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Isolation of the Nitrate Reductase Structural Gene in Cucurbita Maxima

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ISOLATION OF THE NITRATE REDUCTASE STRUCTURAL GENE
IN CUCURBITA MAXIMA

by

JOYCE JEAN OSTBERG

A Thesis Submitted to the Faculty of the Graduate School of
Loyola University of Chicago in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

AUGUST

1987

TO BRIAN

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VITA

The author, Joyce Jean Ostberg, is the daughter of Robert H. Ryan and Lucille (Mills) Ryan. She was born in Chicago, Illinois on February 18, 1961.

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In 1986, while attending Loyola University of Chicago, Ms. Ostberg was elected vice-president of the Biology Graduate Student Association and became a member of the American Society of Plant Physiologists.

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS	ii
VITA	iii
LIST OF TABLES	iv
LIST OF FIGURES	v
Chapter	
I. INTRODUCTION	1
II. REVIEW OF RELATED LITERATURE	4
III. MATERIALS AND METHODS	31
IV. RESULTS	55
V. DISCUSSION	91
LITERATURE CITED	112

LIST OF TABLES

<u>Table</u>	<u>Page</u>
I. Squash Genomic DNA Isolations	56
II. Genomic Southern Blots	67
III. Cloning Efficiencies	69
IV. Clone DNA Isolations	71
V. Clone Restriction Enzyme Digests	80

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Proposed Structure of Squash Nitrate Reductase Holoenzyme	7
2. Structure of λ gt11	24
3. Restriction Enzyme Digestion of Squash Genomic DNA	58
4. Isolation of pCmc1	61
5. Squash Genomic Southern Blot of Eco RI and Hind III Digests	63
6. Squash Genomic Southern Blot of Eco RI and Bam HI Digests	65
7. Dot Blot Analysis of Four Putative Positive Clones	72
8. Clone and Squash Genomic Southern Blot Probed with cDNA Insert of pCmc1	75
9. Restriction Enzyme Digestion of Four Putative Positive Clones	78
10. Southern Blot Analysis of Putative Positive Clones Probed with Intact pCmc1	81
11. Southern Blot Analysis of Putative Positive Clones Probed with cDNA Insert of pCmc1	83
12. Proposed Structure of λ Osnr	86
13. Soybean RNA Dot Blot	89
14. Proposed Regulation of the Nitrate Reductase Structural Gene in <u>Neurospora crassa</u>	105

CHAPTER I

INTRODUCTION

The majority of nitrogen assimilated by higher plants is in the form of nitrate. Nitrate reductase is the first enzyme in the nitrogen assimilation pathway of plants and has been shown to catalyze the reduction of nitrate to nitrite (Evans and Nason, 1953). This reaction is generally believed to be the rate-limiting step in the conversion of inorganic nitrogen to protein (Beevers and Hageman, 1969; Campbell and Smarrelli, 1986; Hageman, 1979). The activity of nitrate reductase has been shown to generally increase when exogenous nitrate is present (Nelson et. al., 1983; Robin et. al., 1985), while the presence of reduced nitrogen has been shown to decrease or abolish the activity of nitrate reductase (Curtis and Smarrelli, 1986; Radin, 1985; Langendorfer, personal communication). The increased activity of nitrate reductase in the presence of nitrate has been positively correlated with the presence of nitrate reductase transcripts (Cheng et. al., 1986; Crawford et. al., 1986; Martino, 1987; Smarrelli et. al., in press).

The majority of higher plant assimilatory nitrate reductases are homodimers with subunit molecular weights of approximately 115,000 daltons (Campbell and Smarrelli,

1978). Each subunit consists of an apoprotein and a non-covalently bound molybdenum cofactor (Campbell and Smarrelli, 1978; Dunn-Coleman et. al., 1984; Kleinhofs et. al., 1985). Nitrate reductase apoprotein mutants in both fungi and higher plants have been linked to a single nuclear locus, while mutations involved in the molybdenum cofactor biosynthesis have been linked to multiple loci (Dunn-Coleman et. al., 1984; Kleinhofs et. al., 1985).

A greater understanding of the genetics involved in the regulation of nitrate reductase could be obtained following isolation of the nitrate reductase structural gene. Also, once efficient plant transformation procedures are available, the gene for a more efficient nitrate reductase enzyme could be substituted for a gene encoding a less efficient nitrate reductase enzyme, which could increase plant growth and yield (Campbell and Smarrelli, 1986; Cocking et. al., 1981).

The work described in this thesis involves the isolation a portion of the genomic sequence for the nitrate reductase structural gene from squash (Cucurbita maxima). Genomic DNA from squash was isolated and digested with the restriction enzymes Eco RI and Bam HI. Southern blots of these digests were probed with the plasmid, pCmc1, which contains a 1.2 kb squash nitrate reductase cDNA insert (Crawford et. al., 1986). The results of these blots were used to determine if the restriction enzyme digests produced

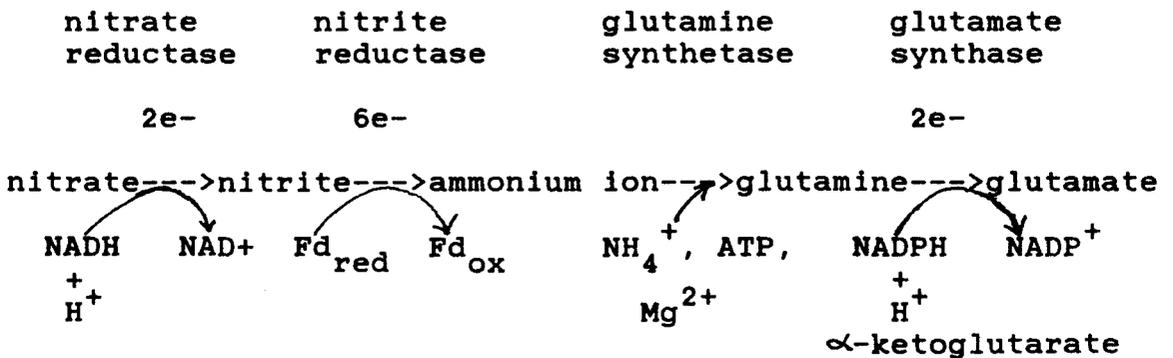
fragments containing sequences homologous to pCmc1 suitable for cloning in the lambda vectors, λ gt11 and EMBL3 (Frischauf et. al., 1983; Huynh et. al., 1984). Genomic libraries were constructed and screened with the plasmid pCmc1, to isolate recombinant phage containing sequences of the nitrate reductase structural gene. The positive nature of clones that continued to hybridize to pCmc1 after a series of rescreening procedures was confirmed by DNA dot blot and Southern blot analyses.

CHAPTER II

REVIEW OF RELATED LITERATURE

Nitrate Reductase

Growth and protein production in most plants is limited by soil nitrogen. Each year approximately 2×10^{10} tons of inorganic nitrogen is fixed by plants (Guerrero, et. al., 1981; Losada, et. al., 1981). The major source of inorganic nitrogen available to plants is soil nitrate (Beevers, et. al., 1980), but most nitrate assimilation occurs in the leaves (Hageman, 1979). Nitrate taken up by the roots of plants is transported through the xylem to the leaves. The nitrogen assimilation pathway in higher plants is shown below (Salisbury and Ross, 1978):



The enzyme nitrate reductase was first isolated by Evans and Nason (1953) and was found to catalyze the reduction of nitrate to nitrite. This step is generally

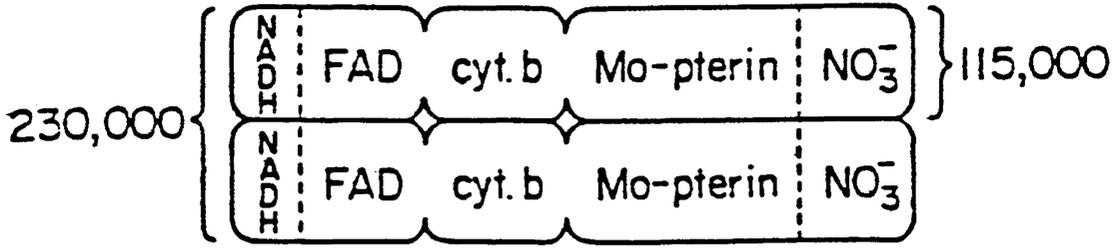
regarded as the rate limiting step in nitrate assimilation (Beevers and Hageman, 1969; Campbell and Smarrelli, 1986; Hageman, 1979). Assimilatory nitrate reductases are soluble electron transferring proteins with molecular weights in the range of 200-300 kilodaltons (kd) (Dunn-Coleman et. al., 1984; Guerrero, et. al., 1981; Hewitt and Notton, 1980). Although the specific structure of nitrate reductase is species specific, most higher plant nitrate reductase enzymes are NADH specific and have a pH optimum of 7.5 (Smarrelli and Campbell, 1981). In particular, squash nitrate reductase is an NADH-specific homodimer with subunit molecular weights of 115,000 daltons (Campbell and Smarrelli, 1978; Dunn-Coleman et. al., 1984, Redinbaugh and Campbell, 1983b). Each subunit of squash nitrate reductase contains one heme-iron, flavin adenine dinucleotide (FAD), and molybdenum-pterin plus one binding site each for NADH and nitrate (Redinbaugh and Campbell, 1985). These prosthetic groups function in the transfer of electrons from the pyridine nucleotide (NADH) oxidation site to the nitrate reduction site (Campbell and Smarrelli, 1978,; Hewitt and Notton, 1980). Enzyme inhibition studies have shown that NADH binds at or near the FAD prosthetic group, while nitrate binds at the Mo-pterin site (Campbell and Smarrelli, 1984; Hewitt and Notton, 1980). Steady-state kinetic studies have indicated that NADH and nitrate have separate, non-overlapping binding sites (Campbell and

Smarrelli, 1984). These results have led Redinbaugh and Campbell (1985) to propose that each subunit of the squash nitrate reductase holoenzyme has three domains. The first domain contains the FAD prosthetic group and is the binding site for NADH. The second domain contains the heme-iron group, while the third domain contains the Mo-pterin prosthetic group and the binding site for nitrate. A diagram of the proposed structure of nitrate reductase is shown in Figure 1 (Redinbaugh and Campbell, 1985).

The reduction of nitrate to nitrite is not the sole reaction catalyzed by nitrate reductase. Sorger (1966), and Wray and Filner (1970) reported cytochrome c reductase activity in nitrate reductase. Warner et. al. (1977) correlated cytochrome c reductase mutants with nitrate reductase mutants in barley. Studies performed by Smarrelli and Campbell (1979) on the NADH:dehydrogenase activity of nitrate reductase indicated that the enzyme acts as a general dehydrogenase and is involved in the reduction of cytochrome c, ferricyanide and other iron chelates. Redinbaugh and Campbell (1983a) showed that ferric citrate is a substrate for the NADH dehydrogenase activity of squash nitrate reductase. Furthermore, ferric citrate reductase activity of crude extracts from squash was found to co-purify with nitrate reductase NADH dehydrogenase activity in non-denaturing polyacrylamide gel electrophoresis. Since ferric citrate is the form of iron transported in the xylem

Figure 1. Proposed Structure of Squash Nitrate Reductase

Holoenzyme. Squash nitrate reductase is a homodimer with subunit molecular weights of 115,000 daltons. Each subunit is proposed to have three domains. The first domain contains the FAD prosthetic group, which accepts electrons from the pyridine nucleotide NADH. The electrons are shuttled from the FAD prosthetic group to a cytochrome b prosthetic group in the second domain, and from the cytochrome b to a molybdenum-pterin cofactor located in the third domain. It is at the site of the molybdenum cofactor that nitrate is reduced to nitrite.



of higher plants, but no enzyme has been identified as the major ferric citrate reductase, it has been suggested that nitrate reductase may play a role in the assimilation of iron by the leaves and roots of higher plants (Campbell, 1985). Squash nitrate reductase has been shown to catalyze the reduction of a broad range of ferrisiderophores (Castignetti and Smarrelli, 1984; Smarrelli and Castignetti, 1986). Reduction of ferrisiderophores by squash nitrate reductase has a pH optimum of 4-5 and is negligible above pH 6, while the nitrate reductase activity of the enzyme has a pH optimum of 7.5 and is abolished at pH 6 or less. Smarrelli and Castignetti (1986) propose that the substrate specificity of nitrate/ferrisiderophore reductase may be controlled by the plant by altering the pH of the environment (i.e., roots or leaves).

The activity of nitrate reductase has been shown to vary in the presence of nitrate and/or reduced nitrogen sources. In the presence of reduced nitrogen alone, little or no nitrate reductase activity has been shown, while the activity increases with increased ratios of nitrate to reduced nitrogen (Oaks, 1974; Nelson et. al., 1984; Langendorfer, personal communication). The events controlling nitrate reductase synthesis at the molecular level have been studied recently. Cheng et. al. (1986) have shown an increase in nitrate reductase mRNA levels in barley in response to nitrate induction. Crawford et. al. (1986)

have reported the appearance of a 3.2 kb mRNA transcript only after nitrate induction. Our laboratory (Martino, 1987) has shown a positive correlation between the activity of nitrate reductase in the presence of varying ratios of nitrate and reduced nitrogen and the presence or absence of mRNA transcripts for nitrate reductase, indicating the control of nitrate reductase appears to lie at the level of transcription.

DNA Isolation

Nitrate reductase has been studied extensively in fungi and, to a lesser extent, in higher plants. The genomic DNA sequence coding for nitrate reductase, however, has yet to be isolated. Crawford et. al. (1986) have isolated a 1.2 kb cDNA clone that encodes part of the squash nitrate reductase enzyme. This clone is approximately one third the length of the 3.2 kb squash nitrate reductase mRNA (Crawford et. al., 1986). A 1.1 kb cDNA clone encoding part of the barley nitrate reductase sequence has also been isolated by Cheng et. al. (1986). This cDNA clone hybridizes to a 3.5 kb mRNA transcript whose translation product is immunoprecipitated by nitrate reductase antiserum. As the term cDNA implies, these clones are copies of their respective nitrate reductase mRNAs and due to the processing involved in producing a mature mRNA, they do not represent the entire genomic sequence coding for the squash nor barley nitrate reductases.

Nuclei Isolation. Procedures for the isolation of plant DNA generally begin with the isolation of plant nuclei and are modifications of procedures used for animal nuclei isolations (D'Alessio and Trim, 1968). In plants, these procedures produce low yields of nuclei due to a low number of nuclei per unit of fresh weight, difficulty in breaking the cell wall, the presence of a large number of chloroplasts and amyloplasts, and the high level of nuclease activity in many plant tissues (D'Alessio and Trim, 1968; Hamilton et. al., 1972; Kislev and Rubenstein, 1980; Mascarenhas et. al., 1974). The presence of a cell wall requires homogenization of plant tissue to be more vigorous and of longer duration than is necessary for animal tissues. This often results in the partial or complete destruction of a large number of the nuclei. Other nuclei are trapped by the cell wall debris, further contributing to low nuclei yields (Mascarenhas et. al., 1974). Nuclei that are isolated must be lysed and the extract subjected to various purification steps to isolate DNA free from contaminants. More problems are encountered at this stage due to the presence of oils, pigments, polyphenols, polysaccharides and secondary metabolites that purify with the DNA (Edelman, 1975; Kislev and Rubenstein, 1980; Loomis, 1974; Segovia et. al., 1965; Sung and Slightom, in press).

Many different procedures have been developed to circumvent the problems encountered in the isolation of

plant DNA. Hamilton et. al. (1972) recommend selecting small leaves (4-8 cm) for isolation procedures due to their low content of chloroplasts and soft primary cell walls. Kuehl (1964) recommends incubating leaves in a medium containing n-octanol and gum arabic prior to homogenization to soften cell walls, although other laboratories have found incubation with proteinase K or snail digestive juice (introduced by vacuum infiltration) to be more effective (Blin and Stafford, 1976; D'Alessio and Trim, 1968; Gross-Bellard et. al., 1973). Mascarenhas et. al. (1974) report that pre-chilling plants decreases enzymatic degradation of nuclei, thereby increasing nuclear yields. Starvation of plants by keeping them in the dark for 24-48 hours prior to use was introduced to decrease starch contamination (D'Alessio and Trim, 1968). Blin and Stafford (1976) indicate that the presence of liquid nitrogen in the homogenization step aids in the disruption of plant tissue and isolation of "nick"-free DNA. Hamilton et. al. (1972) recommend repeating the homogenization procedure several times to increase the efficiency of cell wall breakage.

The content of the isolation medium also plays a major role in the yield of nuclei. The isolation medium of Honda et. al. (1966) is widely used for the maintenance of intact cellular organelles. This medium uses Dextran-40 as a polymer to adsorb the abundant polyphenols present in plant tissue (D'Alessio and Trim, 1968; Jones and Hulme, 1961).

