ISOLATION AND CHARACTERISATION OF SENESCENCE-RELATED GENES IN CARNATIONS

by

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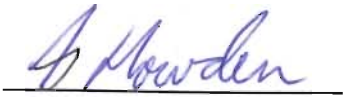
ABSTRACT

Senescence has been the subject of many studies, with the ultimate goal of increasing longevity of cut flowers, and for insight into the process of ageing. Senescence in the carnation is a complex phenomonen involving many physiological and molecular events. The carnation serves as an excellent subject for studies in senescence because of the clearly visible evidence of "in-rolling" of petals indicating the onset of senescence. Senescence in the carnation results in a large number of physiological changes in the flower which in many cases involve the action of the growth regulating hormones, particularly ethylene. Physiological studies of senescence, though exhaustive, have not as yet been able to pinpoint the source of control of senescence in the carnation and the exact controlling mechanism. Senescence has in the past been shown to be partially regulated at the transcriptional level, thus warranting studies in the area of molecular biology. The aim of the present investigation was to isolate genes active during senescence. To achieve this, mRNA from presenescent and senescing carnation petals, receptacles and ovaries was extracted. The mRNA was used to synthesise cDNA which was cloned into λgt10 phage to produce presenescent and senescing cDNA libraries of carnation material. The production of the petal library was the only library that, after boosting levels of petal cDNA using PCR (polymerase chain reaction) technology, was generated successfully.

A subtraction procedure was carried out between the presenescent and senescing libraries to isolate the sequences unique to the senescing library ie. the genes active only during senescence. One sequence of 1 kb in size was isolated. This gene could be used for future research into the influence of the growth regulators on its activity, and also to pinpoint organs other than the petals where this particular gene is active. It would be of interest to investigate the sequence of the gene, for a comparison with other gene isolates in order to elucidate the identity and function of the gene product.

DECLARATION

I hereby declare that this thesis, except where the work of others is acknowleged, was the result of my own investigation carried out under the supervision of Professor J. van Staden and Dr W. Cress, in the Department of Botany, University of Natal, Pietermaritzburg.



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CHAPTER 1

SENESCENCE OF CUT FLOWERS - AN OVERVIEW

Senescence is the final stage in the development of the plant, organ, tissue or cell. Petals often have a short lifespan, senescing rapidly once they have completed their function of attracting pollinators to fertilize the ovary (LAWTON, HUANG, GOLDSBROUGH AND WOODSON, 1989). Many cut flowers have their vase life terminated by the onset of petal wilting, despite being held in water (HALEVY AND MAYAK, 1981). Evidence of petal senescence in the carnation includes petal inrolling (sleepiness), (Plate 1.1.) accompanied initially by ethylene and respiration climacterics (MOR AND REID, 1980).

Many studies have been done to evaluate senescence both for economic reasons in the cut flower trade, and for an understanding of ageing. Carnations have been used for numerous studies as they show well-defined physiological and biochemical symptoms of advancing senescence particularly in response to ethylene (COOK AND VAN STADEN, 1988; SACALIS, 1989).

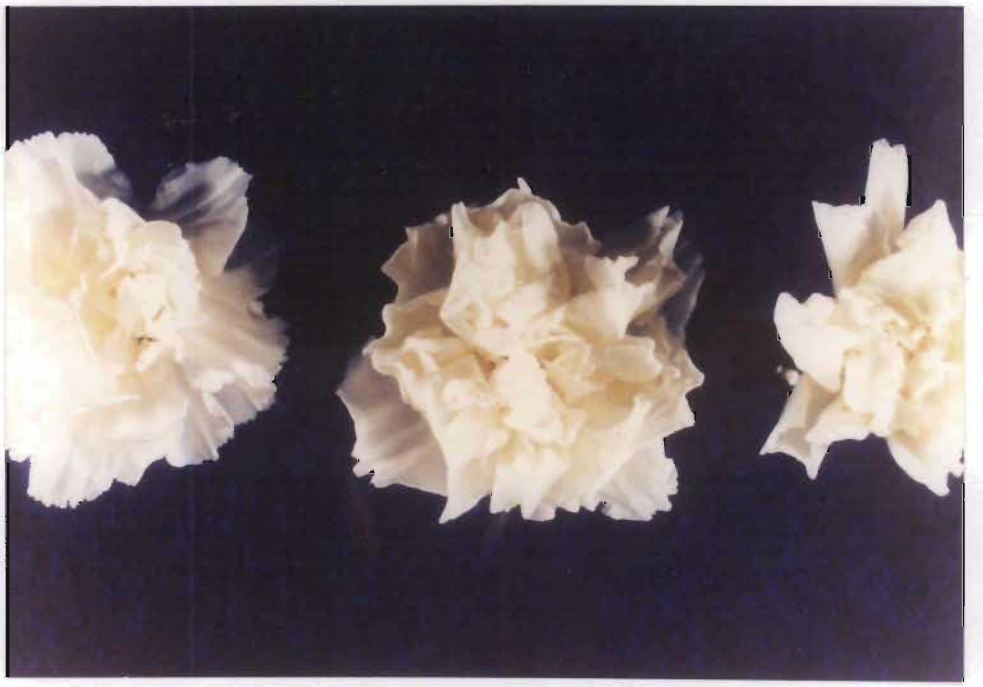


Plate 1.1. The progression of senescence in the carnation, showing a fresh presenescent flower, a climactic ("sleepy") flower and a post senescent flower.

1.1 PHYSIOLOGICAL ASPECTS OF FLORAL SENESCENCE

A major manifestation of senescence in cut flowers is the effect on water relations. Ageing cut flowers are characterized by a decrease in water conductivity from possibly microbial stem blockage or oxidative processes resulting from stem injury. This decrease does not occur on flowers remaining on the plant, indicating a change in physiology after cutting (HALEVY AND MAYAK, 1981). Loss of turgor can result from water loss through stomata in response to light. Water and ion leakage can also result from a loss of semi-permeability through changes in membrane properties (ACOCKS AND NICHOLS, 1979).

During senescence in cut flowers, a gradual decline in osmotic potential can also occur (ACOCKS AND NICHOLS, 1979). Plants that have, while growing, adapted to stress are enabled to produce flowers of increased longevity possibly by increasing water potential and lowering water content (HALEVY AND MAYAK, 1981). Senescence is also characterized by a redistribution of dry matter (NICHOLS, 1971). There is a sharp decline in flower fresh weight but according to DE VETTEN AND HUBER (1990) dry matter levels increased, probably due to increasing ovary size.

Petal senescence is characterised by a number of changes in the cell walls. There is a decrease in ethanol insoluble solids (including pectin, a polygalacturonide) (DE VETTEN AND HUBER, 1990). Wilting in cut carnations occurs much earlier in a flower whose cell walls to begin with, contained less pectic substances. Pectic decrease in such flowers also occurred at a much greater rate (JONA, 1988). The cell walls also showed slight decreases of uronic acid and cellulose during senescence. In addition,there was also a significant loss of non-cellulosic neutral sugars, mainly galactose and arabinose. The molecular size of the hemicelluloses was also reported to alter, with the possible enzymic breakdown of the large polymers. These changes were not accompanied by any apparent changes in texture of the petals (DE VETTEN AND HUBER, 1990).

Changes in the cell membrane occur during senescence, affecting mainly lipid and protein components. One of the clearest manifestations of senescence is the declining levels of phospholipid. This is evidenced by a decrease in fluidity of the membrane (the ease of movement of the phospholipid in its liquid-crystal state) resulting from the formation of gel-phase domains and increased sterol to phospholipid ratios, caused by loss of phospholipid (BOROCHOV AND WOODSON, 1989).

The decrease in membrane fluidity was slow and gradual at the start of senescence and more rapid approaching wilting, however, it always occurred before the appearance of visible senescence symptoms (BOROCHOV AND WOODSON, 1989). Using microsomal membranes isolated from carnation petals, it has been shown that phospholipids are degraded by enzymes active there (BROWN, PALIYATH AND THOMPSON, 1990). Phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl inositol are phospholipids contained in the microsomal membranes of senescing carnation petals. These decline essentially in parallel with natural senescence. Experiments on *in vitro* ageing of isolated microsomal membranes indicated that various molecular species of phospholipids have different susceptibilities to catabolism, determined by the nature of the headgroup and acyl chain composition. Evidence suggests that phospholipid composition of membranes is continuously altered during senescence by acyl chain desaturation, possibly generating molecular species more prone to catabolism, so enhancing senescence (BROWN, CHAMBERS AND THOMPSON, 1991). Examples of enzymes involved in the degradation of the phospholipids are phospholipase D (which releases choline and promotes the formation of phosphatidic acid from phosphatidyl choline), lipoxygenase, lypolytic acid hydrolase (BROWN, PALIYATH AND THOMPSON, 1990), phospholipase A and phosphatidic acid phosphatase (BOROCHOV AND WOODSON, 1989).

