4 During Nodule Senescence

Northern analysis demonstrated the increase in the signal of the hybridizing mRNA at 1.4kb to clone SAN 8-4 with senescence of the soybean nodule. By scanning the intensity of the band corresponding to the hybridizing mRNA in a Northern analysis, SAN 8-4 expression was greater in the natural than in the induced system.

Northern analysis is not sensitive enough for quantitation of mRNA present. Autoradiographic film exhibits a saturation limit, thus, the measurement of total disintegrations in radioactivity versus the scanned value is non-linear (Johnston *et al.*, 1990). Although a trend can be demonstrated, the amount of the hybridizing mRNA cannot be quantitated accurately.

The collection of more time points during the initiation of senescence, especially in the induced system will aid in the detection of transient expression of SAN 8-4. Clearly, a more accurate quantitation of mRNA present may allow the comparison of mRNA species between the naturally and induced senescent systems. A proposed method of circumventing the inaccuracy of quantitation is by quantitating specific mRNAs using the technique known as

competitive PCR with the use of reverse transcriptase (Galliland et al., 1990). The strategy involves the co-amplification of a competitor template with the same primers as the target cDNA. The products of the amplification can be distinguished by size or by differences in the restriction patterns on an agarose gel. The relative amounts of the PCR products can be determined by the counting of the radioactive signal incorporated into the product if the reaction was performed with a radiolabelled dNTP. By amplifying the target template alongside a dilution series of competitor DNA of known concentration, the original concentration of the target cDNA is the point of equivalence where the amount of products for both templates is the same (1:1 ratio).

SAN 8-4 expression was not detected in root or leaf tissue by Northern analysis. This suggests that SAN 8-4 is a nodulin gene.

In section 3.6, it was found through Southern analysis that SAN 8-4 belongs to a small multigene family. Northern analysis only showed one hybridizing band at 1.4kb. This may either be a result of one mRNA species or several mRNA species of similar size, from the expression of various members of the gene family.

4.1.2 Southern Analysis of SAN 8-4

Southern analysis of SAN 8-4 with four cultivars of soybean of known genotype, revealed no polymorphism in the BamHI, EcoRI or HindIII sites in or flanking the gene. This gene is well conserved amongst the cultivars.

The EcoRI digestion gave four hybridizing bands, all of equal intensity. These hybridizing sequences may represent genes at four different loci. This suggests that the SAN 8-4 gene belongs to a small gene family. Since there were no EcoRI sites within the cDNA clone, the sites may exist in the introns or in the regions flanking the gene. Because the hybridizing sequences were greater than 3.0 kb, it is unlikely that the EcoRI sites are in the introns because that would imply that the introns are greater than 1.6kb. This is not likely since typically, plant introns are between 70bp to 1kb in length (Hawkins, 1988).

Digestion with HindIII resulted in a complicated pattern of six bands. Varying band intensities may be caused by sequence variations in the members of the multigene family. Members of the gene family with HindIII sites at identical positions in the gene or in the flanking regions of the gene may give bands of greater intensity. Weaker bands may be a result of fewer DNA fragments from members that have unique HindIII sites. Partial digestion of the genomic DNA is not possible since digestion was performed twice, with excess amounts of each restriction

enzyme. These bands may be a result of weak hybridization of the probe to fragments in the digest that contain shorter regions corresponding to the cDNA. The standard conditions for hybridization and washing may have been too stringent for the binding of the probe to this short region.

Hybridization of the probe to the BamHI digest of the genomic DNA gave a single band of high molecular weight. Although this result does not show that SAN 8-4 belongs to a multigene family, it may suggest the presence of a methylated C residue in the BamHI sites flanking the gene. Soybean genomic DNA is known to be highly methylated at C residues in the sequences CpG and CpNpG (McLelland, 1983).

Because of the band patterns shown on this Southern analysis, it is speculated that SAN 8-4 belongs to a gene family of four members. Four bands of equal intensity were seen in the digest with EcoRI. The band patterns observed in the Southern analysis is typical of multigene families (Blank et al., 1993).