Honda et. al. (1966) point out that control of pH is critical as the release of acidic vascular contents results in the disintegration of cellular organelles, therefore their medium contains 0.025 M Tris-HCl (pH 7.8) to prevent organelle disruption. However, at higher pH's phenolic components of plant extracts become oxidized, reducing the effectiveness of phenol adsorbents (Loomis, 1974). For this reason the isolation medium in many procedures is adjusted to pH 6.7-7.2 (Ikuma and Bonner, 1967; Marei and Romani, 1971; Romani et. al., 1969). Tautvydas (1971) has found that an even lower pH of 6.0-6.1 produced optimal yields of pea nuclei. Honda et. al. (1966) found a low osmoticum (0.08 osmolar) obtained with 0.025 M sucrose to be more effective in preventing organelle swelling than the higher osmoticums recommended by Granick (1938). The presence of Ca^{2+} or Mg^{2+} has been shown to be essential to prevent breakage and aggregation of nuclei (Hamilton et. al., 1972; Mascarenhas et. al., 1974). Several laboratories report the addition of detergents to be beneficial in solubilizing cytoplasm and disrupting the membranes of chloroplasts and mitochondria and the outer nuclear membrane (Bard and Gordon, 1969; D'Alessio and Trim, 1968; Mascarenhas et. al., 1974). Holtzman et. al. (1966) report that the detergent used should be a mixture of non-ionic and ionic compounds, such as Tween 40 and sodium deoxycholate, as a non-ionic

detergent alone will not remove cytoplasmic material and an ionic detergent alone will lyse nuclei.

The presence of ethidium bromide in the isolation medium is recommended by Kislev and Rubenstein (1980) to inhibit the activity of nucleases. Hamilton et. al. (1972) recommend using a large ratio of isolation medium to tissue and rapid isolation of nuclei in order to reduce the effect of nucleases. Tautvydas (1971) recommends filtering the plant homogenate through a series of nylon screens with diminishing pore sizes, thus eliminating the preliminary centrifugation steps. This decreases the amount of time between cell rupture and the start of a differential density gradient centrifugation to fifteen minutes.

DNA Extraction. Following the isolation of plant nuclei, DNA isolation is generally performed by the method of Marmur (1961) or a modification of this method (Green and Gordon, 1967; Gross-Bellard et. al., 1973). Lysis is obtained through the action of sodium dodecylsulfate (SDS) and heating to 65 C. The presence of ethylenediaminetetraacetate (EDTA) and high pH is used in conjunction with the SDS and heat to prevent degradation by DNase (Marmur, 1961; Wells and Ingle, 1970). The extract is then deproteinized by extractions with chloroform:isoamyl alcohol (24:1). Chloroform causes surface denaturation of proteins which then form a complex with the chloroform and settle out due to their increased density; isoamyl alcohol reduces foaming

and aids in the separation of the organic and aqueous layers (Marmur, 1961; Sevag et. al., 1938). The deproteinized solution is then treated with ribonuclease and re-extracted with chloroform:isoamyl alcohol. DNA is then precipitated by the addition of 0.54 volumes of isopropanol (Marmur, 1961) which selectively precipitates DNA, leaving RNA, oligonucleotides and polysaccharides in solution. While isopropanol is more selective for DNA, it also requires higher concentrations of DNA in the purified extract for good yields (Howell, 1973). Maniatis et. al. (1982) recommend precipitating DNA by the addition of 0.1 volumes 3 M sodium acetate, 2 volumes 100% ethanol and overnight incubation at -20 C. Gross-Bellard et. al. (1973) omit the chloroform:isoamyl alcohol deproteinization and substitute proteinase K digestion, followed by phenol extractions to deproteinize the extract. Residual phenol is subsequently removed by dialysis.

DNA Purification. Affinity chromatography is another approach that has been used to improve yields of plant DNA. Edelman (1975) reports that applying a DNA extract to a concanavalin A column removes polysaccharide contaminants. Concanavalin A is a plant hemagglutinin that has an affinity for carbohydrates containing glucose, fructose or mannose as their terminal groups (Aspberg and Porath, 1970; Edelman, 1975; Lloyd, 1970). Stein and Thompson (1978) employ gel chromatography to remove pigment contaminants, followed by a

hydroxyapatite column to remove proteins, polysaccharides and RNA. Walbot and Goldberg (1979) eliminate the isolation of intact nuclei and apply a plant homogenate to a hydroxyapatite column. Polysaccharides, proteins and RNA are eluted from the column using an 8 M urea, 0.24 M phosphate buffer (Meinke et. al. , 1974; Walbot & Goldberg, 1979). Urea is thought to disrupt the secondary and tertiary structure of proteins and single-stranded nucleic acids, thereby reducing their affinity for hydroxyapatite (Britten, et. al., 1970). The double-stranded plant DNA is then eluted from the hydroxyapatite column in a 0.5 M phosphate buffer (Meinke et. al. , 1974; Walbot & Goldberg, 1979).

Cesium-chloride density gradients were introduced by Meselson et. al. (1957) as a means of separating and purifying nucleic acids. By centrifuging a solution containing a low molecular weight solute until equilibrium is obtained, the opposing actions of sedimentation and diffusion produce a stable concentration gradient. The combined action of the concentration gradient and compression of the liquid due to centrifugal force cause the density of the solution to increase continuously along the direction of the centrifugal force. When macromolecules are present, the centrifugal force drives the macromolecules into a region where the net force acting on a given molecule is zero (Meselson et. al., 1957). Thus, macromolecules of

similar weight form a band where their density is equal to that of the surrounding medium (Lehninger, 1982). Flamm et. al. (1966) report the use of a fixed angle rotor in cesium-chloride density-gradient centrifugation provides five to ten times better resolution than is obtainable with swinging bucket rotors. The geometry of fixed-angle rotors also reduces the column height compared to swinging-bucket rotors, which in turn reduces the time required to generate a density gradient (Howell, 1973). When the density of the starting cesium chloride solution is adjusted to the density of DNA in cesium chloride (1.700 g/ml at pH 8.5) (Howell, 1973), RNA settles to the bottom of the gradient, DNA collects near the middle of the gradient, and proteins float on the top (Maniatis et. al., 1982; Marmur, 1961).

DNA Cloning

Isolation of eukaryotic genes is generally accomplished through cloning DNA fragments. The DNA may be genomic and isolated directly from the organism of study, or it can be complementary (cDNA) in which a DNA copy is made from messenger RNA. The DNA fragments, whether genomic or cDNA, may be cloned in a "shotgun" fashion in which random fragments from a heterogenous pool of DNA are inserted into the cloning vector (Benton and Davis, 1977; Cameron et. al., 1975). Alternatively, the DNA fragments are "enriched" for the appropriate size, generally by sucrose density gradients, prior to insertion into the vector (Benton and

Davis, 1977; Cameron et. al., 1975; Gerlach et. al., 1982; Maniatis et. al., 1978).

Effective cloning of DNA fragments requires three basic steps. First, the DNA fragment must be inserted into a second DNA molecule that is capable of autonomous replication, such as a plasmid or phage (Bahl et. al., 1976; Jones and Murray, 1975). Second, the chimeric DNA molecule must be able to transform competent bacterial cells (Bahl et. al., 1976; Hohn and Murray, 1977; Lederberg and Cohen, 1974; Morrow et. al., 1974). Finally, the DNA fragment must be replicated within the prokaryotic host (Bahl et. al., 1976; Hohn and Murray, 1977).

Autonomous replication and transformation ability are only two of the characteristics desired in a cloning vector. Hohn and Murray (1977) state that ideal vectors should contain one or only a limited number of target sites for a particular restriction enzyme and that insertion of a DNA fragment should not destroy a gene essential to the function of the vector. Blattner et. al. (1977) add that the vector should be able to accept a variety of DNA fragment sizes, allow cloning with several different restriction enzymes, control transcription of the DNA fragment from a promoter on the vector, replicate in high yield, allow easy recovery of the cloned DNA and possess features that contribute to its biological containment. Furthermore, both Hohn and Murray (1977), and Blattner et. al. (1977) indicate that

recombinant vectors should be easily distinguished from non-recombinant vectors. A variety of plasmid and lambda phage vectors have been engineered to meet these requirements.

Plasmid Vectors. Plasmid vectors have been used to clone several eukaryotic genes. Some examples of these genes include two chicken skeletal muscle myosin light chains (Nabeshima et. al., 1982), rat preproinsulin (Villa-Komaroff et. al., 1978), human terminal deoxynucleotidyltransferase (Peterson et. al., 1984), and the maize alcohol dehydrogenase gene (Adh1) (Gerlach et. al., 1982). All of these genes utilized the E. coli plasmid pBR322 as a cloning vector, although many other plasmid vectors exist (Calos et. al., 1983; Grunstein and Hogness, 1975; Vieira and Messing, 1982; Yanisch-Perron et. al., 1985).

Plasmid genomes are found within bacterial cells as circular duplexes of DNA and at a characteristic copy number dependent upon the type of plasmid. Single copy plasmids are present in only one copy per cell and consequently make poor cloning vectors (Lewin, 1985). Multicopy plasmids are generally maintained at 10 to 20 copies per host cell (Lewin, 1985). Some multicopy plasmids are under relaxed replication control and when the host cells are treated with chloramphenicol to prevent replication of bacterial DNA, these plasmids may accumulate 1000 to 3000 copies per cell (Lewin, 1980). Relaxed copy plasmids, such as pBR322 and

the pUC series (Vieira and Messing, 1982; Yanisch-Perron et. al., 1985) make good cloning vectors due to their high DNA yields.

Plasmids are capable of accepting and replicating large fragments of foreign DNA (Lewin, 1980). Furthermore, once they are taken up by bacterial cells, the chimeric plasmids can be maintained within the host as long as conditions for their selection are maintained. Plasmid vectors are often engineered to possess multiple cloning sites. The plasmid pBR322 and the pUC plasmids have unique cleavage sites for several different restriction enzymes (Lewin, 1985; Vieira and Messing, 1982; Yanish-Perron et. al., 1985). Consequently, a variety of restriction enzymes may be used to obtain fragments of the DNA to be cloned.

Selection of plasmid vectors is often accomplished by means of antibiotic resistance. The vector generally possesses two genes that confer resistance to two different antibiotics. The first antibiotic is used to screen for bacteria cells that are transformed by the plasmid. The gene for the second antibiotic often contains the cloning site within its sequence. Insertion of a DNA fragment into the cloning site disrupts the sequence of the second antibiotic causing cells containing chimeric plasmids to be sensitive to this antibiotic (Lewin, 1985). Thus, by replica plating (Hayes, 1964; Nester et. al., 1978) colonies of transformed bacteria onto agar plates containing the

second antibiotic, cells containing chimeric plasmids can be identified by their failure to grow (Lewin, 1980; Lewin, 1985).

Phage Vectors. A variety of genes have been cloned through the use of phage vectors. Some of these genes include the genes for Adh from Arabidopsis thaliana (Chang and Meyerowitz, 1986), Adh 2 from maize (Dennis et. al., 1985), the cell wall degrading enzyme polygalacturonase from tomato (DellaPenna et. al., 1986), and rabbit B-globin (Maniatis et. al., 1978).

Most phage vectors have been engineered through manipulations to the genome of bacteriophage lambda (Lewin, 1980). Phage vectors have the advantages of easy amplification, purification, maintenance and screening (Lewin, 1980; Karn et. al., 1983). They are however, limited in the size of DNA fragments they can accept (Hohn and Murray, 1977; Lewin, 1980; Karn et. al., 1983; Lewin, 1985).

Wild-type lambda phages are packaged as linear, nonpermuted, double-stranded DNA molecules 47 kilo-bases in length with specific complementary single-stranded sequences 12 base pairs in length located at each 5'-phosphate end of the DNA molecule (Blattner et. al., 1977; Sternberg and Weisberg, 1975; Weil et. al., 1972). The single-stranded sequences are called cos sites (Sternberg and Weisberg, 1975; Weil et. al., 1972) and join soon after infection of

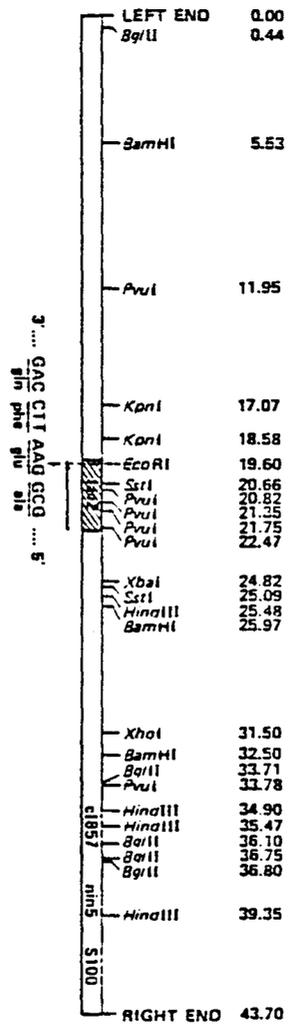
an E. coli. cell to form a circular DNA molecule (Lewin, 1985). Through the action of the cro gene product, or in cI⁻ mutants, integration of the lambda DNA into the host genome is prevented and the lytic cycle ensues leading to the synthesis of phage progeny and cell lysis (Belfort and Wulff, 1973; Lewin, 1985). During the later stages of the lytic cycle, rolling circle replication (Lewin, 1985) of lambda DNA occurs producing large catenated, linear DNA molecules (Maniatis et. al., 1982). The products of genes A and D, act with the phage head precursor to cleave the lambda DNA molecules at the cos sites (Blattner et. al., 1977; Sternberg and Weisberg, 1975; Weil et. al., 1972). The DNA between adjacent cos sites is then highly condensed and packaged into a phage head (Lewin, 1985; Sternberg and Weisberg, 1975).

The genes essential for lambda phage lytic cycle are contained within approximately sixty percent of the lambda genome (Karn et. al., 1983). The remaining non-essential genes are grouped in the center of the lambda DNA molecule and can be deleted without affecting the lytic cycle (Blattner et. al., 1977; Maniatis et. al., 1982). Lambdoid phages, however, have a lower and upper DNA size limit of 70 to 108 percent of the wild-DNA length for the packaging reaction to occur (Karn et. al., 1983). Consequently, if all the non-essential genes are deleted from a lambda vector, a DNA fragment of 9 to 22 kilo-bases must be

inserted into the vector for packaging to occur (Blattner et. al., 1977).

Construction of a lambda vector that requires the insertion of a foreign DNA fragment to meet the minimum length required for packaging is one method of selecting for chimeric phage. A second selection method is exemplified by the expression vector λ gt11 which can accept DNA fragments up to 7.2 kilobases in length (Huynh et. al. , 1984). The E. coli. gene for B-galactosidase (lac Z) is located on the genome of λ gt11 and contains a unique Eco RI cleavage site 53 base pairs upstream of the lac Z termination site (Blattner et. al., 1977; Clarke and Carbon, 1975; Galas et. al., 1980; Snyder and Davis, 1985; Young and Davis, 1983) A diagram of λ gt11 is shown in Figure 2 (Huynh et. al., 1984). Interruption of the lac Z gene by the inserted DNA fragment produces an inactive B-galactosidase fusion protein (Young and Davis, 1983). Thus, recombinant phage can be easily identified by their inability to form blue plaques on a lac Z⁻ host on agar plates containing 5-bromo-4-chloro-3-indolyl B-D-galactoside (X-Gal) as a chromogenic indicator (Blattner et. al., 1977; Young and Davis, 1983). Use of a lac Z⁻ host is necessary due to the presence of the lac operator located on the λ gt11 genome. In a lac Z⁺ host, the phage operator can bind the lac repressor during phage growth causing derepression of the host lac operon and pale blue plaques

Figure 2. Structure of λ gt11. A single Eco RI cleavage site is located 53 base pairs upstream of the termination site of the E. coli lac Z gene. Insertion of foreign DNA fragments up to 7.2 kb in size into the Eco RI site produces an inactive B-galactosidase fusion protein. Recombinant phage used to transfect lac Z⁻ host cells form clear plaques on agar plates containing X-gal, while blue plaques are formed by non-recombinant phage.



will be produced by recombinant phage (Blattner et. al., 1977).

Gene Isolation. Isolation of specific DNA sequences inserted into a cloning vector can be accomplished by screening the recombinant library with antibodies or radiolabelled nucleotide probes. Screening with antibodies is generally performed on cDNA libraries since most eukaryotic genes contain intervening sequences that must be removed from the transcript prior to translation and the prokaryotic hosts used in cloning do not process RNA transcripts (Lewin, 1985). When screening is performed with radiolabelled nucleotide probes equal success can be obtained with cDNA or genomic libraries.

The expression vector λ gt11 has been used to isolate a variety of cDNA clones by antibody screening. Crawford et. al. (1986) have isolated a 1.2 kb cDNA clone of squash nitrate reductase, while a 1.1 kb cDNA clone of barley nitrate reductase has been isolated by Cheng et. al. (1986). Landau et. al. (1984) have isolated a cDNA clone of terminal deoxynucleotidyltransferase. A cDNA clone coding for human factor X has been isolated by Leytus et. al. (1984). Few, if any, intervening sequences are present within yeast genes (Snyder et. al., In press) which allows these genes to be expressed in E. coli (Goto and Wang, 1984). Consequently, genomic clones for yeast DNA topoisomerase II (Goto and

Wang, 1984) and DNA polymerase I (Johnson et. al., 1985) have been isolated by antibody screening of λ gt11 libraries.