Two of the lipid degrading enzymes present in membranes, phospholipase D and phosphatidic acid phosphatase are stimulated by 10 to 15 μM Ca^{2+}. This stimulation is mediated by calmodulin. If there was a reduced capability to maintain Ca^{2+} at homeostatic levels, lipid breakdown, and thus senescence would be facilitated. During early senescence, before the ethylene climacteric, a decline of ATP-dependent Ca^{2+} pumping capability (microsomal uptake) occurs which could result in an accumulation of Ca^{2+} in the cytoplasm, possibly resulting in the stimulation of Ca^{2+} sensitive enzymes and thus lipid degradation. This decline in Ca^{2+} also precedes the onset of deteriorative changes in the microsomal membrane lipids, although the reduced activity of the pump may be brought about by changes in the lipid environment around it. Exogenous ethylene applications at the first stage of senescence is able to induce the decline in pumping, possibly indirectly (PALIYATH AND THOMPSON, 1988).

Senescence-mediated changes, such as decreases in phospholipids and membrane fluidity, appear to be induced by the activity of free radicals since in carnation petal membranes there is an increase in free radical content before any visible symptoms of senescence are apparent (MAYAK, LEGGE AND THOMPSON, 1983).

This is supported by findings that compounds typical of free radical-mediated lipid degradation, such as aldehydes (malonaldehyde), are produced by senescing carnation petals. Application of anti-oxidants reduced the radical pool resulting in the reduction of fatty acid saturation and increased flower longevity (BOROCHOV AND WOODSON, 1989).

Quantitative and qualitative changes in the membrane proteins also occur during petal senescence. Age-related changes of the membrane-bound enzymes have been reported (ADAM, BOROCHOV, MAYAK AND HALEVY, 1983). There was an overall decline in the total protein content of petals, with a similar trend occurring in membrane proteins which could be explained by the general decline in metabolic rate which accompanies senescence. New polypeptides were detected in senescing petals while the relative abundance of various polypeptides present changed. This serves as an indication that protein synthesis or degradation, occurs during senescence (WOODSON, 1987; BOROCHOV, DRORI, TIROSH, BOROCHOV-NEORI AND MAYAK, 1990). Protein and lipid levels appear to decline in parallel (BOROCHOV AND WOODSON, 1989). The membrane proteins have their hydrophobicity affected by senescence. Another important qualitative change involves their free thiol groups.

These groups are known to be involved in many enzyme reactions through participation in substrate binding or as part of sites of action. Changes in these groups have been correlated with environmental stress (BOROCHOV, WALKER, KENDALL, PAULS AND MCKERSIE, 1987), and developmental stages of plants (DANIEL AND GAFF, 1980). It was found that free sulphydryl groups existed in a lipophilic environment. Possibly changes in the fluidity of the membrane could alter the vertical position of these thiol groups, lowering the number in contact with the aqueous environment surrounding the membrane. There is an age-related decrease in reactive protein thiols of membranes (occurring after lipid modifications). Non-specific oxidation, or thiospecific protease activity may be responsible for this. Another possibility is that the new proteins produced during senescence have a lower reactive thiol content. This, as well as changes in their lipid environment, could account for the decline in membrane enzyme activity during senescence, since many enzyme activities of the plant membranes are thiol-dependent (BOROCHOV, DRORI, TIROSH, BOROCHOV-NEORI AND MAYAK, 1990).

Another important event that occurs during senescence, is the movement of sucrose from wilting petals to the surrounding organs ie. the ovary and receptacle. Sucrose is the only mobile sugar in petals and is immobilised in the form of glucose and fructose through the hydrolytic activity of the enzyme invertase.

During senescence in carnations, a protein invertase inhibitor is formed in the petals. This inhibitor appears to be synthesized in many varieties of senescing flowers at the beginning of wilting, in cut and non-cut flowers (HALABA AND RUDNICKI, 1989).

Senescence and the associated changes in water relations and metabolism, is the final phase in flower development and as such can be influenced by growth regulators (HALEVY AND MAYAK, 1981). Hormonal treatments that modify the rate of senescence also lead to corresponding alterations in membrane properties (BOROCHOV, DRORI, TIROSH, BOROCHOV-NEORI AND MAYAK, 1990).

1.2 HORMONAL REGULATION OF FLORAL SENESCENCE

Growth regulators encompass five groups of plant hormones with the most extensive work, with respect to flower senescence having been done on the gaseous hormone, ethylene (HALEVY AND MAYAK, 1981). The other hormones involved appear to have their effect on senescence through their interrelationship with ethylene. Cytokinins are considered to have an anti-senescent effect through their prevention of ethylene biosynthesis, while the senescence promoting action of abscisic acid (ABA) and auxins are connected to ethylene synthesis (COOK AND VAN STADEN, 1988).

Gibberellins may, at certain concentrations, be able to delay senescence by the inhibition of enzymes involved in ethylene synthesis (ARIE AND FERGUSON, 1990). The ethylene level is stimulated to rise from a low basal level just before the onset of flower senescence, to a climacteric peak of ethylene production accompanied by petal senescence characterized by "inrolling". The ethylene climacteric in carnation petals appears to be under autocatalytic regulation since exposure to ethylene induces ethylene biosynthesis (HALEVY AND MAYAK, 1981). Petal "inrolling" during the climacteric was thought to be at a fixed rate and irreversible but has recently been found to be reversible (WANG AND WOODSON, 1989). This reversal occurs during inhibition of ethylene action after the petals have entered the climacteric stage (HALEVY AND MAYAK, 1981).

Extensive studies have been done to find the possible regulatory steps involved in controlling the ethylene effect, and thus locating the trigger of senescence. According to MAYAK, VAADIA AND DILLEY (1977) factors which cause a rise in the internal level of ethylene in the carnation, initiate some event, exhibiting a lag phase of about two hours followed by a log linear increase in ethylene production. This is followed by an increase in tonoplast permeability about two hours later with a coincident decline in water uptake (MAYAK, VAADIA AND DILLEY, 1977).

A number of studies of the ethylene biosynthetic pathway were therefore made. The sequence of the pathway in apple tissue was proposed by ADAMS AND YANG (1979). A modified version of this pathway can be seen in Figure 1.1.

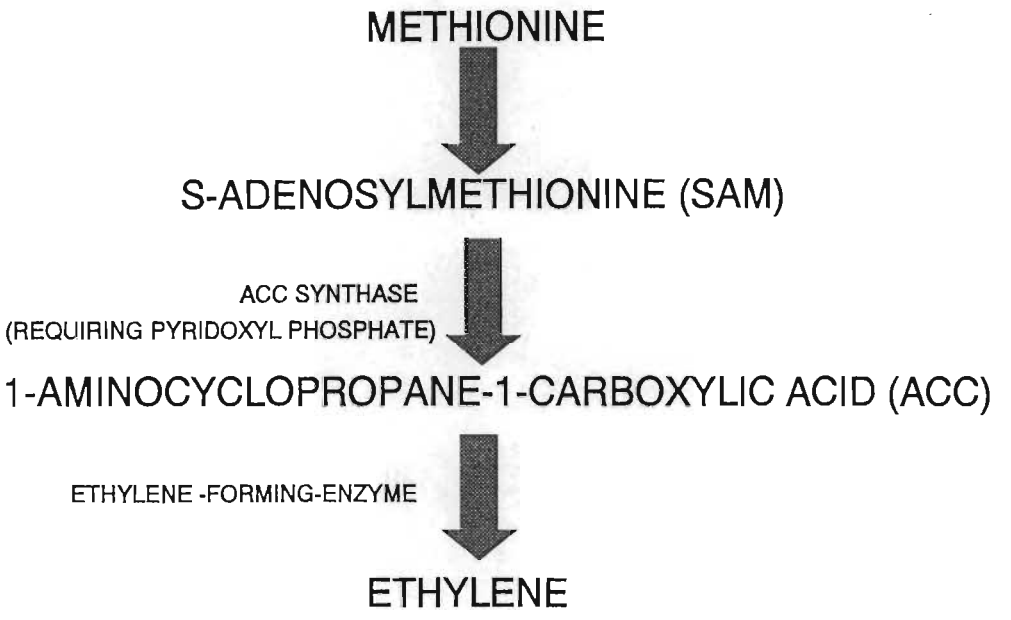


Figure 1.1. The metabolic path of synthesis of ethylene from methionine.