The evidence provided in this Southern only suggests that SAN 8-4 is a member of a multigene family. To provide unequivocal evidence of the number of members, the genomic clones whose band patterns are represented in this Southern must be isolated.

4.2 The Conceptual Protein Sequence of SAN 8-4

The translation of SAN cDNA 8-4 in the +3 reading frame produced the longest ORF which aligned with the amino acid sequence of F3H from *Dianthus caryophyllus* (see Section 3.7, sequence X72592). Interestingly, this ORF is interrupted by 13 stop codons in a span of 210 nucleotides (nt). The alignment of SAN 8-4 amino acid sequence and X72592 showed that these 210 nt divide the proposed F3H amino acid sequence.

This insertion could not be a result of coligation during the cloning procedures (ie. into λ gt11 or M13mp19). The chance that three individual DNA fragments were coligated, two of which code for the same protein preserving an ORF at position +3 is unlikely.

Recently, an attempt to PCR amplify this region from genomic DNA of soybean produced a fragment whose size is identical to that in the sequence of SAN cDNA 8-4 (personal communication, D.A. Johnson). The result suggests that this region exists in the soybean genome.

There is no previous documentation of introns being present in a cDNA. Upon closer inspection of the cDNA sequence there is no polyA tail but a possible polyadenylation signal is found at nucleotide position 915 in Figure 3.5 (Joshi, 1987). SAN cDNA 8-4 may not be a bonafide cDNA clone but the clone of a DNA fragment. The total RNA samples used to construct the cDNA libraries were

not digested with DNase, thus it is possible that the RNA extraction procedure did not remove the DNA. If the 210 nt region represents a plant intron, the consensus sequences of the intron splice sites should be present: GT at the 5' end, and AG at the 3' end (reviewed in Hanley and Schuler, 1988). In Figure 3.5, a GT is present at positions 237-238, and an AG is present at positions 493-494. Upon closer inspection, the nucleotides surrounding the splice sites are also consensus sequences AGGT...PyNCAGGN (Py=pyrimidine, N=nucleotide). From the -3 to -20 positions from the 3' splice site, there are 9 purines, suggesting that this intron may belong to the Class III group of introns. Assuming these splice sites, the +3 ORF is maintained. Not only will the removal of the proposed intron sequence eliminate the nonsense sequence, but an alignment of SAN 8-4 with the F3H conceptual protein sequence of *Dianthus caryophyllus* is improved with the elimination of a gap. In Figure 3.6, elimination of amino acid residues 79-93 result in an alignment of 14 residues without the introduction of gaps. Three amino acids are conserved at the same positions between SAN 8-4 and F3H of *Dianthus caryophyllus* and 3 are conservative amino acid substitutions. (Hanley and Schuler, 1988). Sequence comparison of F3H genes will provide information on the splice sites in various members of the gene family and amongst different plants. To date, no genomic copy of the F3H gene has been isolated.

SAN cDNA 8-4 could also represent an expressed pseudogene. As reviewed by W. Li, 1983, a pseudogene is a DNA segment that shows high homology to a functional gene but contains defects such as nonsense and frameshift mutations that prevent it from producing a functional product. The majority of pseudogenes studied to date have features suggesting that they were derived from gene duplication and subsequent loss of function of redundant copies. Since SAN 8-4 was shown to belong to a small multigene family (Section 3.), it is possible that SAN 8-4 is an expressed pseudogene arising from gene duplication.

SAN 8-4 may code for a non-functional protein. The probe used for Northern and Southern analysis may contain an intron. This does not affect the outcome of the analyses for the differential expression of the gene corresponding to the hybridizing message. Northern analysis did demonstrate that a hybridizing mRNA of 1.4kb was up-regulated with senescence. Since SAN 8-4 belongs to a multigene family, northern analysis may have also detected the expression of another member of the same family. It is possible that the hybridizing mRNA may code for a functional protein of another member of the gene family. A possible role in nodule senescence will be discussed in Section 4.4.