Immunoscreening of recombinant expression vectors requires a rapid increase in copy number and high levels of transcription of the foreign DNA as a response to induction. Furthermore, both the vector and host should possess features that minimize the degradation of foreign proteins (Young and Davis, 1983). These characteristics are all met by λ gt11. During lytic infection of E. coli, λ gt11 propagates to high copy numbers (Snyder and Davis, 1985; Snyder et. al., In press; Young and Davis, 1983). The strong E. coli promoter of the lac Z gene possessed by λ gt11 allows the B-galactosidase fusion proteins to comprise a significant portion of the total E. coli cellular protein (Snyder and Davis, 1985; Snyder et. al., In press). Fusion of the eukaryotic protein to all but a small portion of the B-galactosidase protein has been shown to increase the stability of the foreign protein (Stanley, 1983; Young and Davis, 1983). The E. coli strain, Y1090, used in antibody screening, is deficient in the lon protease enabling the B-galactosidase fusion proteins to accumulate to higher levels than are possible in wild-type cells (Young and Davis, 1984).

While immunoscreening can facilitate the isolation of cDNA clones, there are several disadvantages to this procedure. First, relatively pure polyclonal antibodies or

several monoclonal antibodies recognizing different determinants are needed to prevent isolation of clones other than the one of interest (Snyder et. al., In press). Second, for most eukaryotic genes a nearly full length cDNA clone is preferred to provide the most antigenic determinants. This is further complicated by the fact that nonabundant mRNAs are rarely represented in cDNA libraries and, if present, the cDNAs often encode the 3' terminal portion of the gene which contains fewer antigenic determinants (Snyder et. al., In press). Finally, the inserted DNA fragment must be in the proper orientation and reading frame with respect to the B-galactosidase protein for detection of the protein by antibody screening (Young and Davis, 1983). For a 1 kb fragment from a genome 14 kb in size, the correct in-frame fusion will occur in one out of every 8.4×10^4 recombinants (Snyder and Davis, 1985).

To circumvent the problems encountered with immunoscreening, cDNA and genomic libraries constructed in plasmid or phage vectors can be screened with nucleic acid probes. Prior to screening, the DNA must be bound to a nylon or nitrocellulose filter. When plasmid vectors are used, colonies of transformed bacteria are grown on the filter, replica plated (Hayes, 1964; Nester et. al., 1978) to form a reference set, and then lysed on the filter in the presence of 0.5 M NaOH. The sodium hydroxide simultaneously lyses the host cell and denatures the DNA contained within the

cell which is allowed to dry and become fixed onto the filter (Grunstein and Hogness, 1975). The filter is then screened by hybridization to a nucleic acid probe that has been radiolabelled by the process of nick-translation (Feinberg and Vogelstein, 1983; Maniatis et. al., 1982, Rigby et. al., 1977). For organisms of small genome size such as Drosophila melanogaster (1.8×10^8 bp) (Snyder and Davis, 1985), a 1 to 2 kb gene sequence will be isolated by this method in one out of 16,000 colonies, assuming an average insert size of 10 kb (Grunstein and Hogness, 1975).

A more rapid method of radio-nucleotide screening can be performed by in situ plaque hybridization. E. coli cells transfected with aliquots from phage libraries are plated on agar plates and incubated for at least 12 hours at 37 C to allow plaque formation to occur (Benton and Davis, 1977). DNA from the transfection plate is transferred to nitrocellulose or nylon filters by making direct contact between the agar plate and filter (Benton and Davis, 1977; Sanzey et. al., 1976), thus omitting the need to make replica plates. A single plaque has been shown to contain sufficient DNA to allow detection of the insert sequence by hybridization to a radiolabelled nucleic acid probe (Benton and Davis, 1977; Jones and Murray, 1975). The likelihood of isolating a particular sequence by plaque hybridization is equivalent to that found with colony hybridization. The advantages of plaque hybridization result from the ability

to screen larger numbers of plaques per plate (Maniatis et. al., 1982) and the decreased time from cell transformation to hybridization due to the omittance of replica plating (Benton and Davis, 1977; Maniatis et. al., 1978).

Gene Characterization. To demonstate that clones isolated by antibody screening or radiolabelled nucleic acid probes contain the sequence of interest further tests must be performed on the clones. When clone isolation has been performed by antibody screening, the clone can be used to affinity-purify the antibody from the screening serum. The purified antibody is then used to probe immunoblots of the desired protein (Snyder and Davis, 1985). When clones are isolated with radiolabelled nucleic acid probes, the clones may be radiolabelled and used to probe genomic DNA blots (Southern, 1975; Maniatis et. al., 1982) or RNA blots (Maniatis et. al., 1982) and the results compared to genomic DNA blots or RNA blots probed with the original nucleic acid probe (Maniatis et. al., 1978). Hybrid-selection translation (Bunemann and Westhoff, 1983) and/or hybrid-arrested translation (O'Farrell, 1975) may be performed by hybridizing the clone insert to poly (A⁺) mRNA from the organism of study. Finally, if the amino acid sequence of the desired protein is known, the DNA sequence of the clone insert can be determined and the amino acid sequence deduced and compared to the known amino acid sequence (Snyder and Davis, 1985).

CHAPTER III

MATERIALS AND METHODS

DNA Isolations

Squash seeds (Cucurbita maxima var. buttercup) were grown in vermiculite in a 25 C growth chamber that received artificial illumination for 16 hours per day. The seeds were watered with tap water. Cotyledons or primary leaves were randomly harvested at seven days or approximately twenty-one days, respectively. Choice of tissue was determined by the procedure used. After the initial DNA isolations, all plants were placed in a dark cabinet overnight prior to harvesting to reduce starch levels.

Procedure 1. Squash nuclei were isolated by a modified method of Honda et al. (1966) from which DNA was isolated by a modified method of Maniatis et. al. (1982). Ten grams of shelled squash seeds, seven day cotyledons or primary leaves were frozen in liquid nitrogen and pulverized with a mortar and pestle. The ground tissue was then suspended in a buffer containing 25mM Tris-HCl (pH 7.8), 250 mM sucrose, 1 mM magnesium chloride, 1 mM sodium acetate, 4 mM beta-mercaptoethanol, 5% Dextran sulfate, 2.5% glycerol. The homogenate was filtered through four layers of cheesecloth and centrifuged in an SS34 rotor (Sorvall/DuPont) at 5000 rpm for 15 minutes at 4 C.

The pellets were resuspended in 4 ml (each) of a solution containing 25 mM Tris-HCl (pH 7.8), 300 mM sucrose, 1 mM magnesium chloride, 1 mM sodium acetate, 5 mM beta-mercaptoethanol and loaded onto density gradients. The density gradients consisted of the above solution containing an additional 20%, 40% and 60% sucrose layered from highest to lowest density (bottom to top). The gradients were placed in a Sorvall OTD65B ultra-centrifuge in a T864 rotor (DuPont) and centrifuged at 23,000 rpm for 2 hours at 4 C. The supernatants were discarded and the nuclei - containing pellets frozen at -20 C overnight. The next day, the pellets were resuspended in a solution containing 25 mM Tris-HCl (pH 7.8), 300 mM sucrose, 1 mM magnesium chloride, 1 mM sodium acetate, 5 mM beta-mercaptoethanol. The suspension was centrifuged in an SW41 rotor (Sorvall/DuPont) at 5000 rpm for 10 minutes at 4 C and the supernatant discarded. After washing the nuclei-containing pellets for two hours as described above, the pellets remained green, and clear supernatants were obtained. The final pellet was suspended in 5 ml of 10 mM Tris-HCl (pH 7.5), 10 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), and incubated at 50 C for thirty minutes with periodic swirling. Proteinase K was added to a final concentration of 1 mg/ml and the solution incubated at 50 C for four hours. The solution was then stored at 4 C overnight. Subsequently, the solution was

extracted with phenol saturated with 10 mM Tris-HCl (pH 7.5), 10 mM sodium chloride, 1 mM EDTA until no particles were present at the interphase. The phenol layers were reextracted with 10 mM Tris-HCl (pH 7.5), 10 mM sodium chloride, 1 mM EDTA. All aqueous layers were combined and extracted twice with chloroform:isoamyl alcohol (24:1) and the DNA ethanol precipitated with 0.1 volume 3 M sodium acetate and 2 volumes 100% ethanol.

This procedure yielded DNA that was resistant to digestion with various restriction enzymes. Consequently, the following modifications were made. Cotyledons were washed, patted dry and chopped three times with razor blades in the presence of the initial buffer, filtering the homogenate through cheesecloth between each chopping repetition. Nuclei-containing pellets were washed four times and the proteinase K digestion was performed at 37 C. The saturated phenol solution was extracted before use with 10 mM Tris-HCl (pH 7.5), 10 mM sodium chloride and 1 mM EDTA until the pH of the phenol was approximately 7.5. Finally, 3 M ammonium acetate was used in place of 3 M sodium acetate in the ethanol precipitation. These procedures were unsuccessful in yielding DNA that could be cut by the restriction enzymes Eco RI or Hind III.

Procedure 2. The DNA isolation procedure of Zarowitz (1984) was performed. Fifty grams of squash cotyledons were frozen in liquid nitrogen and ground to a powder with the

aid of a mortar and pestle. The ground tissue was resuspended in a buffer containing 10 mM Tris (pH 7.6), 1.14 M sucrose, 5 mM magnesium chloride, 10 mM beta-mercaptoethanol. The suspension was filtered through four layers of cheesecloth followed by vacuum filtration using coarse filter paper. The filtrate was centrifuged in a swinging bucket (HB4) rotor at 5000 rpm for 12 minutes at 4 C. Pellets were resuspended in the above buffer and an aliquot stained with aceto-oracin and viewed under a light microscope to confirm the presence of nuclei. The nuclear suspension was centrifuged as described above and the nuclei were resuspended in the above buffer plus 0.25% Triton X-100. The centrifugation step was repeated again with the nuclei resuspended in the above buffer without the Triton X-100. The nuclear suspension was loaded onto glycerol gradients consisting of 30%, 50%, 70% and 90% glycerol diluted with a solution containing 10 mM Tris (pH 7.6), 1.14 M sucrose, 5 mM magnesium chloride, 10 mM beta-mercaptoethanol. The gradients were centrifuged in an HB4 rotor at 7000 rpm for 30 minutes at 4 C. The layers from each gradient containing the nuclei were determined by aceto-oracin staining, pooled, diluted five times with the above solution and centrifuged in the HB4 rotor at 1000 rpm for 10 minutes at 4 C. The nuclei were then washed twice with the above solution to remove residual glycerol and resuspended in a solution containing 10 mM Tris (pH 7.5), 10

mM EDTA, 10 mM sodium chloride, 0.5% SDS. Four milligrams of Proteinase K (dissolved in the same solution) was added and the suspension incubated overnight at 37 C with gentle shaking. The suspension was centrifuged in an SS34 rotor at 13000 rpm for 20 minutes at 4 C. The supernatant was extracted three times with a solution containing a 1 to 1 ratio of phenol saturated with 1 mM Tris (pH 8.6), 100 mM sodium chloride, 1 mM EDTA, and chloroform:isoamyl alcohol (24:1), followed by one extraction with chloroform:isoamyl alcohol (24:1). The DNA was ethanol precipitated as described, centrifuged in an SS34 rotor at 10000 rpm for 20 minutes and resuspended in 1 mM Tris (pH 7.6), 0.1 mM EDTA. RNase T₁ was added to a final concentration of 5000 units per milliliter and the solution incubated on ice for thirty minutes. The solution was extracted and the DNA ethanol precipitated as described above. The method of Zarowitz (1984) produced negligible amounts of DNA.

Procedure 3. The DNA isolation procedure of Walbot and Goldberg (1979) was followed. Squash leaves and cotyledons were frozen in liquid nitrogen, homogenized and resuspended in an extraction buffer containing 8 M urea, 0.12 M sodium phosphate monobasic, 0.12 M sodium phosphate dibasic (final pH 6.8), 0.01 M EDTA, 2% SDS. The suspension was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and applied to a hydroxyapatite column. The hydroxyapatite was packed in a buffer containing 0.25 M

sodium phosphate monobasic, 0.25 M sodium phosphate dibasic (pH 6.8). The column was equilibrated with a solution containing 8 M urea, 0.12 M sodium phosphate monobasic, 0.12 M sodium phosphate dibasic (pH 6.8) prior to the application of the sample. RNA, polysaccharides, and proteins were eluted from the column with a buffer containing 8 M urea, 0.12 M sodium phosphate monobasic, 0.12 M sodium phosphate dibasic (pH 6.8). Urea was removed from the column with a solution containing 5 mM sodium phosphate monobasic, 5 mM sodium phosphate dibasic (pH 6.8). The DNA was eluted in a buffer containing 0.25 M sodium phosphate monobasic, 0.25 M sodium phosphate dibasic (pH 6.8). The DNA was then dialyzed overnight against 0.3 M sodium acetate, ethanol precipitated as described above, and resuspended in distilled, deionized water. The DNA was further purified in an ethidium bromide - cesium chloride gradient. Cesium chloride was added to the DNA solution at a 1:1 ratio of grams per milliliter of solution. Ethidium bromide was added to a final concentration of 0.1 mg/ml and the refractive index adjusted to 1.3990. The gradients were centrifuged in a Sorvall OTD65B ultracentrifuge in a T864 rotor at 33,000 rpm for 48 hours at 20 C. Cesium chloride creates a density gradient during centrifugation causing the DNA to form a band where its density is equal to that of the surrounding medium. The ethidium bromide intercalates the DNA double helix and fluoresces brightly when exposed to

ultraviolet light (LePecq and Paoletti, 1967). This enables the DNA to be easily visualized and harvested from the gradient. The ethidium bromide was removed by extraction with isopropanol saturated with cesium chloride followed by a second ethanol precipitation in an SW41 rotor at 30,000 rpm, 0 C for 1 hour to concentrate the DNA. The DNA was resuspended in a small volume of distilled, deionized water and stored at 4 C. DNA obtained from this procedure could be cut by restriction endonucleases.

Plasmid DNA Preparation

1. Transformation. A 1.2 kb squash nitrate reductase cDNA clone in pUC19, a gift from N. M. Crawford, (Crawford et al., 1986) was used to transform E. coli JM105 cells by the method of Lederberg and Cohen (1974). Fifty milliliters of LB broth (10 g bacto-tryptone, 5 g yeast extract, 10 g sodium chloride, pH 7.5,) containing 1 mM magnesium chloride was inoculated with E. coli JM105 cells from an overnight culture and incubated with gentle shaking at 37 C in a Lab-Line Orbit incubator shaker until an OD₅₉₅ of 0.6 was reached. The cells were chilled on ice for 10 minutes and centrifuged in an SS34 rotor for 10 minutes at 5000 rpm and 4 C. The cells were resuspended in 20 ml cold 0.1 M calcium chloride, chilled on ice for 25 minutes and centrifuged as described above. The cells were then resuspended in 0.5 ml 0.1 M calcium chloride. A 100 ul aliquot of the cell suspension was added to a solution

yielding final concentrations of 8 mM Tris-HCl (pH 8.0), 8 mM sodium chloride, 0.4 mM EDTA, 140 mM calcium chloride. Forty nanograms of the plasmid pCmc1, containing a 1.2 kb cDNA segment of the squash nitrate reductase gene was added to the cell suspension. The suspension was incubated on ice for 10 minutes, then at 37 C for five minutes. One milliliter of LB broth containing 1 mM magnesium chloride was added to the cell suspension. The culture was incubated at 37 C with gentle shaking for 1.5 hours to allow amplification of the plasmid. Transformed cells were selected by plating the culture (100 ul per plate) on LB agar plates containing 25 ug/ml ampicillin and incubating the plates at 37 C overnight.

2. Isolation. A large quantity of pCmc1, the pUC19 plasmid containing the cDNA clone was obtained by a modified method of Maniatis et. al. (1982). The transformed cells were grown overnight in LB broth containing 50 ug/ml ampicillin at 37 C with gentle shaking. Ten milliliters of the overnight culture was added to one liter of M9 medium containing 17 mM ammonium chloride, 22 mM potassium phosphate, 42 mM sodium phosphate dibasic, 0.5 mM magnesium chloride, 0.5% casamino acids, 0.4% glucose, 20 mg/l vitamin B₁, 50 ug/ml ampicillin. The culture was shaken at 37 C until it reached an OD₅₉₀ of 0.6, at which time 250 mg chloramphenicol was added and the culture incubated overnight at 37 C with gentle shaking to allow amplification

of the plasmid. Subsequently, 10 ml chloroform was added to the culture and the culture was kept at 37 C with shaking for ten minutes. The culture was centrifuged in a GSA rotor at 8000 rpm for 20 minutes at 4 C. The cells were resuspended in cold 0.01 M Tris-HCl (pH 7.9), 0.001 M EDTA and the centrifugation step repeated. The cells were then resuspended in cold 25% sucrose, 0.05 M Tris-HCl (pH 8.0) and homogenized in a glass homogenizer to break up clumps of cells. Fifty milligrams of lysozyme dissolved in 0.25 M Tris-HCl (pH 8.0) was added to the cell suspension and the mixture stirred slowly on ice for 10 minutes. The mixture was placed in a 37 C water bath for three minutes, after which cold 0.25 M EDTA (pH 8.0) was added and the mixture stirred on ice for five minutes to stop the lysozyme reaction. A solution containing 1.0% Brij 58, 0.4% sodium deoxycholate, 0.0625 M EDTA, 0.05 M Tris (pH 8.0) was added and the mixture kept on ice with stirring for an additional 30 minutes. The suspension was centrifuged at 35,000 rpm for 1 hour at 0 C in a T864 rotor to precipitate chromosomal DNA, cell walls, and other cellular debris. Cesium chloride - ethidium bromide gradients were prepared as described above with a few modifications. Prior to the 48 hour centrifugation the solution was kept at room temperature overnight, filtered through cheesecloth, centrifuged at 33,000 rpm for 1 hour at 20 C in a T864 rotor, filtered through cheesecloth again and the refractive index

readjusted to 1.3990. DNA bands were harvested, pooled and an additional 0.4 mg/ml ethidium bromide added. The refractive index was readjusted to 1.3950 and the ultracentrifugation step repeated as described above. A single DNA band was harvested. Ethidium bromide was extracted, and the DNA ethanol precipitated as described above. The DNA was dried under nitrogen gas and resuspended in 0.6 ml 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. A total of 678 ug of DNA was isolated with an $A_{260/280}$ ratio of 1.64.