End product metabolites are thought to be N-malonyl ACC, CO_2 and CN which is detoxified by conversion to asparagine (COOK AND VAN STADEN, 1988). Another proposal is the direct degradation of methionine to ethylene with the release of free radicals (LIEBERMAN, 1977).

For ethylene evolution to increase from the basal level, an additional factor to the components and enzymes in the pathway mentioned may be required. Some of these components or accompanying enzymes may be deficient, and an increase can only occur when all become present (HALEVY AND MAYAK, 1981). A study by VAN DER WESTHUIZEN AND DE SWARDT (1978) revealed that methionine (and other amino acids) was absent or present in only trace amounts during early vase life, but increased during continued vase life probably due to ethylene production. The intermediate SAM, being formed by the action of the enzyme SAM synthetase, was confirmed by KENDE (1989). It appears that SAM synthetase may not be a regulator of ethylene biosynthesis during senescence (LARSEN, RAGHOTHAMA AND WOODSON, 1990).

A regulatory step in the path is the alternate conversion of SAM to polyamines (eg. putrescine and spermidine) rather than to ethylene as was demonstrated when aminooxyacetic acid (AOA) was used to block the ethylene pathway (EVEN-CHEN, MATTOO AND GOREN, 1982). Polyamines may inhibit ethylene production by preventing protein synthesis and interfering with the continuous synthesis of ACC synthase. They may also delay senescence by binding with the anionic groups on membranes so preventing leakage and stabilizing them (SMITH, 1985).

Another possible rate-limiting step is the conversion of SAM to ACC by ACC synthase (activated by pyridoxal phosphate and located in the cytosol where it is inhibited by AOA). ACC synthase was found to be induced by factors that promote ethylene formation eg. auxin (IAA), stress and wounding (YANG AND HOFFMAN, 1984), and in such situations is produced by *de novo* synthesis (BLEECKER, ROBINSON AND KENDE, 1988). Ethylene exposure increases ACC synthase activity markedly in the basal portion while hardly affecting that in the upper portion of the petal (COOK AND VAN STADEN, 1988). ACC increased at the climacteric with a high level persisting after a reduction in ethylene production. The biosynthetic pathway, therefore, does not appear to change during senescence (COOK AND VAN STADEN, 1988). ACC synthase is inactivated by its substrate SAM by the covalent linkage of the enzyme with the 2-amino-butyrate portion of SAM to form a vinyl-glycine complex (SATOH AND YANG, 1989).

ACC appears to increase in other parts of the flower before the petals, so translocation of ACC from those parts of the flower to the petal may occur. Possibly, this non petal increase is induced by a factor other than ethylene since silver thiosulphate (STS), which inhibits ethylene binding, does not prevent the increase in ACC content from basal level, as it does in the petals (BUFLER, MOR, REID AND YANG, 1980). Carnation petals, however, do synthesise their own ACC, as was shown in detached petals (MOR AND REID, 1980). ACC levels are lower in the basal portion of the petals compared to the upper flag region (MOR, HALEVY, SPIEGELSTEIN AND MAYAK, 1985). The onset of senescence is probably associated with an increase of the membrane bound enzyme converting ACC to ethylene (ethylene forming enzyme, EFE) as well as that of the enzyme involved in ACC synthesis (BUFLER, MOR, REID AND YANG, 1980) in its early stages of development, or possibly compartmentalisation may limit penetration of ACC into the subcellular system containing EFE (COOK AND VAN STADEN, 1988). Evidence indicates that the EFE is a membrane-bound enzyme, requiring membrane integrity to function (BOROCHOV AND ADAM, 1984). It has been found to be active in vacuoles where it is bound on the inner surface of the tonoplast. The vacuole thus forms an ethylene-forming compartment. There may be other such compartments (KENDE, 1989).

ACC, being an amino acid, is probably actively transported into the cell compartment, coupled to proton transport (BOROCHOV AND ADAM, 1984). It was observed that the upper flag region is less sensitive to ethylene than the basal portion (MOR AND REID, 1985). This led to the proposal that the transport of an "ethylene sensitivity factor" was responsible for increasing the ethylene sensitivity of the flag region (MOR AND REID, 1980) and the corolla during pollination (HALEVY AND WHITEHEAD, 1988). OVERBEEK AND VAN WOLTERING (1990) indicated that although ACC is readily translocated within the whole flower, there may be an alternative explanation to the "ethylene sensitivity factor" theory to explain the increased sensitivity of the flag. They proposed that, during natural senescence, the base of the petal provides the upper flag with both ACC and ethylene. This ethylene, induces EFE activity, which in turn promotes the production of ethylene from ACC. They demonstrated that the upper petal parts were in fact more sensitive to ethylene than originally thought, as even small amounts produce changes in enzyme activity, however, for wilting to occur ACC must be supplied. HALEVY AND WHITEHEAD (1988) alternatively put forward that the "ethylene sensitivity factor" function could be accomplished by two short chain fatty acids, possibly octanoic acid and decanoic acid, that were able to increase sensitivity to ethylene in the corolla of petunia after being applied to the stigma (as would occur during pollination).

The mode of action was not elucidated, but since the fatty acids increase membrane permeability, they may also modify the activities and binding sites on membranes through changes in structure and properties (HALEVY AND WHITEHEAD, 1988). A naturally occurring inhibitor of the ACC to ethylene conversion step, found in the cytosol, was identified as 1-O-feruloyl-B-D-glucose. High levels of the inhibitor were present in presenescent flowers with a maximum occurring during flower opening. A decrease in concentration of the inhibitor occurs at the same time as an increase in ethylene (in cell free extracts). The mechanism that controls ethylene synthesis may thus be modulated by endogenous levels of this inhibitor (CHING YU SHIH, DUMBROFF AND THOMPSON, 1988).

The production of ethylene from ACC in microsomal membranes as opposed to *in vivo* systems, has been found not to be the result of *in situ* EFE activity but rather to be mediated and enhanced by the non-enzymic activity of the hydroperoxides generated by a lipoxygenase in the membranes (LYNCH, SRIDHARA AND THOMPSON, 1985). This system may play some role in ethylene production and senescence (possibly contrary to the conclusions of WANG AND YANG, 1987) *in vivo*, since senescence induced symptoms (which are similar to those occurring in stress conditions) such as alterations in membrane properties and lipid catabolism could bring about the increased activity of lipoxygenase.

This could result in enhanced formation of lipid hydroperoxides and free radicals, thus increasing the rate of formation of ethylene from ACC. This alternative pathway may explain some similarities between wound/stress and senescence phenomena (KACPERSKA AND KUBACKA-ZEBALSKA, 1989).

An alternative to the formation of ethylene from ACC, is the conjugation of ACC into N-malonyl-ACC (MACC) (HOFFMAN, YANG AND MCKEON, 1982). MACC is able to be converted to ethylene both in vegetative tissue and in petals. In some systems it may be the inactive endproduct of ACC, not being oxidised to ethylene (HANLEY, MEIR AND BRAMLAGE, 1989). MACC has been detected in all parts of the flower during early senescence (first 3 days). Increased MACC is often associated with a blockage of ACC to ethylene conversion and may be a means of reducing cytoplasmic ACC accumulation (KACPERSKA AND KUBACKA-ZEBALSKA, 1989). HANLEY, MEIR AND BRAMLAGE (1989) reported that since synchrony occurs amongst all the ageing flower parts, it was unlikely that mobilization of an ethylene precursor from one flower part to another is necessary for the synchronous production of ethylene observed within the flower. The synchronous changes of MACC may be related to the synchronous changes in polysome populations in ageing carnation flower parts observed by BUFLER AND ROMANI (1983).

Malonylation of ACC takes place in the cytosol and the MACC formed is taken into the vacuole via carrier-mediated transport (BOUZAYEN, LATCHE, PECH AND MARIGO, 1989). The possibility that the plasma membrane may be a site of EFE activity in cooperation with the cell wall in ethylene production (PORTER, BORLAKOGLU AND JOHN, 1986) was refuted by BOUZAYEN, LATCHE AND PECH (1990), from work on grape protoplasts with or without cell walls. The latter authors suggested that there may be two sites of ethylene production. There are indications of an external site converting apoplastic ACC, located at the plasma membrane and very sensitive to high osmotica, and an intracellular site converting internal ACC and unaffected by even severe plasmolysis.

The conversion of ACC to ethylene also produces CO_2 and CN (LIEBERMAN, 1979). The CN is detoxified by β-cyanoalanine (CAS) which is then metabolised to asparagine (MANNING, 1986). CAS may also have a role in EFE activity since its activity has some correlations with those of the EFE in ethylene production. Induction by ACC and ethylene, requires protein synthesis at the translation level, and is also more active in the basal portion of the petal (MANNING, 1986). The CAS enzyme, however, is located in the mitochondria unlike the membrane-bound EFE (COOK AND VAN STADEN, 1988).