4.3.1 Protein Sequence Alignment for the identification of the conceptual protein of SAN 8-4

The DNA sequence of SAN cDNA 8-4 was translated in all six reading frames and used to search SWISSPROT and PIR protein banks available through the BLAST algorithm. Entries up to March 23, 1995 were used in the search. The substitution matrix used was PAM120. The conceptual protein of SAN cDNA 8-4 showed most significant similarity to a protein known as flavanone 3-hydroxylase (F3H) in *Dianthus caryophyllus* (Stich et al., 1992). Because SAN cDNA 8-4 contained a stretch of 210 nt of 13 stop codons, the amino acid sequence was divided into two blocks. The first block of sequence showed 50% identity and 69% similarity over a span of 69 amino acids to the F3H of *D. caryophyllus*. The second block showed 55% identity and 62% similarity over a span of 29 residues. The significance of the match was based on a method proposed by Doolittle, 1986 which measures the level of significance of a match according to the lengths of the sequences in the alignment and its score calculated from the number of identities. According to this method, SAN 8-4 is significantly similar to F3H in *Dianthus caryophyllus* and in seven other plant species whose cDNA or protein has been isolated and sequenced (discussed in Section 4.4).

F3H belongs to a class of enzymes known as the 2-oxoglutarate dependent dioxygenase (E.C.# 1.14.11.9). Enzymes in this group require ferrous iron and a reducing

agent (usually ascorbate) and utilize 2-oxoglutarate and molecular oxygen as co-substrates (reviewed in Prescott, 1993). F3H catalyzes the 3 β -hydroxylation of 2S-flavanones to 2R,3R-dihydroflavonols which are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins in plants (Heller and Forkmann, 1988). With the incorporation of molecular oxygen, the substrates naringenin and eriodictyol are converted to dihydrokaempferol and dihydroquercetin, respectively.

Both the cDNA and enzyme of F3H had been isolated and sequenced in *Petunia hybrida* (Britsch et al, 1992). The amino acid sequence showed 75% similarity to F3H protein sequence in *Hordeum vulgare* (Meldgaard, 1992). cDNAs from *D. caryophyllus*, *Callistephus chinensis* and *Matthiola incana* have been isolated and sequenced (Britsch et al., 1993). The protein search also found conceptual protein sequences of F3H from *Malus* sp., *Vitis vinifera*, *Zea mays*, and *Medicago sativa*, all of which are unpublished.

The next most similar sequences to SAN 8-4 found in the protein banks, were also 2-oxoglutarate dependant dioxygenases (2-ODDs) or were closely related to this class of enzymes. Hyoscyamine 6-dioxygenase (Matsuda et al., 1991), gibberellin 20-oxidase (Lange et al., 1994) and flavonol synthase are 2-ODDs. 1-aminocyclopropane 1-carboxylate oxidase (ACC), although having significant sequence similarity to F3H (Hamilton et al. 1990) is not

dependent on 2-oxoglutarate (McGarvey and Christoffersen, 1992). The cDNAs for the above enzymes have been isolated. F3H had been shown to exhibit 30% amino acid similarity to the mentioned 2-ODDs and related enzymes (Britsch et al. 1993).

Motifs were found amongst the various sequences in the alignment. Conserved histidine and aspartate residues were found in the sequences of SAN 8-4, F3H and other 2-ODDs and ACC. Spectroscopic investigation of purified isopenicillin-N synthase (IPNS) strongly suggested the involvement of three histidines and an aspartic acid in the iron-binding site of this enzyme (Ming and Que, 1991). IPNS is also a 2-ODD. All 2-ODDs and ACC are dependent on ferrous iron for its activity. Because SAN 8-4 was not a full-length clone, the 5' end is missing; therefore, the first conserved histidine is absent in the alignment. Other motifs common in 2-ODDs include a leucine 230/glutamine 231 (X60512) dipeptide, and conserved prolines at positions 151 and 206. These residues were predicted to be important in the folding of the peptide (Britsch et al., 1993). Instead, SAN 8-4 has an alanine/glutamine dipeptide and it is unknown whether conserved prolines are in SAN 8-4 since the sequence was not full length. An alanine-leucine exchange in the dipeptide consensus sequence is a conservative amino acid exchange since both have aliphatic side chains. It is suspected that the dipeptide in SAN 8-4 may maintain the same function.