Confirmation of the pUC19 plasmid isolation was determined by restriction enzyme digests and agarose gel electrophoresis. Ten micrograms of the plasmid DNA were digested with 20 units of the restriction enzyme Eco RI, and, as molecular weight standards, 2.5 ug of lambda DNA were digested with Hind III as described by Maniatis et. al. (1982) and modified by Bethesda Research Laboratories (BRL). The digests were analyzed by loading 0.4 ug lambda Hind III, 0.9 ug pCmc1 Eco RI and 1.1 undigested pCmc1 onto a 0.8% agarose submarine slab gel and electrophoresed according to the procedure described by Maniatis et. al. (1982). After electrophoresis, the gel was exposed to ultraviolet light and a polaroid picture was taken.

Southern Blotting

Squash DNA was digested with the restriction enzymes Eco RI, Hind III and Bam HI according to the procedure described by Maniatis et. al. (1982) as modified by BRL.

Fifteen micrograms of genomic squash DNA were digested with twenty units of Eco RI and Hind III, while thirty units of Bam HI were used. Lambda DNA was digested with Hind III and pCmc1 was digested with Eco RI as described above. Twelve micrograms of each squash DNA digest, 0.4 ug lambda Hind III and 0.3 ug pCmc1 Eco RI were loaded onto 0.8% agarose submarine slab gels and electrophoresed according to the procedure described by Maniatis et. al. (1982). The gels were exposed to ultraviolet light and polaroid pictures were taken to confirm separation of the DNA fragments. The gels were then soaked in a solution containing 0.4 M sodium hydroxide, 0.6 M sodium chloride for thirty minutes. The DNA in the gels was then transferred to a piece of GeneScreenPlus (DuPont NEN) that had been soaked in distilled, deionized water for a minimum of one hour and in a solution containing 0.4 M sodium hydroxide, 0.6 M sodium chloride for fifteen minutes. Transfer of the DNA from the gels to GeneScreen was performed by the method of Maniatis et. al. (1982) using the 0.4 M sodium hydroxide, 0.6 M sodium chloride solution as the transferring solution. After the transfer of DNA was complete, the piece of GeneScreen was soaked in a solution containing 0.5 M Tris-HCl (pH 7.0), 1 M sodium chloride for fifteen minutes to neutralize the sodium hydroxide. The GeneScreen was subsequently dried at room temperature.

Detection of the DNA fragments containing the genomic sequences of squash nitrate reductase was performed as described by Maniatis et. al. (1982) with the modifications made by DuPont New England Nuclear (NEN). The 1.2 kb squash nitrate reductase cDNA clone was radiolabelled with ^{32}P -dCTP using the nick translation method of Maniatis et. al. (1982) as modified by BRL, and subsequently used as a probe. The blots were placed in sealable plastic bags and incubated at 37 C with constant shaking for 15 minutes in 0.2% SDS, 0.05 M Tris-HCl (pH 7.5), 1 M sodium chloride, 50% formamide. Hybridization was performed by adding 5 mg denatured herring sperm DNA and 0.25 ug denatured probe to the above solution. The herring sperm DNA and probe were denatured by heating in a boiling water bath for 5 minutes. The blots were hybridized overnight at 37 C with constant shaking.

The blots were washed following hybridization as follows: two 5 minute washes consisting of 0.3 M sodium chloride, 0.03 M sodium citrate at 22 C; two 30 minute washes containing the above solution plus 2% SDS at 60 C followed by two 30 minute washes of 0.015 M sodium chloride, 0.0015 M sodium citrate at 22 C. Homologous sequences in the squash DNA fragments to which the probe bound were determined by autoradiography. Squash DNA Eco RI fragments containing sequences homologous to the nitrate reductase cDNA clone and less than 7 kilobases (kb) in length were

desired for cloning into λ gt11, while Bam HI fragments from 9 to 22 kb were desired for cloning in EMBL3.

Cloning

Squash DNA fragments were cloned as described by Maniatis et. al. (1982) with the modifications made by Promega Biotec. Squash Eco RI DNA fragments were inserted into the single Eco RI site of the expression vector λ gt11 and ligated for 2 hours at 16 C using T4 DNA ligase in a buffer containing 30 mM Tris-HCl (pH 7.8), 10 mM magnesium chloride, 10 mM DTT and 0.4 mM ATP. The chimeric λ gt11 were then packaged in a packaging extract obtained from Promega Biotec. Packaging efficiency was determined by infecting cells of the E. coli strain Y1090 with the packaged phage and plating the infected cells on LB plates (LB broth plus 15 grams per liter bacto-agar) containing 100 ug/ml ampicillin with 0.8% LB top agar containing 10 mM magnesium chloride, 0.16 ug/ml isopropyl beta-D- thiogalactopyranoside (IPTG), 0.4 ug/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Recombinant phage were identified by their inability to form blue plaques. The packaged phage were then used to infect cells of the E. coli strain LE392. The infected cells were plated on LB plates with 0.8% LB top agar containing 10 mM magnesium chloride and screened as described below.

The lambdoid vector EMBL3 was prepared to clone squash DNA Bam HI fragments of approximately 22 kb in length as

described by Frischauf et al. (1983) and modified by Promega Biotec. Five micrograms of EMBL3 DNA was digested with the restriction enzyme Bam HI as described above. The digested vector was ethanol precipitated with 0.1 volume 3 M sodium acetate and 2 volumes 100% ethanol, placed at -70 C for 30 minutes and microfuged 10 for minutes. The EMBL3 DNA was resuspended in 10 mM Tris-HCL (pH 8.0), 1.0 mM EDTA, and digested with a 3-fold excess of the restriction enzyme Eco RI as described above. This step was performed to remove short sequences at the 5' and 3' ends of the central fragment to prevent subsequent religation of the EMBL3 DNA onto itself. The digest was extracted with phenol, followed by an ether extraction and precipitated with 0.15 volumes 3M sodium acetate (pH 6.0) and 0.6 volumes isopropanol, incubated on ice for 15 minutes and microfuged as described above. The DNA pellet was washed with 500 ul of 0.3 M sodium acetate (pH 6.0): ethanol (1:2.5), microfuged, rewashed and microfuged again. The EMBL3 DNA was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA at a concentration of 0.5 ug/ul. Although the EMBL3 vector was prepared for cloning squash Bam HI fragments, cloning was not performed with this vector.

Detection of λ gt11 clone(s) containing the genomic sequence of squash nitrate reductase was performed by plaque hybridization as described by Maniatis et. al. (1982) with the modifications made by DuPont NEN. Colony/PlaqueScreen

membranes (DuPont NEN) were placed "A" side down on top of a cooled, plaque-containing agar plate for 5 minutes, scored with a 20 gauge needle and carefully removed without disturbing top agar. The membranes were placed "A" side up on 2 sheets of 3M paper saturated with 0.5 M sodium hydroxide for 2 minutes. The membranes were moved to a fresh sheet of 3M paper soaked with 0.5 M sodium hydroxide for 2 minutes. The two-step process was repeated with 1 M Tris (pH 7.5) to neutralize the sodium hydroxide. The membranes were allowed to dry at room temperature for 1 hour. Prior to hybridization, the membranes were placed in a sealable plastic bag and incubated at 37 C with constant shaking for 3 hours in 1 M sodium chloride, 0.2% polyvinylpyrrolidone (M.W. 40,000), 0.2% ficoll (M.W. 400,000), 0.2% BSA, 0.1% SDS, 0.05M Tris-HCl (pH7.5), 0.001 mM EDTA, 50% formamide, 150 ug/ml denatured (as described above) herring sperm DNA. The above solution was prepared for hybridization of the membranes with the addition of the denatured, radiolabelled 1.2 kb nitrate reductase cDNA clone as described above. Hybridization and washes were performed as described above. Plaques produced by recombinant phage containing sequences homologous to the 1.2 kb nitrate reductase cDNA probe were detected by autoradiography.

Fourteen plaques containing recombinant phage that hybridized to the 1.2 kb cDNA probe were eluted by the method of Maniatis et. al. (1982). The top agar and hard

agar surrounding and including a positive plaque were drawn into a sterile pasteur pipett equipped with a rubber bulb. The agar core was transferred to a sterile 1.5 ml Eppendorf tube containing 600 ul 0.1 M sodium chloride, 0.01 M Tris-HCl (pH 7.8) and 0.01 M magnesium sulfate to which 25 ul chloroform was added. The phage elutions were incubated at 4 C overnight. Aliquots of the phage elutions were plated, transferred to Colony/PlaqueScreen membranes, hybridized to the nitrate reductase cDNA probe as described above. Positive plaques were again identified by autoradiography. The process of picking and rescreening positive phage was repeated three times.

Four of the fourteen putative positives continued to hybridize to the 1.2 kb nitrate reductase cDNA containing plasmid after three rescreening procedures had been performed. Phage DNA was isolated from the four remaining recombinant phage by the small scale plate lysate method of Maniatis et. al. (1982). E. coli LE392 cells were grown overnight with constant shaking at 37 C in NZCYM medium containing 10 g NZ amine, 5 g sodium chloride, 5 g yeast extract, 1 g casamino acids and 2 g magnesium sulfate per liter and adjusted to pH 7.5. Aliquots of the LE392 cells corresponding to 10^9 cells (assuming 1 OD₆₀₀ equals 8×10^8 cells) (Maniatis et. al., 1982) were withdrawn, centrifuged at 4000 g (5700 rpm, SS34 rotor) for 10 minutes at 4 C, and resuspended in 500 ul of a buffer containing 0.1 M sodium

chloride, 0.01 M Tris-HCl (pH 7.8) and 0.01 M magnesium sulfate. Approximately 10^5 plaque forming units (pfu) of the lambda phage recombinants were added to each aliquot of cells, incubated for 20 minutes at 37 C and plated on NZCYM hard agar containing 1.5 % agarose with 3 ml NZCYM top agar containing 0.7 % agarose. The agar plates were incubated at 37 C overnight. The following day the phage were eluted from the top agar by adding 5 ml of phage dilution buffer (0.1 M sodium chloride, 0.01 M Tris-HCl (pH 7.8) and 0.01 M magnesium sulfate) directly onto each plate. The plates were then incubated overnight at room temperature with constant gentle shaking. The next day the buffer was removed from each plate and transferred to 12 ml Corex tubes. Bacterial debris was removed by centrifugation at 8000 g for 10 minutes at 4 C and the supernatants transferred to fresh 12 ml Corex tubes. RNase A and DNase I were added to a final concentration each of 1 ug/ml and the tubes incubated at 37 C for 30 minutes to degrade bacterial DNA and RNA. Bacteriophage particles were precipitated by adding an equal volume of a solution containing 20% polyethylene glycol 8000 and 2 M sodium chloride in phage dilution buffer to each tube and incubating the tubes at 0 C (ice water) for one hour. The tubes were then centrifuged at 10,000 g for 20 minutes at 4 C and the supernatants discarded. The bacteriophage particles were resuspended in 0.5 ml of phage dilution buffer, vortexed and centrifuged at 8000 g for 2

minutes at 4 C to remove debris. The supernatants were transferred to 1.5 ml Eppendorf tubes to which 5 ul 10 % SDS and 5 ul EDTA (pH 8.0) were added. The tubes were incubated at 68 C for 15 minutes to release the phage DNA by degradation of the protein coat. Protein contaminants were removed by extracting one time each with phenol, phenol:chloroform (1:1) and chloroform, transferring the aqueous phases to fresh Eppendorf tubes between each extraction. Phage DNA was precipitated by adding an equal volume of isopropanol. The tubes were placed at -70 C for 20 minutes, thawed and microfuged for 15 minutes at 4 C. The pellets were washed with 70 % EtOH, dried under a stream of nitrogen gas and resuspended in 10 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA. DNA yields and purities for the four positive clones are given in Table IV.

Confirmation of the positive nature of the four clones was performed by dot blot analysis as described by Thomas (1980) and modified by DuPont New England Nuclear. Ten micrograms of each phage DNA and 1.13 micrograms of the nitrate reductase cDNA containing plasmid were denatured in 0.25 N sodium hydroxide for 10 minutes, chilled on ice and diluted with an equal volume of 0.0375 M sodium chloride and 0.0375 M sodium citrate to yield final concentrations of 0.125 N sodium hydroxide, 0.01875 M sodium chloride and 0.01875 M sodium citrate. The DNA solutions were transferred using sterile capillary tubes to the "B" side of

a piece of GeneScreenPlus membrane that had been soaked for 30 minutes in 0.4 M Tris-HCl (pH 7.5). The membrane was allowed to air dry at room temperature. The dot blot was subsequently hybridized to the radiolabelled nitrate reductase cDNA containing plasmid and washed as described previously. Autoradiography indicated that all four clones hybridized to the nitrate reductase cDNA probe.

Clone Characterization

1. Insert Length. The DNA isolated from all four clones by the method of Maniatis *et. al.* (1982) proved to be undigestable by the restriction enzyme Eco RI. DNA from the first clone was purified on a prepacked column of the resin NACS-52, supplied by BRL. Forty micrograms of the clone DNA was placed in a polypropylene tube and diluted with four volumes of a binding buffer containing 0.5 M sodium chloride, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA. The solution was mixed, incubated in a 70 C water bath for 10 minutes and transferred to a 42 C water bath for 10 minutes. The solution was then applied to a NACS-52 Prepac column that had been hydrated with a solution containing 2.0 M sodium chloride, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA and equilibrated with 3-5 ml of the above binding buffer. After the sample solution had passed through the column, the column was washed with 3-5 ml of 42 C binding buffer to remove impurities. The bound DNA was then eluted in three 200 ul aliquots of 2.0 M sodium chloride, 10 mM Tris-HCl (pH

7.2) and 1 mM EDTA. The eluent was diluted by adding an equal volume of distilled, deionized water to prevent subsequent salt precipitation. The DNA was then precipitated by adding 10 ug tRNA and two volumes of pure ethanol. The samples were placed at -70 C for thirty minutes, microfuged at 4 C for 15 minutes and washed with 75% ethanol. The pellets were dried under vacuum and resuspended in sterile distilled, deionized water. A total of 18 ug of the clone DNA was obtained with an $A_{260/280}$ ratio of 2.0.

Ten micrograms of the purified clone DNA was digested with 20 units of the restriction enzyme Eco RI as described by Maniatis et. al. (1982) and modified by BRL. Five micrograms of the clone DNA digested with Eco RI was electrophoresed on a 0.8% agarose submarine slab gel. The clone DNA was barely visible when the ethidium bromide stained gel was exposed to ultraviolet light. Two bands of high molecular weight DNA were faintly visible, indicating the clone DNA had been digested with Eco RI.

It was subsequently discovered that the pUC19 plasmid containing the 1.2 kb squash nitrate reductase cDNA fragment also contains the E. coli lac Z gene possessed by the λ gt11 cloning vector (Yanisch-Perron et. al., 1985). Thus, it was necessary to determine if only the lac Z gene was hybridizing to the clone DNA dot blots, or if the clones contained squash DNA inserts that hybridized to the cDNA

insert of pCmc1. Squash genomic DNA and the first clone DNA were digested with Eco RI, electrophoresed and Southern blotted as described previously. The blot was probed with the 1.2 kb nitrate reductase cDNA insert of pCmc1. The 1.2 kb cDNA fragment was freed from the pUC19 DNA by digesting 14 ug of pCmc1 with 20 units of Eco RI as described by Maniatis et. al. (1982) and modified by BRL. The cDNA fragment was separated from the pUC19 DNA by electrophoresing the Eco RI digest of pCmc1 on a 0.8% low melting agarose gel. The gel was stained with ethidium bromide and exposed to ultra-violet light which allowed the 1.2 kb cDNA fragment to be visualized and cut out of the gel with a razor blade. The gel slice was placed in a polypropylene tube and incubated in a 70 C water bath until the agarose was completely melted. The DNA fragment was purified from the molten agarose on a prepacked NACS column as described previously. A total of 3.8 ug of the 1.2 kb cDNA fragment was obtained with an $A_{260/280}$ ratio of 2.1.