During the conversion of ACC to ethylene, there may be the production of free radicals such as O_2- from the microsomal membranes (from loss of fluidity causing phospholipid breakdown and peroxidation of fatty acids). These radicals may be involved in triggering senescence through stimulating ethylene biosynthesis and membrane deterioration (MAYAK, LEGGE AND THOMPSON, 1983). Evidence in support of this proposed activity is that suppression of ethylene biosynthesis does not prevent lipid breakdown (SYLVESTRE AND PAULIN, 1987).

The manner in which ethylene exerts its effects has been postulated to involve the binding of the ethylene molecule to a specific binding site. In petals, molecules with a higher affinity for ethylene than the rest of the cytoplasmic milieu were discovered. This interaction could result in the release of a second message (transligand) (SISLER AND GOREN, 1981) which directly or indirectly results in the transcription of new mRNA from the genome. Some evidence for this mechanism is that compounds which mimic ethylene ie. norbornadiene or propylene inhibit ethylene action through competitive binding (SISLER, REID AND YANG, 1986). Cycloheximide, an inhibitor of protein synthesis on 80-S ribosomes, can also inhibit the effects of ethylene, supporting the possibility of production of a second message (FRENKEL, KLEIN AND DILLEY, 1968).

It has been suggested that ethylene-metabolizing hormones may also function as receptors for ethylene binding, although recent evidence indicates that ethylene metabolism is not linked to its mode of action (SANDERS, SMITH AND HALL, 1989).

Another model proposed is that the binding site of the ethylene receptor interacts with ethylene, so modulating the response induced by the ethylene. Three binding sites for ethylene (in the mung bean) have been located, one apparently being a copper-containing glycoprotein (SANDERS, SMITH AND HALL, 1989; NAPIER AND VENIS, 1990). There is evidence, however, that ethylene does not interact with preformed factors but alternatively may activate gene expression in at least some genes by mediation by labile protein factors synthesized on cytoplasmic ribosomes (LAWTON, RAGHOTHAMA AND WOODSON, 1990).

Ethylene-binding reaches a maximum just before the rise in ethylene production, after which, it declines, possibly because of the reduction of binding sites through the degradation of membranes (BROWN, LEGGE, SISLER, BAKER AND THOMPSON, 1986). The action site may be located between the plasma membrane and the middle lamella of the receptacle (VEEN, HENSTRA AND DE BRUYN, 1980).

In addition to the initial binding, ethylene action in the flower petals may be mediated at some other point in the chain of signal transduction events. Little, however, is known about the sequence of events linking ethylene binding to senescence in petals (BOROCHOV AND WOODSON, 1989).

Cytokinins are known to delay senescence of cut carnations, causing petals instead to burn and become necrotic at the edges. They are able to do this through their ability to maintain RNA and protein synthesis and possibly through lowering of the respiratory rate (MACLEAN AND DEDOLPH, 1962; COOK AND VAN STADEN, 1988). Biosynthesis and action of ethylene may also be blocked by cytokinins (EISINGER, 1977), possibly at the stage of initial conversion of SAM to ACC . This may account for cytokinins only having an effect immediately after harvest, before the rise in ethylene production occurs (COOK AND VAN STADEN, 1988). MEYER AND WOODSON (1990) noted that while benzyladenine (BA) delays ethylene senescence, it does not block the ability of the petals to perceive ethylene. The conversion of ACC to ethylene by EFE has also been shown to be affected by cytokinins (EISINGER, 1977), though the effect might not be the same *in vivo* where penetration of cytokinins and ACC to the membrane-bound enzyme has to occur. According to BOROCHOV AND WOODSON (1989) cytokinins do not prevent ethylene production once climacteric is reached.

At this stage they may not inhibit the enzyme activity but rather their synthesis. Cytokinins do appear to be capable of countering low amounts of ethylene (VAN STADEN, 1989). Since they act in two places, they may act via a second messenger (MOR, HALEVY, SPIEGELSTEIN AND MAYAK, 1985). They may also alter tissue sensitivity to ethylene rather than directly affecting its biosynthesis (COOK AND VAN STADEN, 1988). It is possible that declining levels of cytokinins may trigger senescence (EISINGER, 1977), since during natural senescence, cytokinins decrease in petals, stems and ovaries (VAN STADEN, 1989). According to VAN STADEN AND DIMALLA (1980) there was an increase in cytokinins in the ovary during senescence (at the onset of petal wilting), followed by a decrease. The effect was different in the petals and rest of the plant, whose endogenous levels increased and then decreased during senescence, and increased again after the wilting of the petals. This could be as a result of translocation of cytokinins from petals to the ovary (KELLY, STABY AND CHISM, 1985). This was suggested to be related to the mechanism which mobilizes metabolites to the developing ovary as a preferential sink (VAN STADEN AND DIMALLA, 1980). Other evidence does not seen to support this and it may be possible that sucrose and cytokinins (kinetin or isopentenyladenine) are able to reduce sensitivity to ethylene (MAYAK AND KOFRANEK, 1976).

The stem and receptacle may act as reservoirs from which applied or natural cytokinins may be released into the petals (KELLY, STABY AND CHISM, 1985; VAN STADEN AND BOSSE, 1989; VAN STADEN, BAYLEY, UPFOLD AND DREWES, 1990).

There are many different cytokinins, each differing in their physiological action, eg. dihydrozeatin (DHZ) and some of its derivatives are effective in delaying senescence, zeatin is ineffective and isopentenyladenine and its derivatives of intermediate efficacy (UPFOLD AND VAN STADEN, 1990; VAN STADEN, UPFOLD, BAYLEY AND DREWES, 1990). The cytokinin pool would therefore be expected to change as well as its role in regulating senescence (COOK AND VAN STADEN, 1988). Metabolism of endogenous and exogenously applied cytokinin also can occur which could also affect the levels in flower parts. The rate of metabolism depends on the type of cytokinin (KELLY, STABY AND CHISM, 1985).
Benzyladenine (BA), for example, is apparently metabolised to ribosylbenzyladenine (BAR), which in turn is metabolised to ribosylbenzyladeninemonophosphate (BARMP), with interconversion occurring. Each of these metabolites seems to affect senescence similarly to BA (KELLY, STABY AND CHISM, 1985; VAN STADEN, UPFOLD, BAYLEY AND DREWES, 1990). The glucosides e.g. benzyl adenine-9-glucoside and isopentenyladenine-9-glucoside may be physiologically inactive storage products (VAN STADEN, UPFOLD, BAYLEY AND DREWES, 1990).

There is evidence that cytokinins may act as radical scavengers in the cells (HALEVY AND MAYAK, 1981). It is of interest to note that STS causes an unexplained decrease in cytokinins in all flower parts (VAN STADEN, 1989).

Auxins e.g. indole-3-acetic acid (IAA) may cause premature senescence in carnations, when present in moderate amounts through stimulation of ACC synthesis (SACALIS, 1989), increasing the duration and amount of ethylene production (WULSTER, SACALIS AND JANES, 1982). It was demonstrated that ACC could be oxidised to yield ethylene by horseradish peroxidase in the presence of IAA. In research on other plants e.g. poinsettias, auxins were found to delay senescence possibly by inducing the synthesis of peroxidase, which then was thought to prevent the accumulation of free peroxide associated with ageing (GILBART AND SINK, 1971). The synthetic auxin 2,4 D was found to increase the longevity of petals when in high concentrations, due to death of the ovary (SACALIS, 1989). There may be an early and later response to auxin in producing ethylene: the early response may be through stimulation of oxidising activity of peroxidases in the cell wall, while the later response may act through the induction of *de novo* synthesis of ACC synthase (OSSWALD, SHUTZ AND ELSTNER, 1989).

SHERER AND ANDRE (1989) showed that in zucchini hypocotyls, biologically active auxins were able to rapidly stimulate phospholipase A_2 *in vitro* and *in vivo*, possibly aiding lipid catabolism.

ACC in the petals, resulting in the production of ethylene, may have been transported to the petals or may arise there as a result of IAA transport (SACALIS, WULSTER AND JANES, 1983). The IAA, though reaching all the parts of the petal is only able to accelerate ACC synthesis at the petal base, where it requires a secondary messenger, e.g. ethylene to wilt the rest of the petal (COOK AND VAN STADEN, 1988). Auxins have been shown (mainly from research on plant development), to rapidly alter the expression of specific sets of genes, resulting in increases in ribosomal RNA and proteins. A similar situation may arise during senescence (KEY, 1989).

The role of auxins in the carnation flower appears to be the mobilization of assimilates and therefore they may be involved in the shift of carbohydrates from the petals to the ovary at senescence. This conclusion may be reached since an IAA-like auxin was detected in the ovary (JEFFCOAT AND HARRIS, 1972).