Blocks of sequences highly conserved amongst 2-ODDs and related proteins were highlighted. The same blocks were found in SAN 8-4. The sizes of F3H cDNAs isolated so far range from 1.3-1.4kb. The hybridizing mRNA of SAN 8-4 is 1.4kb which falls within the expected range. Although the cDNA of SAN 8-4 is not full length, the alignment of its conceptual protein sequence showing high similarity with F3H and the presence of motifs characteristic of 2-ODDs and related proteins show that SAN 8-4 is an F3H related protein that may belong to the class of enzymes known as 2-ODDs. To date, the consensus sequence for a 2-oxoglutarate binding site has not been identified in 2-ODD protein sequences; thus, we cannot predict whether SAN 8-4 is 2-oxoglutarate dependent by means of searching for this signature.

A search through Prosite Version 5.10 (November 1993) to look for sites and signatures in SAN cDNA 8-4 conceptual protein identified a possible tyrosine sulfation site and phosphorylation site. Tyrosine sulfation is a post-translational modification (reviewed Huttner, 1987). The reaction is catalyzed by tyrosylprotein sulfotransferase in the Golgi apparatus. Most tyrosine-sulfated proteins are secretory.

Phosphorylation by protein kinase C (reviewed Pinna, 1990) and by casein kinase II (Kishimoto et al., 1985) involves the addition of phosphate groups to protein

substrates. There is no experimental evidence for these modifications.

4.4 The Biochemistry of Flavanone 3-Hydroxylase (F3H) and other Related Proteins

Flavanone 3-hydroxylase (F3H) catalyzes the hydroxylation in the 3-position of flavanones such as naringenin, eriodictyol and 5,7,3',4',5'-pentahydroxy flavanone to yield dihydroflavonols. These compounds are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins. Compounds of the flavonoid pathway have been shown to affect the host-*Rhizobium* specificity, *nod* gene regulation and flower pigmentation.

F3H is an enzyme involved in flavonoid biosynthesis. Also known as naringenin 2-oxoglutarate 3-dioxygenase (E.C.# 1.14.11.9), F3H is a 2-oxoglutarate dependant dioxygenase requiring ferrous iron, oxygen and ascorbate (Heller and Forkmann, 1988). The purified protein from *Petunia hybrida* is a dimer composed of two identical subunits of 37 kilo Daltons (kD) (Britsch and Grisebach, 1986; Britsch, 1990; Britsch et al., 1992). Loss of expression of F3H in mutants of *Hordeum vulgare* deficient in anthocyanin and proanthocyanidin biosynthesis suggested that *ant 17* is the structural gene for a barley F3H (Meldgaard, 1991). As yet no F3H genes have been cloned. In petunia, the full length cDNA clone has been expressed in transformed *E. coli*

producing a fully functional protein (Britsch et al., 1992). This study did not determine the protein structure although the amino acid sequence showed 73.5% similarity to the conceptual protein sequence of barley F3H.

Previous studies which attempt to elucidate the biosynthetic pathway of flavonoids utilize the flower as the test system. Flavonoids have also been isolated from root exudates. Thus, these plant compounds are distributed in various plant tissues. This study is the first to find differential expression of a proposed enzyme involved in flavonoid biosynthesis with nodule senescence in soybean.

Hyoscyamine 6 β -hydroxylase (H6H) converts hyoscyamine to 6 β -hydroxyhyoscyamine during the biosynthesis of tropane alkaloids by *Hyoscyamus niger* L. (Hashimoto and Yamada, 1986).