The 1.2 kb squash nitrate reductase cDNA fragment was nick-translated and hybridized to the Southern blot of the squash genomic DNA and the clone DNA. Squash genomic DNA digested with Eco RI gave the same pattern of hybridization obtained when the intact pCmc1 plasmid was used as a probe. A single band of hybridization corresponding to a DNA fragment 1.2 kb in length was obtained in the lane containing the clone DNA digested with Eco RI.

The low yields and subsequent poor gel visibility of the clone DNA purified on a prepacked NACS column would make further characterization of the clone difficult. Consequently, the size of the clone inserts were determined by digesting the four putative positive clones with the restriction enzymes Kpn I and Sst I as described by Maniatis *et. al.* (1982) and modified by BRL. The DNA digests were electrophoresed, Southern blotted and hybridized to the nick translated 1.2 kb squash nitrate reductase cDNA insert and the intact pCmc1 plasmid as described previously.

The restriction digests and Southern blot results indicated the four putative positive clones were identical. A 4.4 kb DNA fragment hybridized to both the 1.2 kb cDNA insert and the intact pCmc1 plasmid. This fragment was isolated by digesting 100 micrograms of the clone DNA with Kpn I and Sst I and electrophoresing the double digest in several lanes of a 0.8% low melting agarose submarine slab gel. The 4.4 kb fragment was cut out of the gel and purified on a prepacked NACS column as described previously. A total of 32 ug of the insert DNA was obtained with an $A_{260/280}$ ratio of 2.0.

2. RNA Dot Blot. Confirmation of the positive nature of the clone insert fragment was obtained by using the insert fragment to probe dot blots of soybean RNAs that had been isolated from cotyledons of seedlings twenty-four hours after treatment with nitrate, glutamine, a combination of

nitrate and glutamine and from cotyledons prior to treatment. Dot blots of the soybean RNAs had been probed with the 1.2 kb squash nitrate reductase cDNA containing plasmid (pCmc1) and had shown hybridization to occur with RNA isolated from seedlings twenty-four hours after treatment with nitrate and a combination of nitrate and glutamine (Smarrelli et. al., in press).

RNA dot blot analysis was performed as described by Thomas (1983) and modified by DuPont New England Nuclear. From each treatment group, 4.5 ug of soybean RNA and 5 ug of pCmc1 DNA were diluted with DEPC-treated water to 5.0 ul. Each sample was dissolved in 50 % dimethyl sulfoxide (DMSO), 1 M deionized glyoxal, 12.5 mM sodium phosphate (pH 6.5). The samples were incubated at 50 C for 15 minutes, chilled on ice and transferred with sterile capillary tubes to the "B" side of a piece of GeneScreenPlus membrane that had been soaked in distilled, deionized water for fifteen minutes. The membrane was allowed to dry at room temperature for thirty minutes. The glyoxal reaction was then reversed by soaking the membrane in 50 mM sodium hydroxide for fifteen seconds and neutralizing the sodium hydroxide by soaking the membrane in 0.15 M sodium chloride, 0.015 M sodium citrate, 0.2 M Tris-HCl (pH 7.5) for thirty seconds. The blot was allowed to air dry at room temperature.

The RNA dot blot was subsequently probed with the purified 4.4 kb insert containing clone fragment that had

been labelled with ^{32}P -dCTP as described previously. The hybridization solution used was identical to that used for the DNA dot blot, although due to the use of a heterologous probe, the stringency was reduced by performing the hybridization at room temperature for 48 hours. Washes were performed as for the DNA dot blot with the following changes: (1) The first two washes were performed for 15 minutes each. (2) The second two washes at 55 C. Autoradiography indicated the clone fragment hybridized to all the RNA samples and to the plasmid DNA.

Materials

Squash seeds (Cucurbita maxima var. buttercup) were obtained from W. Altee Burpee Company. Restriction enzymes, T4 DNA ligase, RNase T₁, the nick translation kit and NACS Prepac columns were obtained from Bethesda Research Laboratories (BRL). Cloning vectors, packaging extracts and E. coli strains were obtained from Promega Biotec. The 1.2 kb squash nitrate reductase cDNA clone was a gift from N. M. Crawford (Crawford, et. al., 1986). Deoxynucleotide [^{32}P] cytosine triphosphate, GeneScreenPlus and Colony/Plaque Screen hybridization transfer membranes were obtained from DuPont New England Nuclear (NEN). RNase A and DNase I were obtained from Sigma Chemical Company. All chemicals used were reagent grade or better. All solutions were prepared with distilled, deionized water.

CHAPTER IV

RESULTS

DNA Isolations

Three different DNA isolation procedures were performed in attempts to obtain genomic DNA from squash tissue that was digestible by various restriction endonucleases. Table I gives the yields of DNA obtained from each procedure in micrograms of DNA per gram of fresh weight tissue. Concentrations of DNA were determined by the absorbance of the DNA solutions at a wavelength of 260 nanometers (nm). At this wavelength, an OD of 1 corresponds to a concentration of 50 ug/ml for double-stranded DNA (Maniatis et. al., 1982). Also included in Table I are the ratios of the absorbances of the DNA solutions at wavelengths of 260 nm and 280 nm. This ratio indicates the purity of the DNA solution with an $A_{260/280}$ of 1.8 representing a pure DNA solution. Ratios less than 1.8 are due to contamination from proteins or phenol (Maniatis et. al., 1982).

Isolation of genomic DNA from squash embryos, cotyledons and primary leaves was performed by modified procedures of Honda et. al. (1966) and Maniatis et. al. (1982). Although embryos yielded the highest concentration of DNA per gram of tissue and the highest $A_{260/280}$ ratio of the three tissue types, the DNA was heavily contaminated and

TABLE I

SQUASH GENOMIC DNA ISOLATIONS

TISSUE	A _{260/280}	YIELD*
Embryos ¹	0.99	15.2
Cotyledons ¹	0.79	2.2
Cotyledons ²	1.41	1.3
Leaves ¹	0.97	0.73
Leaves and cotyledons ³	1.84	7.5

* Microgram DNA per gram fresh weight of tissue.

¹ Modified Honda et. al. procedure.

² Zarowitz procedure.

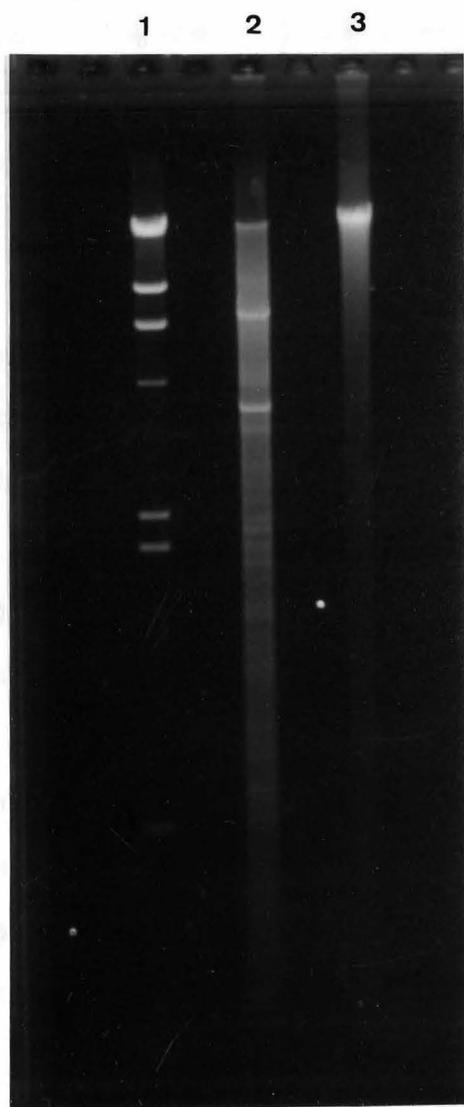
³ Hydroxyapatite chromatography.

undigestible by restriction enzymes. The procedure of Zarowitz (1984) was used to isolate genomic DNA from squash cotyledons. This procedure yielded a relatively low concentration of DNA, but a higher $A_{260/280}$ ratio. DNA isolated from this procedure, however, was still resistant to digestion by restriction endonucleases. The above procedures involved the isolation of intact nuclei prior to preparation of genomic DNA. Due to the problems encountered in the isolation intact nuclei from plants (D'Alessio and Trim, 1968; Hamilton, 1972; Mascarenhas et. al., 1974; Edelman, 1975; Sung and Slightom, In press) the procedure of Walbot and Goldberg (1979) was attempted to circumvent the isolation of nuclei. Table I indicates that when a mixture of primary leaves and cotyledons was used as a source of DNA, this procedure yielded intermediate concentrations of DNA compared to the above procedures. The DNA isolated using hydroxyapatite chromatography and cesium chloride gradient centrifugation had the highest $A_{260/280}$ ratio and was digestible by restriction endonucleases, as depicted in Figure 3.

Plasmid DNA Preparation

A 1.2 kb cDNA clone of squash nitrate reductase in pUC19, obtained from Nigel Crawford (Crawford et. al., 1986), was used to transform JM105 cells by the method of Lederberg and Cohen (1974). A large quantity of the recombinant plasmid, pCmc1, was isolated by a modified

Figure 3. Restriction Enzyme Digest of Squash Genomic DNA.
Lambda DNA digested with Hind III (lane 1), squash DNA digested with Eco RI (lane 2) and undigested squash DNA (lane 3) were electrophoresed on a 0.8% agarose submarine slab gel. The smear of fragments obtained in lane 2 indicates the squash DNA was digested with Eco RI. Bands present within the smear of fragments are due to the presence of repetitive sequences within the squash DNA.



method of Maniatis et. al. (1982). A total of 678 ug of plasmid DNA with an $A_{260/280}$ ratio of 1.83 was isolated.

Confirmation of the plasmid isolation and insert size was performed by restriction enzyme digestion and agarose gel electrophoresis. Figure 4 indicates that purified DNA of the recombinant plasmid, pCmc1, was isolated. The size of the insert fragment is approximately 1.2 kb, while the plasmid DNA minus insert is approximately 2.8 kb in length.

Southern Blots

Southern blots were performed as described in Materials and Methods. Figure 5 shows an autoradiogram of genomic squash DNA digested with the restriction enzymes Eco RI or Hind III. Figure 6 represents the results obtained with genomic squash DNA digested with the restriction enzymes Eco RI or Bam HI. Six bands of hybridization to the nitrate reductase cDNA containing plasmid were given by an Eco RI digestion of squash genomic DNA. Digestions with Hind III and Bam HI produced four and five bands of hybridization, respectively. Table II lists the sizes of the DNA fragments corresponding to the bands of hybridization.

The results of the Southern blots were used to determine if fragments containing sequences homologous to the 1.2 kb nitrate reductase cDNA clone were of an appropriate size to be ligated and packaged in the cloning vectors λ gt11 and EMBL3. The maximum insert size allowing

Figure 4. Isolation of pCmc1. Lambda DNA digested with Hind III (lane 1), pCmc1 digested with Eco RI (lane 2) and undigested pCmc1 (lane 3) were electrophoresed on a 0.8% agarose mini-submarine slab gel. The two bands present in the Eco RI digest of pCmc1 represent the 1.2 kb nitrate reductase cDNA insert and the 2.8 kb pUC19 DNA. Multiple bands are produced by the undigested pCmc1 due to supercoiling of the plasmid.

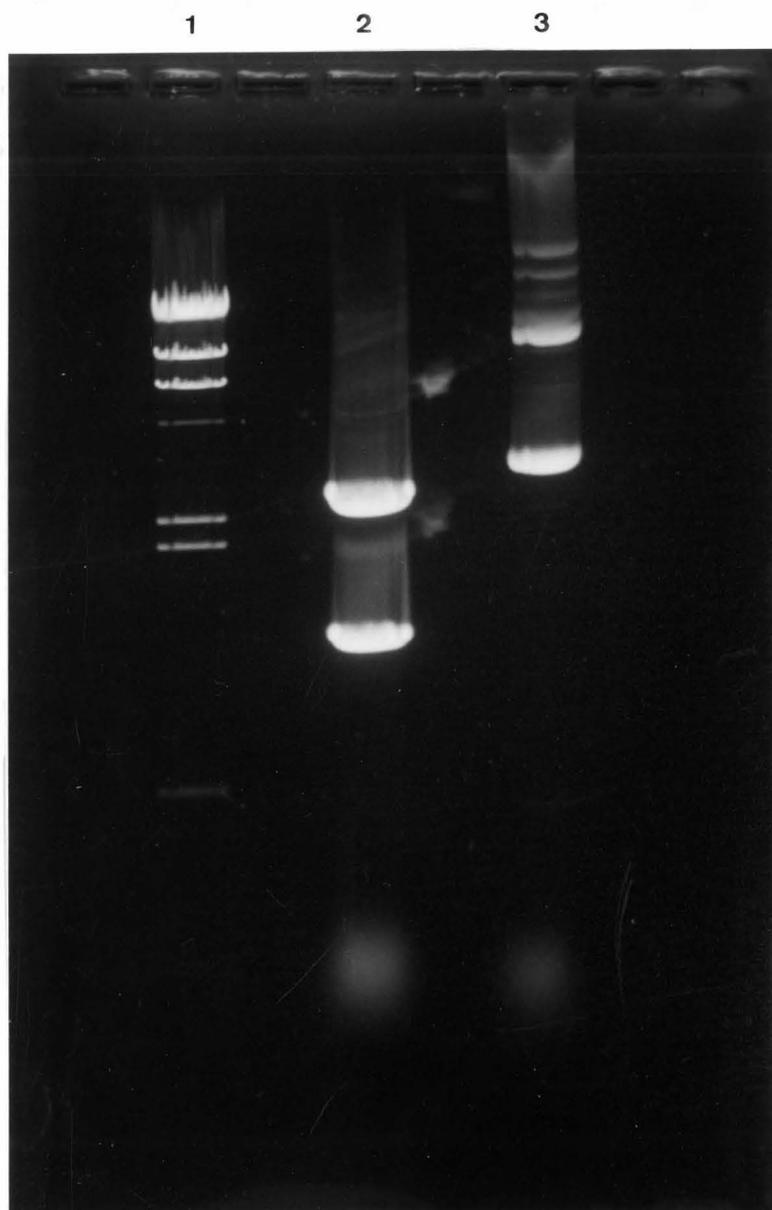


Figure 5. Squash Genomic Southern Blot of Eco RI and Hind III Digests. The Southern blot was probed with pCmc1. Four bands of hybridization were given by a Hind III digest of squash DNA (lane 1). Six bands of hybridization were given by an Eco RI digestion of squash DNA (lane 2). Lane 3 contains undigested squash DNA.

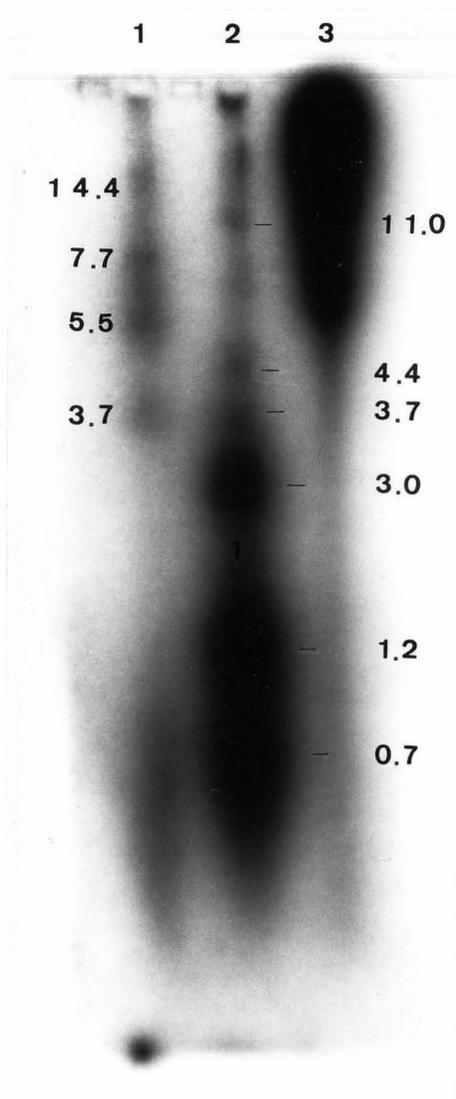


Figure 6. Squash Genomic Southern Blot of Eco RI and Bam HI digests. The Southern blot was probed with pCmc1. Digestion of squash DNA with Bam HI (B) produced five bands of hybridization. Digestion of squash DNA with Eco RI (E) produced six bands of hybridization.