Another auxin, naphthalene acetic acid (NAA) was found to cause increased invertase activity in the ovary, so possibly maintaining the sucrose gradient, while also possibly increasing carbohydrate accumulation by stimulation of chloroplast development in the ovary wall (COOK AND VAN STADEN, 1986).

Auxins may play a role in pollination accelerated senescence, possibly requiring pollen tube growth, to pass through the stigma to reach the ovary where ethylene production would be stimulated. Existing evidence does not support this, especially in the initial stimulation (COOK AND VAN STADEN, 1988). Pollen is known to contain auxin (and ACC), but labelled IAA applied to the stigma in carnation has been shown to move very slowly, therefore is unlikely to be a signal, (REID, FUJINO, HOFFMAN AND WHITEHEAD, 1984).

Auxins and cytokinins often have similar effects on the ovary, such as greening, but very different effects on the petals. Auxins and cytokinins also appear to interact in the process of senescence (VAN STADEN, 1989). IAA and the cytokinin zeatin were found to have a synergistic effect on sucrose metabolism (COOK, 1985). In the ovary, however, the stimulation of ovary dry mass production by IAA adversely affects cytokinin production (FEATONBY-SMITH, VAN STADEN AND COOK, 1987).

Cytokinin increases occurred, when the ovary was not stimulated to increase its metabolism by auxins. This relationship may play a major role in the regulation of senescence by the ovary. If the auxins move into the ovary while cytokinin levels are high, this would produce a strong sink for carbohydrates in the ovary, so stimulating senescence of the petals. If auxins enter first, cytokinin levels would decrease through their metabolism, lessening the dominance of the ovary as a sink (FEATONBY-SMITH, VAN STADEN AND COOK, 1987). In the petals, however, the interaction is antagonistic: a synthetic cytokinin BA prevented IAA induced ethylene production and retarded the metabolism of IAA (COOK, VAN STADEN AND ACKERMANN, 1988).

Abcisic acid (ABA) has a similar effect to ethylene in accelerating senescence (NICHOLS AND MANNING, 1986). According to HALEVY AND MAYAK (1981), ABA increases the sensitivity of the flower to ethylene, enabling the tissue to respond to lower levels of existing ethylene. ABA also induces an earlier increase in ethylene production (MAYAK AND DILLEY, 1976). ABA levels rise in response to water or ion stress which also increases ethylene sensitivity (HALEVY AND MAYAK, 1981). ABA however, has no effect on senescence if ethylene evolution and biosynthesis from ACC synthase is prevented (COOK AND VAN STADEN, 1988).

Some of the effects of ethylene on the level of ABA appear to be mediated through the modification of the cell membrane, a condition which could be countered by cytokinin, thus delaying the rise of ABA levels (SALUNKHE, BHAT AND DESAI, 1990).

Endogenous levels of ABA in the petals and calyx rise steadily from the first day after harvest (NOWAK AND VEEN, 1982), with a small temporary increase in preclimacteric petals (HANLEY AND BRAMLAGE, 1989). In the ovary, the rise occurs right after harvest and reaches a peak three to four days later, after which it declines, possibly having involvement in the carbohydrate sink (TIETZ, LUDEWIG, DINGKUHN AND DORFFLING, 1981; COOK AND VAN STADEN, 1988). This increase in the ovary (and styles) however, did not appear to be triggered by the production of ethylene, or cause the onset of ethylene production in the preclimacteric flower (HANLEY AND BRAMLAGE, 1989). In an immunological study by HANLEY AND BRAMLAGE (1989), increased ABA levels paralleled the increase of ethylene and the onset of irreversible wilting in carnation petals. The receptacle and green tissue showed no signs of wilting although increased ABA levels were detected in them with or after ethylene climacteric in the petals. In the styles and ovary, there was an increase of ABA prior to any increase of ethylene, indicating a separation in the relationship between these two hormones.

ABA may also be able to delay senescence by reducing water loss in flowers by causing stomatal closure in the leaves (MAYAK AND HALEVY, 1972).

ABA, in the final stages of senescence, may influence *de novo* synthesis of ribonuclease, as in *Ipomoea* (BAUMGARTNER, KENDE AND MATILE, 1975). ABA may also regulate novel mRNA synthesis, with Ca^{2+} acting as a second messenger or affecting processing or transport of novel proteins (OWEN AND NAPIER, 1988).

The role of gibberellins in senescence is unclear (COOK AND VAN STADEN, 1988). According to GARROD AND HARRIS, (1978), gibberellic acid (GA) was found to increase flower size and extend longevity of carnations. Gibberellin is thought to stimulate assimilate mobilisation from stems and leaves to the flower (JEFFCOAT AND HARRIS, 1972). Most of its activity, however, may be involved in flower growth rather than senescence (NICHOLS AND MANNING, 1986). It is possible that gibberellins may play a role in ovary growth during senescence (COOK AND VAN STADEN, 1988). Recently, gibberellin has been found to cause a decline in ethylene by inhibiting the ethylene forming enzyme. The decline of ethylene production could also be interpreted as being caused by a loss of membrane integrity through the stimulation of cell death (ARIE AND FERGUSON, 1990).

Although the process of floral senescence has been studied extensively, the means by which it is regulated is still not well understood (SACALIS, 1989). It is thought that petal senescence is regulated by a factor located outside the petals, since all of the petals wilt in synchrony. The source of this stimulation has been difficult to demonstrate and has been a matter of dispute (SACALIS, 1989).

MOR AND REID (1980) showed that young petals removed from the flower had a significantly longer life than those on the intact flower, indicating extrapetal control. This control is probably from the ovary, possibly through competition between the two organs for carbohydrate (MOR AND REID, 1980). The increase in content of ACC in the ovary and receptacle precedes that in the petals (SACALIS, 1989), with low ACC production in the styles (NICHOLS, BUFLER, MOR, FUJINO AND REID, 1983). Excision of the ovary, however, has not been found to have any effect on longevity of vase life of carnations held in water (SACALIS, 1989). The ovary is however, probably the major site of auxin action resulting in petal senescence (with the styles playing a small role) since removal of the ovary at harvest caused suppression of auxin-promoted ethylene synthesis and petal senescence (within a particular concentration range of auxin) (SACALIS, 1989). Cytokinins may also have a role in the regulation by the ovary, as was mentioned earlier (FEATONBY-SMITH, VAN STADEN AND COOK, 1987). Further studies on the source of the regulation is therefore still of interest.

1.3 MOLECULAR APPROACH TO SENESCENCE

Research has shown that ethylene action does not appear to initiate senescence but rather to accelerate it, while events preceding the burst of ethylene occur to prepare the flower for senescence (COOK AND VAN STADEN, 1988). As stated by COOK AND VAN STADEN (1988), efforts in the past have been directed at attempting to find the event that causes the acceleration of ethylene synthesis, while now, it may be more productive to prevent sensitivity sites from forming, particularly through work at the genetic/molecular level. The senescence process is regulated at least partially at the transcription level, or alternatively is an uncontrolled destructive event with loss of cellular compartmentalisation of hydrolytic enzymes which become released to areas of the cell previously separated from their attack (WOODSON, 1987).

The role of genetic expression in senescence and its regulation by ethylene has been examined in depth using mainly fruit ripening as a model (GRIERSON, 1984; MANSSON, HSU AND STALKER, 1985; LINCOLN AND FISCHER, 1988). Through investigations of synthesis and metabolism of macromolecules, the initiation of ripening in tomato fruit regulated by ethylene, was shown to require the switching on of a specific set of genes (GRIERSON, 1984).

The evidence for genetic control of senescence of carnations and cut flowers included the increase of free amino acids (VAN DER WESTHUIZEN AND DE SWARDT, 1978) and proteins, including degradative enzymes, preceding or during wilting (HOBSON AND NICHOLS, 1977), probably as a result of *de novo* synthesis. Treatment of carnation petals with cycloheximide is able to inhibit senescence in ethylene-treated petals (WULSTER, SACALIS AND JANES, 1982). WULSTER, SACALIS AND JANES (1982), however, suggested that regulation could be more at the translational than transcriptional level, since actinomycin D, which inhibits transcription, had no effect on ethylene induced senescence in flowers (although it had been shown to do so in fruit).

Protein synthetic capacities of senescing carnations was assessed through the examination of polysomal populations. Polysomes were found to increase in relation to the progression of onset of senescence, although the complexity of the protein profile had been reduced (probably because of degradative enzymes). This increase in polysomes could result from increased attachment of the ribosomes onto existing mRNA and/or an increase in new mRNA templates (BUFLER, ROMANI AND REID, 1983). These authors therefore advocated further senescence research on carnation petals at the molecular level.