Gibberellins (GA) form a large group of diterpenoid natural products. They function as hormones in plants controlling many aspects of development, including stem extension, fruit set, and seed germination (reviewed by Lange et al., 1994). In later steps of GA biosynthesis, gibberellin 20-oxidase oxidizes the GA skeleton at carbon 20 to produce physiologically active C₁₉ GAs.

Ethylene, a plant growth regulator, is produced upon wounding and senescence of plant tissues, and during seed germination and fruit-ripening (Burg, 1962). The ethylene biosynthetic pathway involves the synthesis of 1-

aminocyclopropane-1-carboxylic acid from S-adenosyl-L-methionine and the conversion of ACC to ethylene (Yang and Hoffman, 1984). Although ACC oxidase activity is dependent on ferrous iron, ascorbate and molecular oxygen, it does not need 2-oxoglutarate; thus, ACC oxidase is not a true ODD although it was shown to have significant similarity to F3H. ACC oxidase from fruit requires CO₂ as an activating agent to produce maximal activity (Dong et al., 1992).

Flavonol synthase is 2-ODD involved in the biosynthetic pathway of flavonoids. The enzyme converts dihydroflavonols to flavonols (Britsch et al., 1981).

In summary, the activities of these enzymes are strictly dependent on the presence of molecular oxygen. Although this is a preliminary study in isolating and characterizing SAN cDNA 8-4, perhaps the evidence which suggests that SAN 8-4 has sequence similarity to F3H and related enzymes could suggest that SAN 8-4 activity may also require molecular oxygen.

4.5 Postulated Role for SAN 8-4 in senescent soybean nodules

Protein sequence similarity and the presence of motifs characteristic to various 2-ODDs, including F3H and related proteins suggest that SAN 8-4 may be a 2-ODD or a related protein, such as ACC oxidase. Literature describing the substrates and products of these enzymes was consulted to

provide the background to our understanding of a possible role for SAN 8-4 in nodule senescence.

SAN 8-4 protein sequence was most similar to F3H and less similar to flavonol synthase. Both are involved in flavonoid biosynthesis. Flavonoids have been shown to regulate *nod* gene expression (reviewed Long, 1989). Flavonoids are also involved in the regulation of auxin transport which is essential for plant growth regulation (Jacobs and Rubery, 1988). SAN 8-4 also showed significant similarity, but less so, to gibberellin 20-oxidase and ACC oxidase. Gibberellin 20-oxidase is involved in gibberellin biosynthesis. GAs function as hormones in plants, that control many aspects of development (Crozier, 1983). ACC oxidase is involved ethylene biosynthesis. Ethylene, a plant growth regulator is produced in the senescence of plant tissue, and fruit ripening (Burg, 1962). All of the mentioned enzymes are involved in the regulation of plant development. It may be speculated that the role of SAN 8-4 is also important in the regulation of development, in particular in nodule senescence.

Plant flavonoids play an essential role in the nodulation processes in the plant-Rhizobium symbiosis (reviewed in Long, 1989). In soybean, natural inducers include an isoflavone, daidzein (Kosslak et al., 1987). The specificity of the *Rhizobium* response to inducers is correlated with the *nodD* allele. In *Rhizobium meliloti*,

there are multiple *nodD* genes (Mulligan and Long, 1989) which confer broad responsiveness to various plant hosts (Long, 1989). A model was proposed to describe the *nodD* gene product interaction with the plant signals (Burn et al., 1987). *nodD* is constitutively expressed producing a gene product which displays characteristics of a DNA binding transcriptional activator. According to this model, plant flavonoids in root exudates specific for certain *nodD* alleles of *Rhizobium* promote the expression of *nod* genes. The extent to which variations in *nodD* alleles amongst species and between strains contribute to the response to plant flavonoids is unknown.