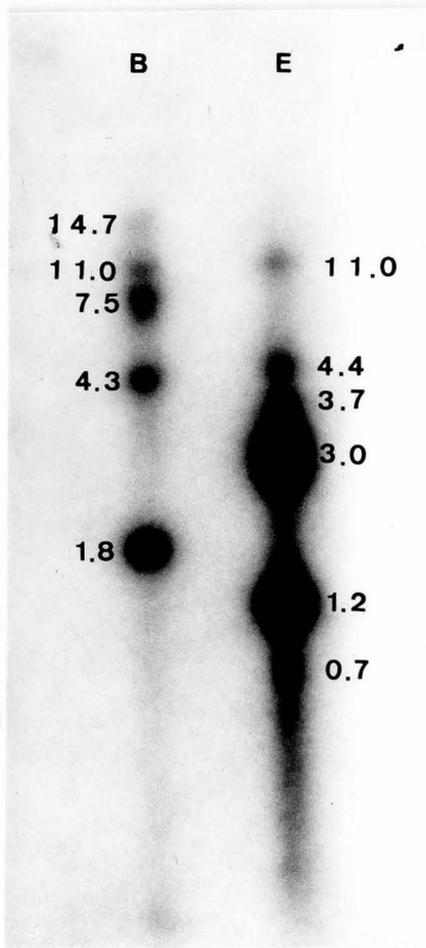


TABLE II

GENOMIC SOUTHERN BLOTS¹Restriction Enzyme Digests

Band Number	<u>Eco RI</u>	<u>Hind III</u>	<u>Bam HI</u>
	Length ²	Length ²	Length ²
1	11.0	14.4	14.7
2	4.4	7.7	11.0
3	3.7	5.5	7.5
4	3.0	3.7	4.3
5	1.2	---	1.8
6	0.7	---	---

¹Probed with 1.2 kb nitrate reductase cDNA containing plasmid.

²Length given in kilobases (kb).

packaging of λ gt11 is 7.2 kb (Huynh et. al., 1984). Digestion of squash genomic DNA with Eco RI yields five bands homologous to the probe that correspond to DNA fragments less than 7.2 kb in length. Thus squash DNA digested as described above is suitable for cloning in λ gt11. The lambdoid vector EMBL3 requires insert DNA fragments digested with Bam HI or Mbo I between 9 and 22 kb in length (Karn et. al., 1983; Kaiser and Murray, 1984). Digestion of squash genomic DNA with Bam HI yields two bands within this range and is suitable for cloning in EMBL3.

Cloning

Squash genomic DNA was digested with Eco RI as described above. Three libraries were constructed by ligating genomic Eco RI fragments into λ gt11 and packaging as described in Materials and Methods. The percentage of recombinant phage and transfection efficiencies for the three libraries are given in Table III.

The three genomic libraries were screened for recombinant phage containing genomic sequences homologous to the 1.2 kb cDNA clone as described in Materials and Methods. A total of 3.1×10^5 plaques were screened, from which fourteen putative positives were found. Four of the fourteen putative positives continued to hybridize to the nitrate reductase cDNA containing plasmid through three rescreening procedures.

TABLE III

CLONING EFFICIENCIES*

LIBRARY	PERCENT RECOMBINANTS	TRANSFECTION EFFICIENCY λ gt11 ¹	TRANSFECTION EFFICIENCY SQUASH ²
1	89.8	7.56×10^4	9.0×10^2
2	93.1	2.95×10^5	3.51×10^3
3	92.7	3.87×10^4	2.47×10^5

* Efficiencies given for E. coli LE392 cells.

¹ Plaque forming units per microgram λ gt11 DNA.

² Plaque forming units per microgram squash DNA.

DNA was isolated from the four remaining recombinant phage by the small scale, rapid phage isolation procedure of Maniatis *et. al.* (1982). Table IV records the yield of DNA in micrograms obtained for each of the four recombinant clones and the purity of the respective DNAs.

Confirmation of the positive nature of the four remaining clones was obtained by dot blot analysis. The DNA isolated from the four recombinant clones was dotted onto a GeneScreenPlus membrane and hybridized as described in Materials and Methods. All four clones hybridized to the plasmid containing the 1.2 kb squash nitrate reductase cDNA clone as indicated by Figure 7.

Clone Characterization.

1. Insert Length. DNA from the four putative positive clones as isolated by the procedure of Maniatis *et. al.* (1982) could not be digested with the restriction enzyme Eco RI even though $A_{260/280}$ ratios of 2.3 were obtained for the clone DNAs. Consequently, 40 ug of clone DNA was purified on a prepacked NACS column. Eighteen micrograms of the clone DNA was recovered from the column with an $A_{260/280}$ ratio of 2.0. The clone DNA was digested with the restriction enzyme Eco RI and electrophoresed as described in Materials and Methods. Two faint bands corresponding to DNA fragments approximately 23 kb in length were visible upon exposure of the ethidium bromide stained gel to ultra-

TABLE IV

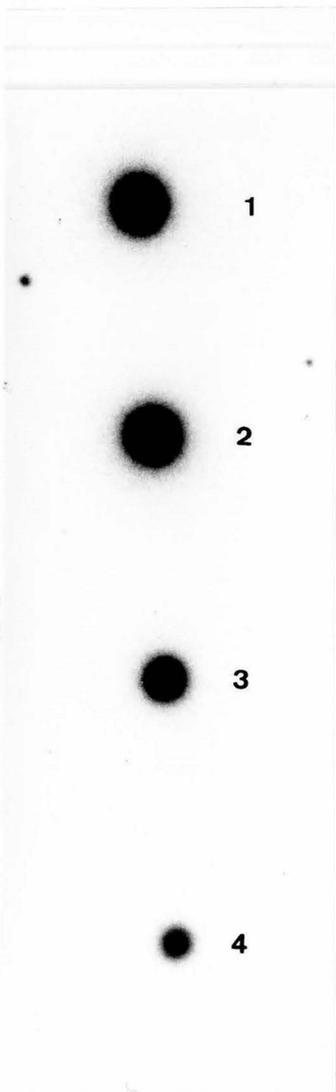
CLONE DNA ISOLATIONS*

CLONE	A _{260/280}	YIELD ¹
1	2.3	13.6
2	2.3	16.5
3	2.3	15.7
4	2.3	11.8

* Maniatis et. al. (1982).

¹ Micrograms of DNA per microliter.

Figure 7. Dot Blot Analysis of Four Putative Positive Clones. DNA isolated from the four putative positive clones (1-4) and pCmc1 (not shown) was dotted onto a positively charged membrane. The membrane was subsequently probed with pCmc1. DNA from all four clones and pCmc1 hybridized to pCmc1.

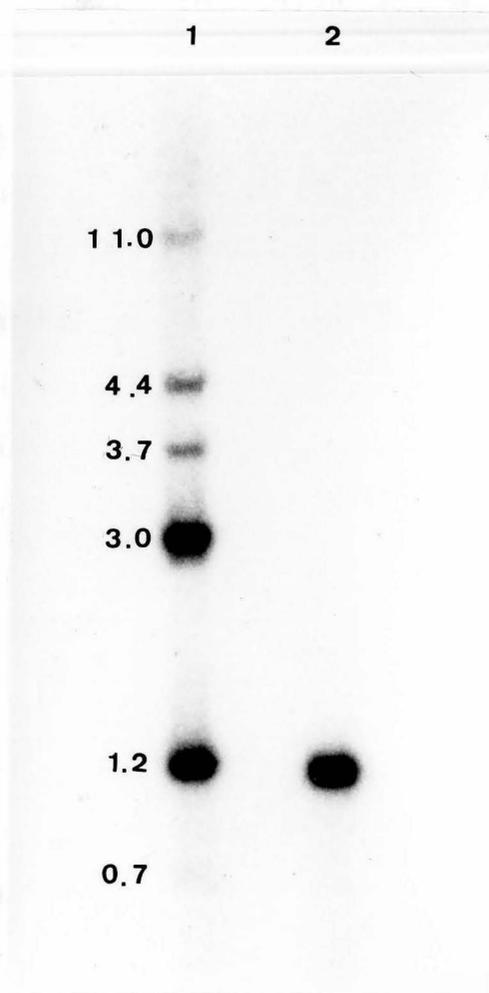


violet light, indicating the clone DNA had been cut by the Eco RI enzyme.

A Southern blot of squash genomic DNA and the clone DNA digested with Eco RI was probed with the nick-translated 1.2 kb nitrate reductase cDNA insert of pCmc1 (Figure 8). Six bands of hybridization were obtained in the lane containing the genomic DNA digested with Eco RI. These bands correspond in size to the six DNA fragments that hybridized to the intact pCmc1 plasmid (Figures 5 and 6, Table II). A single band of hybridization corresponding to a DNA fragment 1.2 kb in length was obtained in the lane containing the clone digested with Eco RI. These results indicate the 1.2 kb cDNA insert of pCmc1 hybridizes to squash genomic DNA. Furthermore, although hybridization of pCmc1 to the clone DNA dot blots could be due to hybridization of the lac Z gene of the pUC19 plasmid to the lac Z gene of λ gt11, the nitrate reductase cDNA insert of pCmc1 also hybridizes to a 1.2 kb fragment of the clone.

The low yields and poor visibility on a gel of the clone DNA purified on prepacked NACS columns made further characterization of the clones by Eco RI digestion difficult. Consequently, DNA from the four clones was digested with the restriction enzymes Kpn I and Sst I, which cut λ gt11 DNA at sites flanking the Eco RI site (see Figure 2). The pattern of DNA fragments produced by single and double digests of the clones with Kpn I and Sst I

Figure 8. Clone and Squash Genomic Southern Blot Probed with cDNA Insert of pCmc1. Six bands of hybridization were given by an Eco RI digestion of squash genomic DNA (1). These bands correspond in size to the DNA fragments obtained when a genomic Eco RI digest was probed with the intact pCmc1 plasmid. A single band of hybridization corresponding to a 1.2 kb DNA fragment was given by an Eco RI digest of the clone DNA (2).

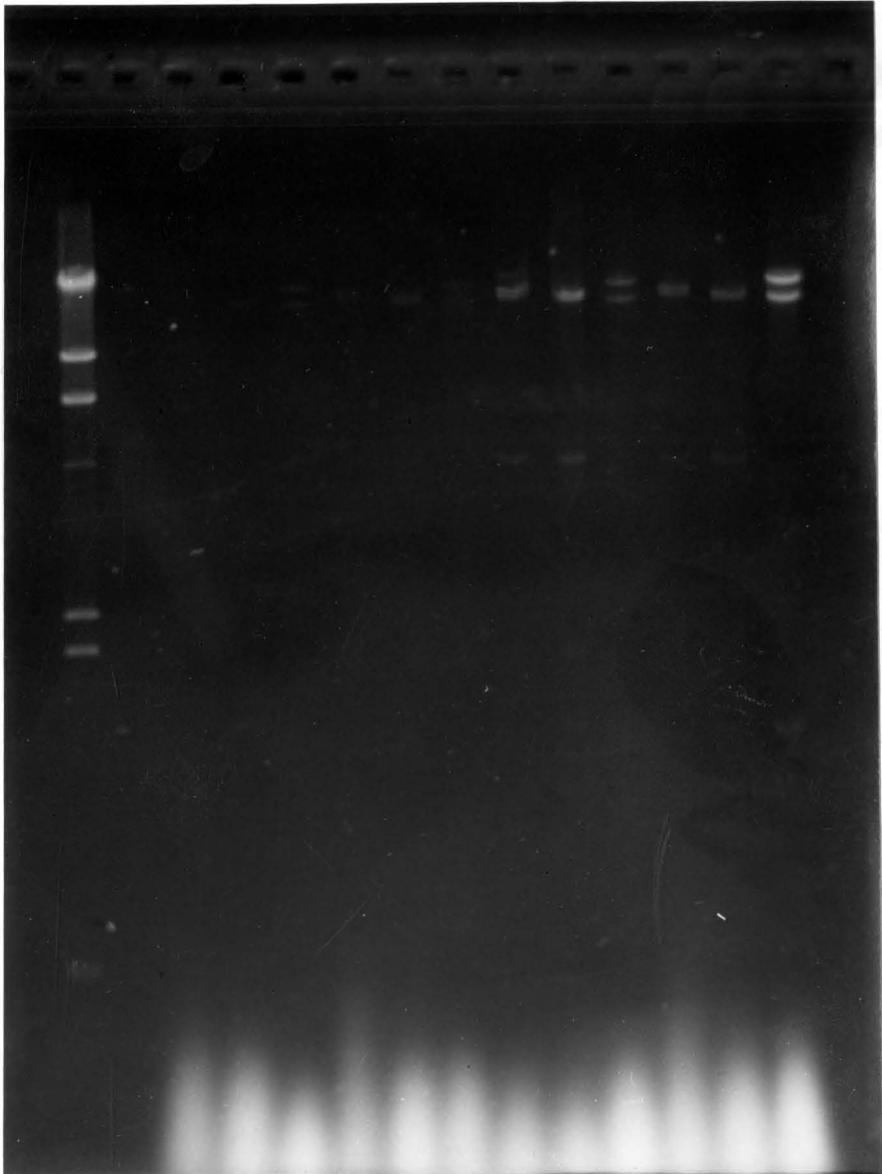


electrophoresed on a 0.8% agarose gel is shown in Figure 9. The digest patterns indicate the four putative positive clones are identical. Table V lists the fragment sizes produced by the single and double digests of the clone DNA.

Southern blots of the clone Kpn I and Sst I double digest were performed. One blot was probed with the nick-translated intact pCmc1 plasmid (Figure 10). The second blot was hybridized to the nick-translated nitrate reductase cDNA insert of pCmc1 (Figure 11). A single band of hybridization corresponding to a DNA fragment 4.4 kb in length was obtained in the clone lanes of both blots. Therefore, hybridization of the intact pCmc1 plasmid to the clone DNA dot blots results not only from hybridization of the lac Z genes, but also from hybridization of the 1.2 kb cDNA insert of pCmc1 to part of the squash DNA insert. Furthermore, in the Southern blots, both the nitrate reductase cDNA fragment and the intact plasmid hybridized to a 4.4 kb fragment of the clone double digest, but digestion of the clone DNA with Sst I produces a 4.4 kb fragment containing only λ gt11 DNA with approximately 2 kb of the lac Z gene (see Figure 2) which would not hybridize to the nitrate reductase cDNA. Consequently, it is proposed that digestion of the clone DNA with Sst I produces two 4.4 kb fragments. One of the 4.4 kb fragments contains only λ gt11 DNA, while the second fragment contains squash genomic DNA

Figure 9. Restriction Enzyme Digestion of Four Putative Positive Clones. DNA isolated from the four putative positive clones (1-4) were digested with Sst I (a), Sst I and Kpn I (b) and Kpn I (c). Digests were electrophoresed on a 0.8% agarose submarine slab gel. The same pattern of fragments was produced by all four clones.

1 2 3 4
a b c a b c a b c a b c



4.4

2.1

1.5

Table V

Clone Restriction Enzyme Digests

Band Number	<u>Sst I</u> Length ¹	<u>Sst I/Kpn I</u> Length ¹	<u>Kpn I</u> Length ¹
1	23.0	23.0	29.5
2	20.7	17.1	17.1
3	4.4	4.4	1.5
4	---	2.1	---
5	---	1.5	---

¹Length given in kilobases (kb).

Figure 10. Southern Blot Analysis of Putative Positive Clones Probed with Intact pCmc1. Sst I and Kpn I double digests of the clone DNA were electrophoresed (lanes 1 and 2) and blotted as described in Materials and Methods. The blot was probed with the intact pCmc1 plasmid. One band of hybridization corresponding to a DNA fragment 4.4 kb in length was produced by the double digests.

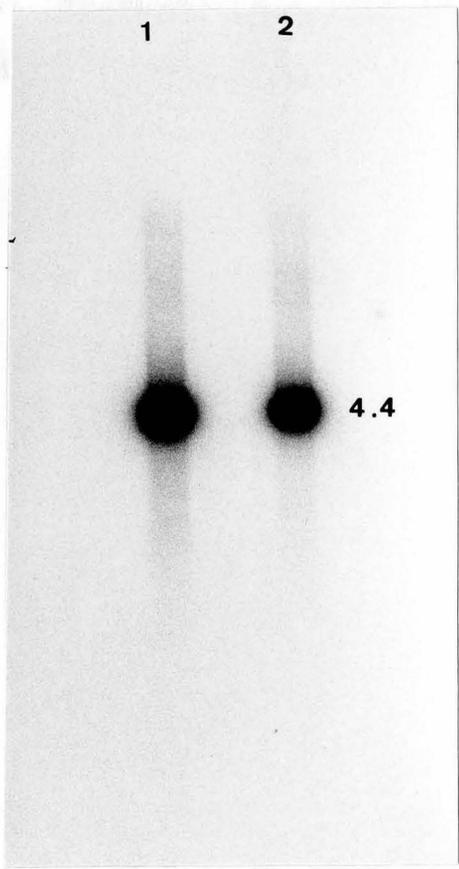
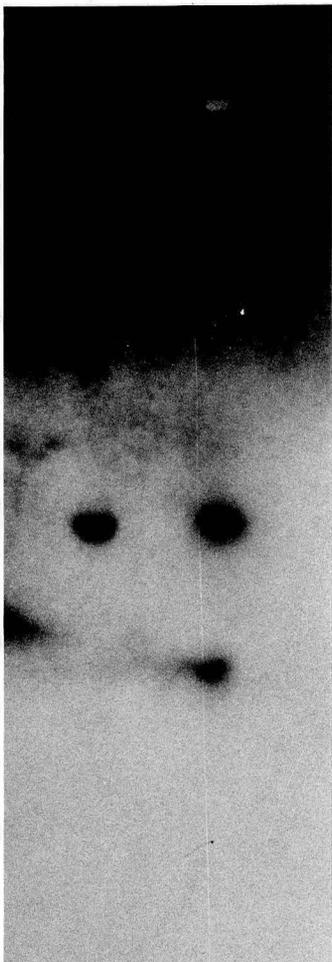


Figure 11. Southern Blot Analysis of Putative Positive Clones Probed with cDNA Insert of pCmc1. Double digests of the clone DNA with Sst I and Kpn I were electrophoresed (lanes 1 and 2) and blotted as described in Materials and Methods. The blot was probed with the 1.2 kb nitrate reductase cDNA insert of pCmc1. One band of hybridization corresponding to a DNA fragment 4.4 kb in length was produced by the double digests.