Genetic expression in the carnation particularly, has been examined by WOODSON and his colleagues at Purdue University. WOODSON (1987) investigated changes in mRNA and protein populations in the carnation during senescence. This work revealed that, although total RNA levels increased during climacteric, mRNA levels remained fairly high. While one class of mRNA remained constitutive, another class of mRNAs increased with the climacteric rise of ethylene accompanying the onset of senescence, and resulted in the synthesis of at least five translation products [as determined by *in vitro* translation of the mRNA, using the rabbit reticulocyte lysate system of PELHAM AND JACKSON (1976)], of approximately 81, 58, 42, 38, and 35 kDA. A third class of mRNAs was found to decrease in abundance during petal senescence. The transition period of mRNA changes occurred mainly during the ethylene climacteric.

The increased levels of polysomes noted by BUFLER, ROMANI AND REID (1983), may be the result of increased message levels of which only a few represent new gene transcription (WOODSON, 1987). The possibility that the mRNA changes could be a result of differences in message stability rather than transcriptional control, however, still remained.

Evidence of redirected protein synthesis in senescing carnations would involve detecting differences in polypeptide populations. The increase of four polypeptides (apparent molecular weights of 76, 62, 35.5 and 24 kDa) and decrease of four polypeptides, (apparent molecular weights of 70.5, 67.5, 46.5 and 31 kDa) was noted, especially between preclimacteric and climacteric petal development and were probably not due to protease induced degradation (WOODSON, 1987).

WOODSON AND LAWTON (1988) examined the relationship beween the autocatalytic ethylene production, petal senescence and mRNA population after exposure to endogenous ethylene, relating tissue responsiveness to ethylene with increasing age, using the inhibitors norbornadiene and STS. Ethylene was confirmed to modulate gene expression in carnation, probably being the major factor in modulation since most of the ethylene induced mRNA s were those previously shown to occur in natural senescence. Developmental changes (such as inrolling of petals), appeared to be the result of rapid alterations of gene expression. It was proposed that though the simplest methods of regulation of developmental responses would be at the level of ethylene binding, the diversity of responses to ethylene may indicate control beyond binding. It was inferred from evidence that the induction of a 58 kDa message by ethylene only occurred in older senescing, ethylene-producing tissue.

Sensitivity and responsiveness of the flowers increases with age with the increased effect of the ethylene not largely as a result of increased ethylene production. The changes are probably associated with branch pathways off the main signal transduction pathway (WOODSON AND LAWTON, 1988).

Further research by this team was aimed at the gene level, with the construction of copy DNA (cDNA) libraries of the carnation, to study gene expression. Similar work had been done along these lines in the characterization of fruit-specific cDNA s from tomato (MANSSON, HSU AND STALKER, 1985) and demonstrations of transcriptional control by ethylene in carrot roots (NICHOLS AND LATIES, 1984). Regulation of plant defence genes by ethylene (or a wound signal) was also demonstrated during plant-pathogen interaction, where four genes, stimulated by ethylene, became involved in coding enzymes for three separate plant-defence pathways (ECKER AND DAVIES, 1987).

LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989), were able to isolate several senescence related genes from carnation petals. These were used to examine the expression of senescence-related, RNAs during flower petal senescence in response to ethylene.

To accomplish this, poly $(A)^+$ RNA (mRNA) was isolated from maximally senescent, day six petals (a critical stage in the coordination of biochemical events) (WOODSON AND LAWTON, 1988). A cDNA library was then constructed from this using a method described by GUBLER AND HOFFMAN (1983). The cDNA was restricted with EcoR1 endonuclease and ligated into phage λgt10 and allowed to infect host *Escherichia coli*. The library was screened by differential hybridisation using ^{32}P labelled single stranded cDNA, prepared from preclimacteric and climacteric (six days) petal poly $(A)^+$ RNAs. A number of recombinant preclimacteric cDNA molecules which hybridized more intensely with climacteric cDNA were selected. After purification, the cDNA was isolated and subcloned onto the EcoR1 site of the plasmid pUC 18. Messenger RNA expression was examined by hybridisation with cDNA probes from different stages of senescence and translated *in vitro* using rabbit reticulocyte lysate.

The cDNA s were found to represent three gene families and were confirmed to represent RNA's which increase in abundance in senescing tissue. Only one of the clones however, was able to be translated *in vitro* to an 81 kDa product. Using the clones, it was established that mRNA's for the senescence related (SR) genes were most abundant during senescence.

Two of the SR genes were stimulated by ethylene but the third not, indicating that it was stimulated by other age-related factors (such as radicals). It was also found that the continued prescence of ethylene is necessary for expression at the molecular level of those genes examined. Exogenous ethylene increased the amounts of senescence related mRNA s, preceding any visible symptoms, which occurred three hours later. The induction appears to take place in a very short time. In efforts to determine organ specificity of the production of senescence-related mRNAs in the whole plant, using RNA extracts subjected to Northern blot analysis with SR clones from petals, maximum accumulation was shown to be in the petals (LAWTON, HUANG, GOLDSBROUGH AND WOODSON, 1989).

The role of ethylene in the process was examined by WANG AND WOODSON (1989), using norbornadiene. Their conclusion was that ethylene is possibly required for the expression of the senescence programme at the molecular level and that the continued presence of ethylene is essential for this expression. Clearly a great deal of research is needed to characterize the action of ethylene at the molecular level in relation to the physiology of petal senescence.

The research direction then proposed by Woodson's team was to examine the control of ethylene regulation of senescence, how the increase of sensitivity development related to age, influences the expression of senescence-related genes, and the control of ethylene regulation (LAWTON, HUANG, GOLDSBROUGH AND WOODSON, 1989).

One of the senescence related genes (SR12), chosen for the specificity of its transcript to petal senescence and response to ethylene, was examined. It was found to be a low copy gene. The genomic clone was isolated and sequenced. From this it was determined that the gene was 12 Kb long and is interrupted by 16 introns ranging in size from 83 to 1120 base pairs. The 5' flanking region was found to contain sequences that are interacted with by nuclear protein sequences from the petals. To examine this and other upstream sequences for the expression of the senescence related gene, promoter fusions of the sequences to a GUS reporter gene have been constructed and analyzed in transgenic plants and transient expression assays carried out (RAGHOTHAMA, LAWTON, GOLDSBROUGH AND WOODSON, 1990).

Regulation of the genes by ethylene was examined by inhibiting protein synthesis of the three isolated genes, by cycloheximide treatment. This treatment was shown to prevent ethylene-induced senescence-related transcript accumulation. The researchers concluded from this that ethylene does not interact with preformed factors (such as binding sites) but rather activates gene expression by mediation with labile protein factors synthesised on cytoplasmic ribosomes. Further work was proposed to determine whether this effect is at the transcriptional or post-transcriptional level (LAWTON, RAGHOTHAMA AND WOODSON, 1990).

The enzymes of the ethylene biosynthesis pathway were also examined by LARSEN, RAGHOTHAMA AND WOODSON (1990). A SAM synthetase cDNA clone from *Arabidopsis thaliana* was used to screen a phage λgt 11 library and to isolate a mRNA of 1.7 Kb. A significantly greater amount was found in the roots. The above authors found, however, that the transcript level in the petals remained constant with the onset of ethylene production during petal senescence. This was in agreement with enzyme activity. From this it was inferred that SAM synthetase mediation is probably not a regulated step in the ethylene biosynthesis pathway during climacteric. WANG AND WOODSON (1989) successfully isolated and partially purified ACC synthase from ethylene-induced petals, which could be used in analysis of the λgt 11 library using antibodies of the enzyme.

The interaction of cytokinin and ethylene in the regulation of the three isolated senescence related genes was examined by MEYER AND WOODSON (1990). BA at a concentration of 100 μM was found to prevent climacteric ethylene production, to delay senescence and to prevent transcription of two of the genes. If however, BA-treated petals were exposed to exogenous ethylene, transcription of the clones was again able to occur. Removal of this ethylene resulted in the disappearance of these transcripts. This indicates that while BA delays senescence and prevents ethylene-induced senescence, it does not block the ability of the petals to perceive ethylene (MEYER AND WOODSON, 1990).

Plant gene expression, such as that which occurs during senescence, is a highly regulated activity dependent on many factors (ELLISTON AND MESSING, 1989). The patterns of gene expression can change in a matter of minutes resulting in changes in mRNA production, thus allowing the expressing genes to be examined by the generation of cDNA libraries (GRIERSON AND COVEY, 1989).

Several models for gene regulation have been proposed, all reformulations of the JACOB AND MONOD (1961) model for the bacterial operon. According to this, there are two types of sequence ie. structural genes expressed as RNA or protein; and sequences whose function is to be recognised by regulator molecules or by enzymes of nucleic acid synthesis.