Flavonoids such as naringenin and eriodictyol of the phenylpropanoid pathway have been implicated as *nod* gene inducers or inhibitors depending on the legume-*Rhizobium* system. The expression of *nod* genes is required for the early stages of nodule formation. Some flavonoids have been purified from species such as *Rhizobium meliloti* (Peters and Long, 1988), *Phaseolus vulgaris* (Hungria et al., 1991) and *Glycine max* (Kosslak et al., 1987) and the effects on the induction of *nod* genes have been demonstrated.

The above scenario is just one example of the interactions between plant and *Rhizobium* in the symbiosis. Could flavonoids also be used as regulatory molecules which regulate senescence in the nodule. Metabolic changes during nodule senescence involve the decline in nitrogen fixation

rates. If flavonoids inhibit the expression of *nif* genes in the endosymbiont, this would destroy its ability to sustain itself and result in the degeneration of the symbiotic relationship.

Flavonoids have also been shown to function as auxin transport regulators in plants (Jacobs and Rubery, 1988). Indole-3-acetic acid (IAA, or auxin) is a plant growth regulator produced in shoot and leaf tissue and transported basipetally to the target tissue where it exerts its many developmental effects (Davies, 1987). The mechanism of transport known as polar auxin transport (PAT) can be inhibited by a group of compounds which act by binding to the plasma membrane protein known as naphthylphthalamic acid (NPA) receptor. The result is a net accumulation of IAA in the transporting cells. This study demonstrated that specific structural requirements of the flavonoids dictate the ability of the metabolite to compete for binding to the NPA receptor and inhibit auxin transport in *Cucurita pepo* L. hypocotyl segments. Flavonoids, such as kaempferol, quercetin and apigenin perturbed auxin transport. These flavonoids are all products of successive enzymatic steps following the activity of F3H in the phenylpropanoid pathway. Interestingly, naringenin, the substrate of F3H, showed little effects on PAT.

The active flavonoids may act as natural regulators of PAT and auxin efflux from cells in plants. The flavonoids

would influence auxin distribution, thus modulating auxin-related phenomena from gene expression and ion transport to cell and organ differentiation (Davies, 1987). To date, there is no evidence for the effects of auxins on nodule development or nodulin expression. It is possible that auxin may affect SAN gene expression and ion transport, thus regulating the distribution of nutrients to developing sex organs and the maintenance of nodule tissue during senescence.

Gibberellin 20-oxidase produces a physiologically active GA through the oxidization of carbon-20 leading to loss of this atom and the formation of activated C₁₉ GA (Lange et al., 1994). Gibberellin is a plant hormone which controls many aspects of development including cell elongation (reviewed in Salisbury and Ross, 1985), and food mobilization in seed storage cells (Akazawa and Miyata, 1982). Nodules senesce under natural conditions in preparation for pod filling. Complex carbohydrates are converted to sugars and amino acids and mobilized to the seed. Gibberellins may function as a signal which promotes this process.

Ethylene is a potent plant growth regulator (Burg, 1962). This phytohormone is linked to the ripening of climacteric fruit (Picton et al., 1993) and the senescence of floral organs (Mattoo and Suttle, 1991). Ethylene has

also been shown to coordinate the transcriptional activation of several senescence-related genes (Lawton et al., 1990).

The ethylene biosynthetic pathway has been completely elucidated (reviewed by Kende, 1993). ACC oxidase catalyzes the conversion of ACC to ethylene. Although ACC oxidase possesses sequence motifs of 2-ODDs, it cannot be considered a true dioxygenase because it does not require oxoglutarate to function. Like dioxygenases, the enzyme does require ferrous iron, molecular oxygen and ascorbate.

The sequence homologies of SAN 8-4 and various closely related plant proteins presented in this thesis suggests that the DNA sequence of SAN 8-4 may be similar to a 2-ODD or a related enzyme. What should be emphasized in this comparison to elucidate the identity of SAN 8-4, is that all the proteins which did show significant homology strictly require molecular oxygen in their reactions. It has been shown during nodule senescence, that oxidation of leghemoglobin occurs resulting in its degeneration (Vance, et al, 1986). Leghemoglobin controls the concentration of free oxygen in the nodule. It provides a flow of oxygen to the bacteroids that balances protection of nitrogenase activity from oxygen damage with support of respiration (Nap and Bisseling, 1990). If this equilibrium is disturbed, as in nodule senescence, the level of Lb decreases in the tissue, perhaps at a rate which accomodates the necessary

concentration of oxygen that fuels the reactions which involve a dioxygenase.