1

2

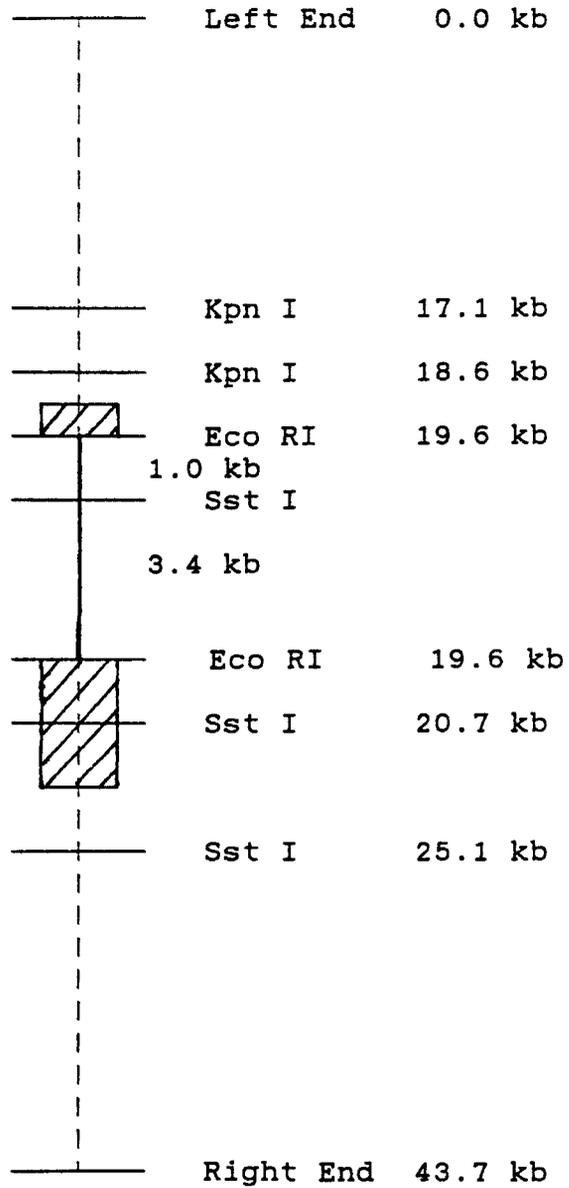


4.4

with sequences homologous to the nitrate reductase cDNA fragment.

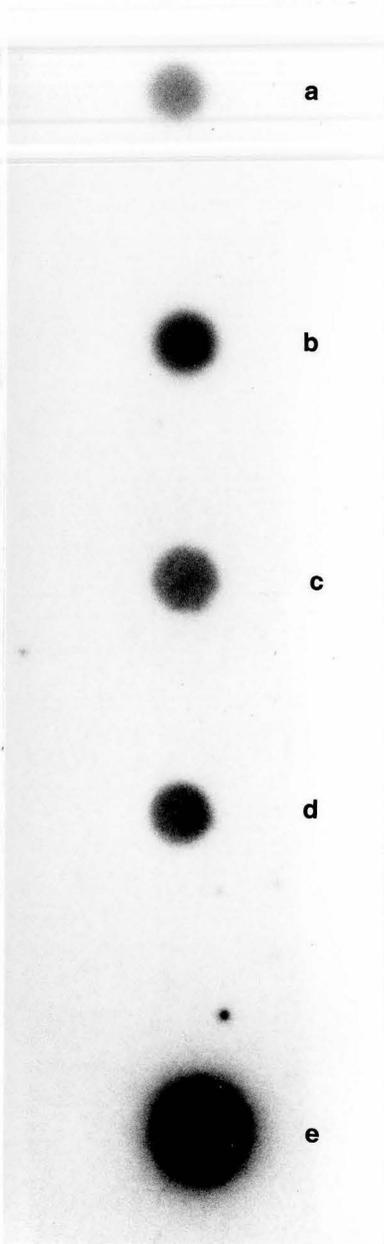
A map of the proposed structure of the clone, λ 0snr, is shown in Figure 12. The squash DNA insert fragment is proposed to be 4.4 kb in length. The length of this fragment agrees with the size of one fragment from the Southern blot of squash genomic DNA digested with Eco RI that hybridized to pCmc1. An Sst I site is proposed to be present in the squash DNA insert 1.0 kb upstream of the 5' end of the squash DNA fragment. Digestion of the clone with Kpn I and Sst I divides the fragment between the λ gt11 Kpn I and Sst I sites flanking the Eco RI site into two fragments 2.1 kb and 4.4 kb in length. These fragments contain approximately 1.0 kb and 3.4 kb of the squash DNA insert. The 1.2 kb nitrate reductase cDNA insert of pCmc1 hybridizes to the 3.4 kb portion of the insert, but does not contain sequences homologous to the 1.0 kb portion of the insert. Thus, only the 4.4 kb fragment containing 3.4 kb of the squash insert produced a band of hybridization in the Southern blot probed with the nitrate reductase cDNA fragment. A second 4.4 kb band is produced by digestion of the λ gt11 DNA with Sst I at the 20.7 kb and 25.1 kb sites. Both 4.4 kb fragments contain part of the lac Z gene and hybridize to the lac Z gene contained in the pUC19 DNA of pCmc1.

Figure 12. Proposed Structure of λ Osnr. Restriction sites for the enzymes Kpn I, Sst I and Eco RI are shown. The squash DNA insert is indicated by the solid line. The box with hash marks represents the lac Z gene. A 2.0 kb Kpn I/Sst I fragment is proposed to contain 1.0 kb of squash genomic DNA. A 4.4 kb Sst I/Sst I fragment that hybridized to the 1.2 kb cDNA insert of pCmc1 is proposed to contain 3.4 kb of squash genomic DNA. Total insert size (Eco RI/Eco RI fragment) is therefore 4.4 kb. The 4.4 kb fragment containing the squash DNA insert and the 4.4 kb Sst I/Sst I fragment of λ gt11 DNA hybridize to the lac Z gene of the intact pCmc1 plasmid.



2. RNA Dot Blots. Dot blot analyses of soybean RNAs probed with the 4.4 kb squash insert containing fragment did not produce the same results obtained when pCmc1 was used as a probe (Smarrelli et. al., in press). The soybean RNA dot blot results are shown in Figure 13. The 4.4 kb clone fragment hybridized to all RNA samples, while pCmc1 hybridized only to the 24 hour nitrate and nitrate-glutamine RNAs (Smarrelli et. al., in press). The 4.4 kb clone fragment contains at least 2.2 kb of squash DNA not present in the 1.2 kb nitrate reductase cDNA insert of pCmc1. The additional DNA sequences present in the 4.4 kb clone fragment may account for the difference in the hybridization pattern when RNA dot blots are probed with intact pCmc1 or the 4.4 kb clone fragment.

Figure 13. Soybean RNA Dot Blot. A 4.4 kb clone fragment that hybridized to the 1.2 kb cDNA insert of pCmcl was used to probe dot blots of soybean RNAs. RNAs were isolated from cotyledons of seedlings treated with (a) no nitrate or glutamine, (b) 10 mM glutamine, (c) 50 mM nitrate and (d) 10 mM glutamine, 50 mM nitrate. DNA from pCmcl (e) was also included. For each sample 4.5 ug of RNA and 5 ug of DNA were applied to the positively charged membrane.



CHAPTER V

DISCUSSION

The cloning vector λ gt11 was utilized to isolate part of the genomic sequence of the nitrate reductase structural gene from squash. Before the cloning process could begin, however, isolation of squash genomic DNA digestible by the restriction enzyme Eco RI was required. When isolated nuclei were used as the source of DNA, predominantly low yields of impure DNA were obtained as shown in Table I. DNA isolated from squash embryo nuclei was an exception to the low yields, but contained numerous contaminants as evidenced by the ratio of absorbances at 260 nm and 280 nm. The low yields of DNA can be explained by the problems encountered in the isolation of intact nuclei from plant tissue (D'Alessio and Trim, 1968; Hamilton *et. al.*, 1972; Kislev and Rubenstein, 1980; Mascarenhas *et. al.*, 1974). The abundance of oils, pigments, polyphenols, polysaccharides and other secondary metabolites in plant tissue that purify with DNA (Edelman, 1975; Kislev and Rubenstein, 1980; Loomis, 1974; Segovia *et. al.*, 1965; Sung and Slightom, in press) can account for the impurity of DNA from isolated nuclei and its resistance to digestion by the restriction enzyme Eco RI.

Chromatography using hydroxyapatite has been used to purify DNA from a variety of plant and animal tissues (Bernardi, 1971; Britten et. al., 1970; Meinke et. al., 1974; Walbot and Goldberg, 1979). Calcium ions present on the surface of hydroxyapatite crystals are involved in the adsorption of nucleic acids due to electrostatic interaction with the negatively charged phosphate groups (Bernardi, 1965; Bernardi, 1971). The secondary structure of the DNA double helix causes the phosphate groups of the sugar-phosphate backbone to protrude further than the phosphate groups of RNA, single-stranded (ss) DNA or RNA-DNA hybrids (Martinson, 1973b). The accessibility of the phosphate groups allows double-stranded (ds) DNA molecules to bind the calcium ions of hydroxyapatite more tightly than RNA, ssDNA or RNA-DNA molecules. Thus, RNA, ssDNA and RNA-DNA hybrids are eluted from a hydroxyapatite column at low salt concentrations, while dsDNA is eluted at high salt concentrations (Martinson, 1973b; Meinke et. al., 1974). When urea is present in the binding and low salt elution buffers, the secondary structure of proteins and polysaccharides in the tissue homogenate is thought to be disrupted, decreasing the affinity of these substances for hydroxyapatite and allowing them to be eluted in the low salt buffer (Britten et. al., 1970, Meinke et. al., 1974). The effect of salt concentration on the interaction between hydroxyapatite and nucleic acids has been linked to the

phosphate anion concentration of the buffer used (Bernardi, 1965; Martinson, 1973a). Phosphate anions in the buffers compete with the phosphate groups of nucleic acids for the calcium ions of hydroxyapatite (Martinson, 1973b). Thus, a lower phosphate molarity is needed to out compete the poorly exposed phosphate groups of RNA and ssDNA, while a higher phosphate molarity is needed to cause the elution of dsDNA due to its exposed phosphate groups.

Isolation of squash DNA using hydroxyapatite chromatography combined with cesium chloride density centrifugation yielded DNA that could be digested with restriction enzymes. Squash DNA isolated by this procedure was digested with the restriction enzymes Eco RI, Hind III and Bam HI. Southern blots of these digests were probed with pCmc1, a 1.2 kb squash nitrate reductase cDNA containing plasmid. The results of the Southern blots indicated multiple bands of hybridization in all three digests.

Several explanations exist to account for the multiple squash DNA fragments hybridizing to pCmc1. First, the digests may have been partial digests, in which case the restriction enzymes did not cleave at all of their respective restriction sites. Second, restriction sites for the enzymes may be located within introns of the genomic sequence encompassed by pCmc1. Since the 1.2 kb nitrate reductase sequence encoded by pCmc1 was produced from a

mature RNA transcript, the introns had been excised during processing of the transcript. Consequently, digestion of the squash genomic DNA with Eco RI, Bam HI or Hind III may have cleaved the squash DNA in introns, dividing the sequence encoded by pCmc1 into multiple fragments. Finally, a combination of these explanations may have produced the multiple bands of hybridization between pCmc1 and the genomic digests.

The results of the genomic Southern blots were used to determine if Eco RI and Bam HI digests could be used for cloning in the vectors λ gt11 and EMBL3, respectively. Five of the six DNA fragments from the Eco RI digest that hybridized to pCmc1 were less than the upper insert limit of 7.2 kb possessed by λ gt11 (Huynh et. al., 1984). Thus isolation of five DNA fragments containing sequences homologous to pCmc1 could be performed by creating genomic libraries in λ gt11 and screening these libraries with pCmc1.

The cloning vector EMBL3 requires insert fragments digested with Bam HI or Mbo I between 9 and 22 kb in length for packaging of the vector to occur (Karn et. al., 1983; Kaiser and Murray, 1984). Two of the five DNA fragments from the Bam HI digest of squash genomic DNA that hybridize to pCmc1 fall within this range. The length of these two fragments reported in Table II is subject to error due to the fact that under the conditions the gel used for the Southern blot was run, the lambda Hind III fragments do not

run linearly according to the \log_{10} of their molecular weights. The two squash genomic DNA fragments generated by the Bam HI digest migrated distances intermediate between the 23.1 kb and 9.4 kb lambda Hind III fragments. Thus, the size of the two Bam HI fragments may be estimated to fall between the 9 and 22 kb limits imposed by EMBL3.

Three genomic libraries were created in λ gt11 and screened with ^{32}P -dCTP - labelled pCmc1. Fourteen putative positive clones were isolated of which four continued to hybridize to pCmc1 after three rescreening procedures had been performed. DNA dot blot analysis of the four remaining clones indicated they did hybridize to pCmc1.

DNA from the four remaining clones as isolated by the method of Maniatis et. al. (1982) proved to be resistant to digestion by the restriction enzyme Eco RI, preventing the excision of the squash DNA insert. Purification of DNA from the first clone on a prepacked NACS column, however, allowed the clone DNA to be digested by Eco RI. Thus, resistance to digestion by Eco RI was due to the presence of a substance inhibitory to Eco RI, although the ratios of absorbances at 260nm and 280nm indicated clean clone DNAs had been isolated.

Hybridization of pCmc1 to dot blots of the clone DNAs did not prove the squash DNA inserts of the clones were homologous to the nitrate reductase cDNA insert of pCmc1. It was subsequently learned that the pUC19 DNA of pCmc1

contained the E. coli lac Z gene (Yanisch-Perron et. al., 1985), which is also possessed by the λ gt11 vector. Thus, it was necessary to show that the clones hybridized to the nitrate reductase cDNA insert of pCmc1.

The cDNA insert of pCmc1 was freed from the pUC19 DNA by digestion with Eco RI and purified on an NACS prepacked column. The purified cDNA insert was labeled with 32 P-dCTP and used to probe Eco RI digests of the clone and squash genomic DNA (Figure 8). The results of this blot indicated that hybridization of pCmc1 to squash genomic DNA digests was due to the 1.2 kb cDNA insert. This was expected since the lac Z gene is an E. coli gene and is not present in the squash genome. The 1.2 kb cDNA insert of pCmc1 produced a single band of hybridization in the lane containing the Eco RI digest of the clone. The band of hybridization corresponded to a DNA fragment 1.2 kb in length. A DNA fragment of this size hybridized to the cDNA insert in the squash genomic DNA digest and was found in the Eco RI genomic blots probed with the intact pCmc1 plasmid (Table II). Thus, the clone contains squash sequences homologous to the nitrate reductase cDNA insert of pCmc1. The squash DNA insert appears to be 1.2 kb in length, but since the Eco RI digest used to create the λ gt11 libraries may have been a partial digest, and the clone fragments were not clearly visible on the gel, the exact length of the insert could not be determined from this experiment.

The low yield of clone DNA from the NACS column and resulting poor visibility on gels was circumvented by digesting the clone DNAs with the restriction enzymes Kpn I and Sst I. A restriction site for Kpn I is located 1.0 kb upstream of the Eco RI site of λ gt11, while an Sst I restriction site is located 1.0 kb downstream of the Eco RI site. The electrophoretic pattern of DNA fragments from single and double digests of the clones with Kpn I and Sst I indicated the four remaining clones were identical.

Southern blots of the clone Kpn I and Sst I double digests were probed with the intact pCmc1 plasmid and the cDNA insert of pCmc1. The clone digests in both blots produced a single band of hybridization corresponding to a DNA fragment 4.4 kb in length. Hybridization between the clone DNAs and the cDNA insert of pCmc1 could only occur if the clones contained sequences homologous to the nitrate reductase cDNA. Thus, the 4.4 kb clone fragment must contain sequences of the squash nitrate reductase structural gene. Hybridization of the clone DNAs to the intact pCmc1 plasmid could occur between the lac Z genes of the pUC19 DNA and λ gt11 DNA, and with squash genomic insert sequences homologous to the cDNA insert of pCmc1. The lac Z gene of λ gt11 is primarily located within a 4.4 kb Sst I/ Sst I fragment of λ gt11 DNA and the fragment containing the squash genomic DNA inserted in the Eco RI site of the lac Z gene. Since a single band of hybridization is produced by the Kpn

I and Sst I double digests of the clone DNAs probed with the intact pCmc1 plasmid, but at least 2 fragments contain sequences of the lac Z gene, both fragments must be the same size.