It was assumed that as in bacteria, recognition elements lie contiguous with the structural genes they control (sequences whose function is to result in translation and transcription of molecules are known as structural genes, while sequences serving only as recognition sites for regulator molecules are described as elements) (LEWIN, 1980). It was confirmed by GOLDBERG (1986), that at least part of the information for the regulation of the genes is found in or close to the genes since plant genes with complete 3′ and 5′ flanking sequences have been transferred to other plants by genetic engineering techniques and have still retained developmental regulation.

The most detailed of the models for eucaryotic gene regulation was proposed by BRITTEN AND DAVIDSON (1969) and elaborated by DAVIDSON AND BRITTEN (1973, 1979). This postulates the existence of four classes of sequence of eucaryotic DNA whose interactions constitute a system of positive control in which genes are inactive unless specifically activated (essentially the opposite mechanism of negative control which occurs in the *Escherichia coli* lactose operon is that genes are active unless specifically switched off). Genes responsible for production of proteins are termed producer (or structural genes). Adjacent to each producer gene is at least one receptor element. The producer gene can be transcribed only when an activator molecule recognizes an adjacent receptor element.

Regulator molecules are proposed to be activator RNA s or proteins. It would be advantageous for this molecule to be RNA so that the whole control network might be contained in the nucleus. Loci which code for these are known as integrator genes (analagous to regulator genes of bacteria). To provide for specific control of gene expression, it is necessary for the activity or synthesis of the regulator molecules to respond to the cell milieu. In the case of bacterial operons, the activity of the repressor proteins is controlled by their interactions with small molecules of the environment. The eucaryotic model proposes that transcription of the integrator genes (producing regulator molecules) can only occur by the activation of the controlling adjacent sensor elements. These sensors are proposed to be the targets that are recognised by agents which change the pattern of gene expression eg. hormone - protein complexes may bind to sensor elements to activate adjacent integrator genes. The model also states that elements and integrator genes may be repeated, which would allow a small number of control elements to activate a large number of genes. There is no evidence, however, of coordination control linkage of eucaryotic genes into clusters as occurs in bacterial operons. Since it is necessary that producer genes be activated in more than one set of circumstances, the model postulates that each gene may possess several different adjacent receptor elements, any of which may respond to its appropriate activator RNA protein (ie. redundancy in the integrator and receptor elements).

A particular activator may thus cause transcription of all structural genes possessing a copy of receptor elements. A particular activator may thus cause transcription of all producer genes possessing a copy of the receptor element that it recognizes thus forming an indirect form of gene linkage.

For extensive changes in gene expression, such as would occur during senescence, it may be necessary to activate many sets of genes. Integrator genes in this case may be in clusters, each falling under one sensor element, thus forming what has been termed a battery. To cause stimulation of a set of genes for more than one stimulus, one integrator sequence may be repeated under the control of more than one sensor, thus its activator RNA may be synthesised for more than one set of cellular conditions (LEWIN, 1980). This model would be consistent with the fact that different repetitive DNA sequence families are preferentially transcribed at different developmental stages (THOMPSON AND MURRAY, 1981).

The primary structure of the gene ie., the linear sequence elements of DNA and RNA, may be responsible for the binding of various cellular factors that influence the expression of any particular gene (VON HIPPEL, BEAR, MORGAN AND MCSWIGGEN, 1984; ELLISTON AND MESSING, 1989).

This influence may be at the DNA level where the transcription of the gene will be affected or at the transcription level itself where the processing and stability of the mRNA will play a role (LEWIN, 1980; ELLISTON AND MESSING, 1989). The two stages of gene expression on which most interest has centred as probable points of control, are the initiation of transcription and processing/splicing of hnRNA (heterogeneous nuclear RNA) and, or, nucleocytoplasmic transport of mRNA. It is very evident that transcriptional control occurs, since not all genome sequences are represented in nuclear RNA (LEWIN, 1980).

The sequences playing a role at the first transcriptional level, are the TATA and to a lesser extent, CAAT boxes (GRIERSON AND COVEY, 1989). The TATA box is the basic primary part of the promoter (ELLISTON AND MESSING, 1989). These sequences are present in all genes and can be expressed constitutively but may also be developmentally regulated. The structure of chromatin may also be important in the selection of genes for transcription since there is a local change in nucleosome conformation both before and after transcription. The aspect of chromatin conformation, is an important feature unique to eucaryotes (LEWIN, 1980). The processing of mRNA, or second level of transcription regulation is influenced by two units of primary structure ie. the mRNA splicing site and the poly (A) signal.

Plants have a distinctive splicing signal that differs from that of animals and there may be distinctions between that of monocotyledonous plants and dicotyledonous plants since monocotyledonous plant mRNA s are not properly spliced when in transgenic dicotyledonous plants (KEITH AND CHUA, 1986). Polyadenylation, which distinguishes mRNA from other types of RNA, is necessary for the export of the mRNA and its subsequent translation, and may be important in its stability (DYER AND LEAVER, 1981). This process requires the poly (A) addition signal which is upstream from the poly (A) tail (ELLISTON AND MESSING, 1989). Some genes may be controlled at transcription initiation, others may be regulated by selection of sequences for processing or transport from the nucleus to the cytoplasm. It is not certain whether there is a functional distinction between genes controlled at one or other level and what type of relationship exists between the two (LEWIN, 1980; GRIERSON AND COVEY, 1989).

Translation is another level at which regulation may occur, since in most cases, a protein is the end-product of gene expression (ELLISTON AND MESSING, 1989). Regulation may influence post-translational transport or modification of the protein product (GRIERSON AND COVEY, 1989). The AUG start codon and the sequences immediately preceding it, plays a direct role in the translation of mRNA and thus has a third level of control on gene expression. Recognition of the AUG start codon by the pre-initiation complex is rate limiting in the translation of mRNA (HEIDECKER AND MESSING, 1986).

There are other signals governing the extent or timing of expression of particular genes. Certain sequences may act as enhancers or silencers. These may stimulate or repress expression in specific developmental situations, such as would occur during senescence. These *cis*-acting signals function similarly to other DNA control sequences by being recognized and bound to by *trans*-acting protein factors encoded by genes located elsewhere. This would result in the direct or indirect regulation of neighbouring genes (GRIERSON AND COVEY, 1989).

The *cis*-acting regulatory elements can be located in many possible sites in the gene including the 5' side of the introns or coding regions or to the 3' side of genes (GRIERSON AND COVEY,1989) and around the AUG initiator codon (ELLISTON AND MESSING, 1989). Gene regulation as a whole may also be affected by the availability of cofactors and metabolites (GRIERSON AND COVEY, 1989).

The work done in this study is similar to that done by LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989). The aim was to isolate genes involved in senescence through the isolation of mRNA which is produced only by genes that are active, from presenescent and senescing carnation tissue. Unlike LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989), not only petals were extracted but also, receptacles and ovaries. Thus, in addition to the petal-specific genes, receptacle and ovary-specific genes would also be isolated so that a better evaluation of the organ in which senescence may originate (with special interest in the receptacle) could be made.

The isolated mRNA was subsequently synthesized into copy DNA (cDNA), and then amplified using the vector phage λgt 10 into an *Escherichia coli* host. This resulted in the contruction of two libraries of presenescent and senescing carnation genes for each of the tissues used (ie. six libraries). To obtain genes unique to senescing tissues, a subtraction library was constructed as opposed to the differential hybridization method which was used by LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989) and stored in a phage vector in an *Escherichia coli* host.

These senescence-related genes, once available can be used to screen mRNA from different organs of the carnation using *in situ* hybridization and dot blots to determine the source organ of gene regulation, and the effects of different hormone treatments on the senescence process in cut carnation flowers.

CHAPTER 2

RNA ISOLATION AND PURIFICATION

2.1 INTRODUCTION

The first step in obtaining the genes involved with senescence of carnations requires the isolation of pure, undegraded RNA from which mRNA (which is the product of the expression of active genes) is extracted.

Early attempts at RNA isolation were variable and largely not very successful for isolating active, intact mRNA. A successful RNA isolation from a cell homogenate requires the removal of unwanted molecules, which can include DNA, proteins and polysaccharides (KIRBY, 1956). A critical factor to be taken into account to isolate undegraded RNA, which was of greatest importance in the development of isolation strategies, is the presence in cell homogenates, of very active ribonucleases (RNAases) (CHIRGWIN, PRZYBYLA, MACDONALD AND RUTTER, 1979).