Future studies in the identification of SAN 8-4 and additional genes associated with nodule senescence will provide more information on the functions involved in cellular maintenance during cellular senescence.

4.6 Future Studies to demonstrate that SAN 8-4 is an F3H-related protein involved in flavonoid biosynthesis

Presently the identification of the insert which contains 13 stop codons in the middle of the SAN 8-4 sequence is being elucidated. As mentioned in Section 4.3, there is evidence that this DNA segment is present in the genome and is not a cloning artifact. Sequence identification of this segment in genomic DNA will undoubtedly prove that this segment is present in the genome. If this is the case, then:

1. why would this pseudogene be expressed during nodule senescence?
2. if this DNA segment represents an intron, then does this mean that we have cloned DNA and that normally the intron would be removed by splicing during the expression of the gene?

The complete gene sequence must be attained for SAN 8-4. This would provide more reliable sequence information for an accurate sequence alignment analysis. In

addition, the regulatory consensus sequences may be identified and a comparison may be made with other members in the F3H multigene family.

Since the probe made from SAN cDNA 8-4 hybridized to an mRNA on a Northern analysis, there is no doubt that an up-regulated gene associated with nodule senescence was found. It is uncertain whether the hybridizing mRNA represents a gene product of SAN 8-4 or a member of the gene family. Since SAN 8-4 was shown to belong to a multigene family, by Southern analysis, it is possible that one of these members was up-regulated.

If SAN 8-4 expression is involved in flavonoid biosynthesis, as in F3H and flavonol synthase, a simple Northern analysis showing the expression of SAN 8-4 during nodule senescence and flower development may give more evidence that SAN 8-4 is F3H related.

The enzymes in the flavonoid pathway are coordinately regulated. It is not well understood how all flavonoid genes are switched on simultaneously, but it seems likely that the different regulators are produced upon a common signal (Weiss et al., 1993). The genes are induced by gibberellic acid (GA) in petunia flowers (Weiss and Halevy, 1989).

The genes and their gene products mentioned have been extensively characterized in several plant species (Melgaard, 1992). To demonstrate the expression of these

genes in the flavonoid pathway, gene induction can be attempted with GA and the expression of the genes involved can be tested in the flower petals of soybean in the same cultivar used for the isolation of *SAN 8-4*. (ie. cv Maple Arrow). In addition to the evidence presented in this thesis, the induction of genes involved in the flavonoid pathway such as those for chalcone synthase, dihydroflavanol reductase and flavanol synthase including *SAN 8-4* would suggest that *SAN 8-4* is an enzymatic step in the synthesis of a flavonoid whose production is associated with nodule senescence.

To attain unequivocal proof for the relatedness of *SAN 8-4* to F3H, a full length cDNA must be attained for expression studies. The production of a functional protein from the clone in an expression vector can then be tested for substrate specificity and activity as has been demonstrated with the putative F3H cDNA clone isolated from *Petunia hybrida* (Britsch et al., 1992).

Antibodies against F3H may be used to identify *SAN 8-4*. Recently, antibodies against F3H from *Petunia* were shown to readily cross react with F3H from a number of other sources (Britsch, 1990).

Clearly, the work presented in this thesis provided possibilities in the identification of a senescence associated cDNA. *SAN 8-4* may be used as a marker for the

process of senescence in nodule tissue and perhaps in other organs. The induction of nodule senescence by the addition of fixed nitrogen is a good model to study the processes of nodule senescence since the initiation of this phenomenon can be controlled and the time in which senescent tissue is attained is much shorter than in the natural system.