The information provided by the clone Kpn I/ Sst I digests (Figure 9), the Southern blots of the clone Kpn I/ Sst I double digests (Figures 10 and 11) and the λ gt11 restriction map (Figure 2) was used to construct a restriction map of the clone λ Osnr, shown in Figure 12. One of the two 4.4 kb DNA fragments contains only λ gt11 DNA and is produced by digestion with Sst I at 20.7 kb and 25.1 kb. This fragment contains part of the lac Z gene and would hybridize to the lac Z gene in the intact pCmc1 plasmid. The second 4.4 kb fragments contains part of the squash DNA insert and part of the lac Z gene. This fragment would hybridize to the lac Z gene of the intact pCmc1 plasmid. This fragment also hybridizes to the cDNA insert of pCmc1, therefore part of the squash DNA insert must be homologous to the nitrate reductase cDNA insert of pCmc1. A 2.1 kb fragment is produced only by a double digest of the clone DNA with Kpn I and Sst I and must therefore lie between a Kpn I site and an Sst I site. To account for the 4.4 kb fragments and the 2.1 kb fragment, this fragment must start at the λ gt11 Kpn I located at 18.6 kb. The Sst I site must therefore lie within the squash DNA insert. The 2.1 kb fragment contains approximately 53 base pairs of the lac Z

gene located at the 3' end of the gene, however this fragment does not hybridize to the intact pCmc1 plasmid. It is possible that the lac Z gene of pUC19 was truncated at the 3' end when the plasmid was engineered. The 3' terminal sequences of the lac Z gene would not be present in the pUC19 DNA of pCmc1 and could not hybridize to this portion of the lac Z gene in λ gt11. The 2.1 kb fragment does not hybridize to the cDNA insert of pCmc1. Thus, the squash genomic sequences contained in this fragment are not found in the nitrate reductase cDNA sequence.

The 4.4 kb clone fragment that hybridized to the cDNA insert of pCmc1 and the intact pCmc1 plasmid was used to probe soybean RNA dot blots. Although both 4.4 kb fragments were present in the probe, the lac Z gene fragments would not be expected to hybridize to soybean transcripts. Consequently, any hybridization that occurred would result from the squash genomic insert. Aliquots of the RNA samples used for these dot blots had previously been used for dot blots probed with the intact pCmc1 plasmid. The earlier dot blots had shown hybridization to occur only between the pCmc1 and the RNAs from nitrate and nitrate - glutamine treated soybean plants (Smarrelli *et. al.*, In press). When the 4.4 kb clone fragment was used to probe RNA dot blots, all RNA samples hybridized to the 4.4 kb clone fragment.

The 4.4 kb clone fragment contains approximately 3.4 kb of squash DNA, which is at least 2.2 kb more squash DNA

than pCmcl contains. Since the extent of hybridization between pCmcl and the 4.4 kb clone fragment is unknown, it is possible that not all of the 1.2 kb cDNA insert of pCmcl hybridizes to the 4.4 kb clone fragment. This increases the amount of additional DNA possessed by the clone fragment which may hybridize to RNAs that do not hybridize to pCmcl.

The additional DNA sequences contained in the clone fragment may not all code for nitrate reductase. If this is true, the non-nitrate reductase sequences may or may not be transcribed. However, since the 4.4 kb clone fragment hybridizes to transcripts from untreated and glutamine treated seedlings, while pCmcl does not hybridize to transcripts from these treatment groups, the additional sequences in the clone fragment must be transcribed. Furthermore, transcription of the additional sequences does not appear to be influenced by the presence of glutamine or the absence of nitrate. Thus, the additional sequences contained within the squash DNA insert may encode a protein other than nitrate reductase, or they may represent one or more repetitive sequences which are prevalent within plant genomes and are transcribed sequences (Britten and Kohne, 1968; Flavell et. al., 1974; Goldberg, 1978; Rivin, 1986).

The presence of non-nitrate reductase sequences within the squash DNA of the 4.4 kb clone fragment raises the question of where the sequences homologous to the nitrate reductase cDNA fragment lie within the squash DNA insert.

The Southern blot results of the NACS-52 purified clone DNA digested with Eco RI and probed with the cDNA insert of pCmc1 (Figure 8) indicate the clone insert sequences homologous to the nitrate reductase cDNA are located within a 1.2 kb DNA fragment. Since the electrophoretic profile and Southern blots of the clone Kpn I and Sst I double digests (Figures 9, 10 and 11) indicate the size of the squash DNA insert is 4.4 kb, the genomic digest used to create the λ gt11 libraries must have been a partial digest. Furthermore, a 3.2 kb Eco RI/Eco RI fragment of the squash insert does not contain sequences homologous to the nitrate reductase cDNA. If the 3.2 kb Eco RI/Eco RI fragment of the squash DNA insert were located at the end of the insert closest to the 18.6 Kpn I site and the 1.2 kb Eco RI/Eco RI fragment was located at the end of the insert closest to the 20.7 kb Sst I site, the results of clone Southern blots and the RNA dot blots could be explained. The non-nitrate reductase sequences within the 4.4 kb Sst I/Sst I clone fragment would lie within the 3.2 kb Eco RI/Eco RI fragment. Also, the squash DNA sequences of the 2.1 kb Kpn I/Sst I fragment would lie within the 3.2 kb Eco RI/Eco RI fragment. This would explain why the 3.2 kb fragment did not hybridize to the cDNA insert of pCmc1 in Figure 8, and why part of the 4.4 kb Sst I/Sst I clone fragment hybridized to all the RNA samples in Figure 13. Location of the 1.2 kb Eco RI/Eco RI fragment within the 4.4 kb Sst I/Sst I fragment would also

explain why this fragment hybridizes to the cDNA insert of pCmc1.

The extent of the clone insert sequences that encode part of the nitrate reductase structural gene can be accomplished by subcloning the insert. Subcloning would allow small pieces of the insert to be used as probes for RNA dot blots. Those subclones containing exons of the nitrate reductase structural gene should hybridize to the RNA dot blots in the same pattern as the intact pCmc1 plasmid.

Isolation of the entire genomic sequence for the squash nitrate reductase structural gene can proceed after nitrate reductase specific subclones are identified. These subclones can be used to probe squash genomic libraries created in the cloning vector EMBL3. The EMBL3 vector has been prepared for acceptance of the squash genomic DNA fragments, although genomic libraries have not been created. The squash genomic DNA fragments that would allow packaging of chimeric EMBL3 molecules would be much larger than those found in λ gt11 genomic libraries, which should facilitate the isolation of bigger sections, or potentially all, of the nitrate reductase structural gene. Furthermore, since the squash DNA fragments used in the creation of λ gt11 and EMBL3 libraries are generated by digestion with different restriction enzymes, the chances of isolating the exact same sequences are greatly reduced. Due to the large DNA

fragments incorporated by the EMBL3 vector, the subcloning process will need to be repeated on the insert fragments of positive clones isolated from EMBL3 libraries. Again, nitrate reductase specific sequences can be determined by using the subclones to probe RNA dot blots.

A greater understanding of squash nitrate reductase at the molecular level can proceed following the isolation of the nitrate reductase structural gene. The number and length of introns and exons within the genomic sequence of nitrate reductase can be determined by S1 nuclease mapping. This procedure will also help to determine transcription initiation and termination sites (Lewin, 1985). Sequencing of the nitrate reductase structural gene would also be possible. Knowledge of the nucleotide sequence and number and length of exons will enable the location of exons and introns within the nitrate reductase structural gene to be established. Finally, the amino acid sequence of the nitrate reductase structural gene could be proposed once the nucleotide sequence and exon locations are known.

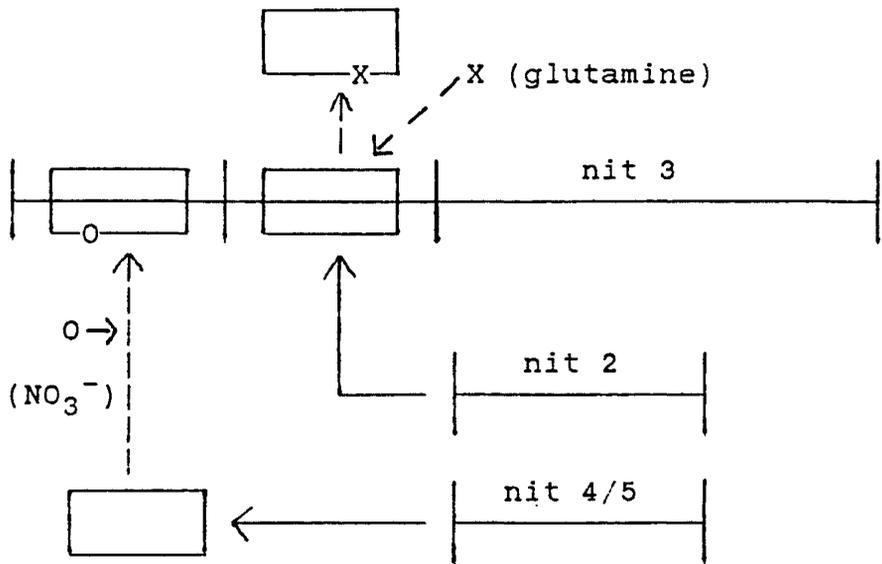
Genetic Regulation of Nitrate Reductase

The genetic regulation of nitrate reductase can be studied more extensively once the nitrate reductase structural gene has been isolated. Studies involving Neurospora crassa have correlated the regulation of nitrate reductase with at least five loci (Dunn-Coleman et. al., 1984; Garrett and Amy, 1978). The nitrate reductase

apoprotein is encoded by the nit-3 gene. The nit-1 gene encodes the molybdenum cofactor, although molybdenum cofactor mutants have also been linked to the nit-7, nit-8 and nit-9 genes (Dunn-Coleman *et. al.*, 1984). The nit-2 gene encodes a positive regulator which is required for the expression of nitrate reductase, but whose action is repressed by the presence of reduced nitrogen. The nit-4/5 locus encodes a second positive regulator required for the induction of nitrate reductase by nitrate (Dunn-Coleman *et. al.*, 1984; Garrett and Amy, 1978). Mutations in any of these genes result in the loss of nitrate reductase activity.

A diagrammatic representation of the proposed regulation of nitrate reductase in *N. crassa* is shown in Figure 14. The nit-2 gene product is thought to bind a regulatory site upstream of the nit-3 gene. When nitrate is present, it is thought to bind the nit-4/5 gene product. The nitrate - nit-4/5 gene product complex subsequently binds a second regulatory site upstream of the nit-3 gene. The presence of the nit-2 and nit-4/5 gene products at their upstream regulatory sites is thought to be required for transcription of the nit-3 gene. When a reduced nitrogen source, such as glutamine, is present, it may bind to the nit-2 gene product causing this regulatory protein to fall off the upstream regulatory site. Removal of the nit-2 gene

Figure 14. Proposed Regulation of the Nitrate Reductase structural Gene in Neurospora crassa. Two regulatory sites are thought to lie upstream of the nitrate reductase structural gene (nit-3). The nit-2 gene product is thought to bind one regulatory site, while a nitrate - nit-4 gene product complex is thought to bind the second regulatory site. The presence of both regulatory proteins at the regulatory sites is proposed to be required for transcription of the nit-3 gene to occur. When glutamine is present, it is thought to bind the nit-2 gene product causing it to fall off the regulatory site, thereby inhibiting transcription of the nit-3 gene.



product would block transcription of the nit-3 gene (Dunn-Coleman et. al., 1984).

The genetics of nitrate reductase regulation in higher plants has been studied through the use of nitrate reductase mutants. Two types of nitrate reductase mutants have been found in plants. The nia mutants are defective in the nitrate reductase apoprotein, while cnx mutants have defective molybdenum cofactors (Campbell and Smarrelli, 1986; Dunn-Coleman et. al., 1984; Kleinhofs et. al., 1985). Studies involving barley (Horedeum vulgare) and pea (Pisum sativum) have linked nia mutants to a single locus, while cnx mutants have been linked to multiple loci (Dunn-Coleman et. al., 1984; Kleinhofs et. al., 1985). Two unlinked loci have been correlated with nia mutants in Nicotiana tobacum, although the presence of duplicate genes for the nitrate reductase apoprotein is thought to be the result of the allodihaploid nature of N. tobacum (Muller, 1983). Thus, in diploid higher plants, a single gene is thought to encode the nitrate reductase structural gene, while more than one loci may encode the molybdenum cofactor.

The nia mutants of higher plants are analagous to nit-3 mutants from N. crassa, while cnx mutants correspond to nit-1, nit-7, nit-8 and nit-9 mutants of N. crassa (Campbell and Smarrelli, 1986; Dunn-Coleman et. al., 1984). Higher plant mutants analagous to the nit-2 and nit-4/5 regulatory genes of N. crassa have not yet been isolated. Repression

of nitrate reductase activity by reduced nitrogen is not strictly analagous between higher plants and fungi (Kleinhofs et. al., 1985). In fungi, the presence of glutamine or other reduced nitrogen sources represses the activity of nitrate reductase even if nitrate is present (Dunn-Coleman et. al., 1984). In higher plants, reduced nitrogen sources have been found to increase nitrate reductase activity in Paul's Scarlet rose (Monhanty and Fletcher, 1976) and radish cotyledons (Ingle et. al., 1966), and decrease, but not abolish, nitrate reductase activity in cotton roots (Radin, 1975), soybean leave (Curtis and Smarrelli, 1986) and squash cotyledons (Langendorfer, personal communication). Thus, a regulatory gene strictly analagous to the nit-2 gene of N. crassa may or may not be found in higher plants (Kleinhofs et. al., 1985).

Induction of nitrate reductase by nitrate in higher plants closely parallels nitrate induction in fungi (Curtis and Smarrelli, 1986; Kleinhofs et. al., 1985). Consequently, it is expected that mutants affecting the regulation of nitrate reductase induction should occur in higher plants (Dunn-Coleman et. al., 1984; Kleinhofs et. al., 1985). To date, only a small number of higher plant nitrate reductase deficient mutants have been fully characterized (Dunn-Coleman et. al., 1984; Kleinhofs et. al., 1985). Thus, nitrate reductase deficient mutants possessing one or more regulatory gene mutants may be found

when a greater number of nitrate reductase mutants are characterized.

The existence of genes affecting the regulation of nitrate reductase could be elucidated prior to their discovery in nitrate reductase deficient mutants by locating regulatory sites upstream of the nitrate reductase structural gene. Discovery of the upstream regulatory sites would necessitate prior isolation of the nitrate reductase structural gene. Isolation of the structural gene would allow the location of the gene and its upstream regions within the squash genome to be determined. Genomic fragments containing the nitrate reductase structural gene and 5' flanking regions could then be manipulated by linker scanning and subsequently assayed for transcriptional activity. Small clusters of point mutations could be substituted for an equivalent number of nucleotides in the 5' flanking region of the nitrate reductase structural gene (McKnight and Kingsbury, 1982). Cell-free extracts from squash plants treated with nitrate, glutamine, nitrate and glutamine and no nitrate or glutamine could then be used to study the ability of the altered and unaltered 5' flanking regions to support transcription of the nitrate reductase structural gene. Similar studies have shown the existence of Sp1, a protein obtained from cultured human cells, to be required for transcription of the SV40 virus (Benoist and Chambon, 1981; Dynan and Tjian, 1983). A similar

interaction has been found between a heat-shock transcription factor (HSTF) from Drosophila and a region upstream of the heat-shock RNA promoters (Pelham, 1982). Initiation of transcription from the silkworm fibroin promoter (Tsuda and Suzuki, 1983), one of the sea urchin histone promoters (Grosschedl and Birnstiel, 1982) and the Drosophila alcohol dehydrogenase promoter (Heberlein et. al., 1985) have been shown to be affected by the presence or absence of DNA binding factors. Multiple binding sites for the same or different binding proteins have been shown to exist (Dynam and Tjian, 1985) and it has been suggested that these factors affect transcription of their respective structural genes by altering the chromatin structure or by directly aiding or preventing attachment of RNA polymerase to the promoter (Darnell, 1982; Weisbrod, 1982).

Conclusion

A 4.4 kb squash genomic DNA fragment that contains sequences of the squash nitrate reductase structural gene has been isolated. Soybean RNA dot blots indicate the 4.4 kb fragment also contains transcribed sequences coding for a protein other than nitrate reductase. Subcloning the 4.4 kb fragment and using the subclones to probe RNA dot blots from seedlings treated with various combinations of nitrate and glutamine should identify the nitrate reductase encoding sequences. These subclones can then be used to probe squash genomic libraries created in EMBL3 to isolate the remaining

sequences of the nitrate reductase structural gene. Isolation of this gene should allow further study at the molecular level of the nitrate reductase apoprotein and its regulation.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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