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APPENDIX I:

In the course of this thesis, a new nodulin cDNA may have been isolated. The cDNA clone 7-2 was isolated through differential screening of the 40 day λ gt11 cDNA library and processed in parallel with SAN cDNA clone 8-4. Northern analysis showed that the hybridizing message was 1.4kb. The signal decreased with senescence and the expression of the putative nodulin 7-2 was undetectable in root and stem tissue (Figure 1).

DNA sequence (Figure 2) of the pGEM-7Zf subclone of 7-2 was used to search the gene bank EMBL using the BLAST algorithm. Entries up to March 27, 1995 were included. The sequence which showed greatest DNA sequence homology is a partial sequence of a cDNA isolated from *Oryza sativa* (accession number D15824). The alignment showed 70% similarity over a stretch of 201 nucleotides. The rice sequence is unpublished but was submitted to EMBL by Yuzo Minobe (1993). The DNA sequence of clone 7-2 also showed 70% similarity over a stretch of 166 nucleotides to a *Arabidopsis thaliana* cDNA sequence (accession number T45758). This unpublished sequence was submitted by T. Newman (1995).

Recently, PCR product of clone 7-2 was used to screen a genomic library from *Glycine max* (Dr D.A. Johnson) and putative clones were isolated. Further sequence analysis

will aid in the identification of a possible function for this cDNA.

FIGURE 1 NORTHERN BLOT ANALYSIS DEMONSTRATING THE DIFFERENTIAL EXPRESSION OF A NEW NODULIN GENE WITH INDUCED (A) AND WITH NATURAL SENESCENCE (B)

10 μ g of total RNA from the nodules at various time points was used for northern analysis versus a probe made from the PCR product of clone 7-2. The analysis was performed under standard hybridization conditions.

(A) The lanes consist of:

c. soybean root control

For the induced nodule senescence system with the addition of 20mM ammonium nitrate, the following samples are shown:

1. 0 hour
2. 12 hours
3. 24 hours
4. 48 hours after induction.

(B) With natural senescence, the following samples are shown:

5. 24 days
6. 39 days
7. 40 days after inoculation.

The size of the hybridizing message is indicated.

A

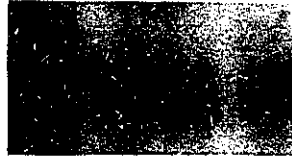
c

1

2

3

4



- 1.4 kb

B

5

6

7



- 1.4 kb

FIGURE 2 SEQUENCE OF THE NODULIN cDNA CLONE 7-2

Shown is the DNA sequence for the putative NOD clone 7-2.

The numbers on the left indicate nucleotide position.

1 ATGGAAGGTG AACAAAGTGTG GTTTGGGAGG TGAGTGATGG AGGGTGGAGG
51 AAGAGAAGGT TACAATGGAA TTGTGATGAC GATGACACGA GACCCAAAGC
101 CGAGGCTACG GTGGACGGCA GATCTTCATG ACCGCTTTGT GGATGCAGTC
151 AAAAAGCTTG GTGGCCCTGA TAAGGCGACT CCCAAGTCTG TTCTGAGGTT
201 AATGGGCTTG AAAGGGCTGA CACTATATCA TTTGAAGAGC CATTTACAGA
251 AGTATAGACT TGGACAGCAA GCTCGGAAAC AAAATGAGGA TATGCACAAA
301 GAAAATAATA GATGTTCGTA TGTAATTTT AGCAATCGTT CTTCAGCACC
351 TAACACCAGT TACAGAGAGA AATCCAATA GCAGAAGCAA TGAGGTGTCA
401 GATTGAAGTT CAAAAGAGAT TGGAAGAGCA GCTAGAGGTA CAGAAGAAAT
451 TGCAGATGAG AATAGAGGCA CAAGGGAAGT ATTTGCAAGC CATGTTAGAG
501 AAAGCACAGA GAAGCCTTTC CCTGGATGGG CCAGGCAGTC TTGAAGCATC
551 GAGAGCTCAG TTGA