Some early workers such as GRINNAN AND MOSHER (1951) and KAY AND DOUNCE (1953) separated RNA from DNA by the removal of cell nuclei. GRINNAN AND MOSHER (1951) then used guanidine hydrochloride to denature the protein followed by heating the preparation at 90 to 100°C. The removal of protein was achieved using extractions with chloroform and butanol. KAY AND DOUNCE (1953) however, extended the purification by adjustment of the pH to an acidic nature (4.5), followed by two treatments with sodium dodecyl sulphate (SDS). Isolation using guanidine hydrochloride was also attempted by VOLKIN AND CARTER (1951) with the temperature this time at 40°C. This however, resulted in a lower yield and a recovery of only 20 to 30 % of total tissue RNA.

The use of phenol for the isolation of RNA became popular in the late 1950's, when an observation by WESTPHAL, LUDERITZ AND BISTER (1952) was further developed by KIRBY (1956). WESTPHAL, LUDERITZ AND BISTER (1952) used a treatment of phenol and water to isolate pyrogenic polysaccharides from bacteria. When the extraction was performed at 68°C, polysaccharides and RNA were found in the aqueous phase (KIRBY, 1956). WESTPHAL, LUDERITZ AND BISTER, (1952) attempted further separation by lengthy precipitation and ultracentrifugation procedures. KIRBY (1956) applied the method to mammalian tissue, achieving better results at room temperature. After centrifugation the molecules distributed in the tube as indicated in Figure 2.1. The function of the phenol was to break the hydrogen bonding of protein to the RNA (KIRBY, 1956).

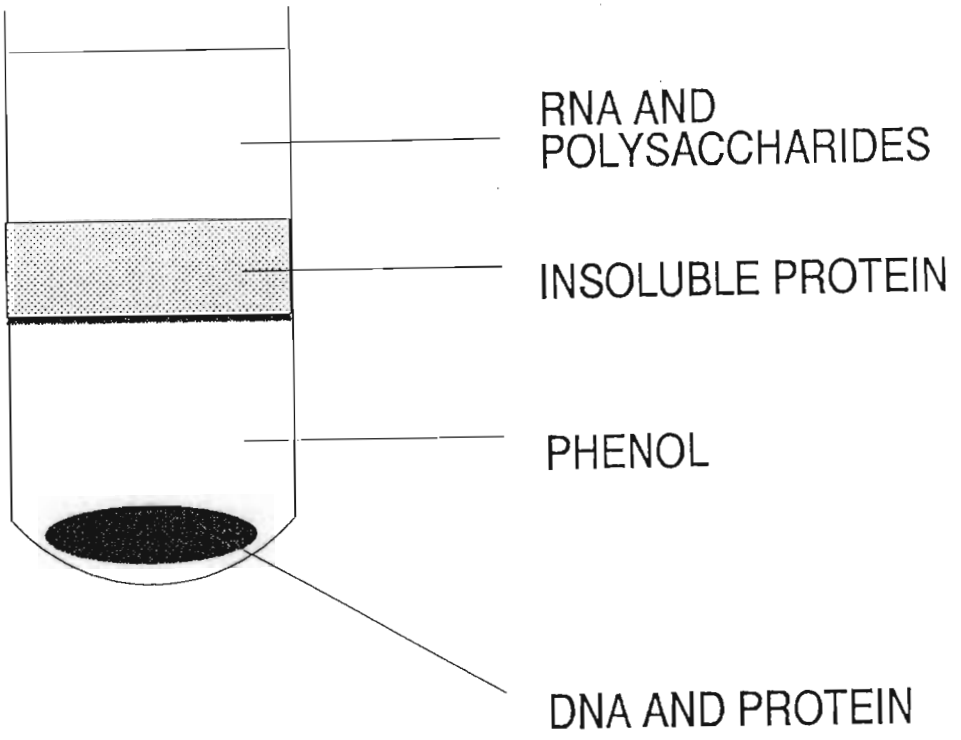


Figure 2.1. Distribution of molecules after centrifugation of homogenized tissue using phenol, after KIRBY (1956).

The problem of separating RNA from the polysaccharides was solved by separating the RNA in a potassium sulphate solution into the organic 2-methoxyethanol phase, leaving the polysaccharides in the aqueous layer. This, however, was not always successful. Centrifugation at 10 000 *x g* for I hour was followed by dialysis and precipitation with potassium acetate and ethanol (KIRBY, 1956). A further advantage of using phenol, was that it was found to some extent to inactivate the very resilient pancreatic ribonuclease (KIRBY 1956, KIRBY 1965). Further purification steps however, still resulted in the degradation of RNA. Failure of the phenol to inactivate certain *Escherichia coli* RNases, however, was noted by LITTAUER AND EISENBERG (I959).

Other strategies that were used by various researchers for the elimination of RNase activity include firstly the use by HUNTER AND BUTLER (1956) of SDS directly after homogenisation, to free RNA from protein, followed later by extraction with chloroform. LASKOV, MARGOLIASH, LITTAUER AND EISENBERG (1959) suggested the use of EDTA for the inhibition of ribonuclease. The addition of 8-hydroxyquinoline by KIRBY (1968) was found to decrease the protein concentration, thus decreasing the level of ribonuclease action. HUPPERT AND PELMONT (1962) realised the importance of reducing RNase contamination from outside sources such as glassware and hands. They therefore advocated treatment of glassware with chromic acid and the wearing of gloves.

Another chemical, later to be used for the same purpose *ie.* diethylpyrocarbonate (DEPC) was advocated by FEDORCSAK, NATARAJAN AND EHRENBERG (1969) as a protein denaturant and general enzyme inhibitor which did not affect "substrate properties of nucleic acids".

PALMITER (1974) selected parts of the various methods and produced a phenol-SDS-chloroform method of extraction. This is fairly similar to the current method used in the present study which was documented by AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL (1989). PALMITER (1974) used diethylene pyrocarbonate (DEPC) in solutions (50 μl/100 ml of solution) for the isolation. The solutions were shaken at room temperature and placed in boiling water for 15 to 30 minutes. While still hot, the solution was shaken to release CO_2 and ethanol. Using this method, PALMITER (1974) found however, that some destruction of mRNA occurred, probably due to alkylation of the RNA. The extraction buffer contained Hepes with sodium acetate (pH 5), SDS (0.5 %) and phenol. PALMITER (1974) carried out the extraction under acidic conditions. An acidic pH minimises RNAase activity (BROWN, 1967), allows the use of EDTA (which would precipitate in ethanol at alkaline pH) and promotes rapid removal of the protein interphase while decreasing DNA recovery (PALMITER, 1974). Liquified phenol was used which PALMITER (1974) had noted was not necessary to redistill unless coloured.

The addition of chloroform was found to be necessary to promote rapid separation of phases and allowed easy removal of the aqueous phase, thus decreasing the transfer of phenol to succeeding stages, which simplified ethanol precipitations. Use of chloroform also prevents retention of water (containing RNA) by phenol thus decreasing losses of RNA by 10 to 15 %. It was emphasised that it is necessary to separate RNA and protein first by shaking with phenol before the addition of chloroform, otherwise loss of RNA will occur through the formation of insoluble protein-RNA aggregates (PALMITER, 1974). The salt concentration used should be sufficient for ethanol precipitation of RNA and should not be a potassium salt as this precipitates SDS. PALMITER (1974) used magnesium as the RNA precipitant in the early steps. In the more recent, modified protocol of AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL (1989) the magnesium was replaced with lithium.

Phenol or guanidine hydrochloride inhibition of RNAase activity is reversible and not completely effective at high concentrations of RNAase or with very active RNAases (HUPPERT AND PELMONT, 1962; CHIRGWIN, PRZYBYLA, MACDONALD AND RUTTER, 1979). This resulted in the development of an alternative extraction technique using guanidinium thiocyanate and guanidine hydrochloride for tissues enriched in ribonuclease (CHIRGWIN, PRZYBYLA, MACDONALD AND RUTTER, 1979). The authors stressed, however, that the critical point in the prevention of degradation by ribonuclease occurs in the initial seconds of the homogenisation of the tissue.

The phenol method, though reportedly not as effective as the guanidine method, is however, more economical. Phenol and guanidine hydrochloride/ guanidinium thiocyanate are very toxic and require careful handling, although phenol, being more volatile is possibly more readily absorbed into the body, through the respiratory tract. The phenol-SDS method [as set out by AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL (1989)] was used for the isolation of RNA for this study.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Standard, white, longstemmed carnations (*Dianthus caryophyllus* L. cv. Kaly) were obtained from Florarcadia, Heidelberg. The carnations were fresh and in a fully mature, presenescent state. On receiving the flowers, the stems were cut under water to a stem length of 10 cm and held in water at room temperature, for dissection and extraction of the RNA the same day. Senescing carnation tissue was obtained by keeping the flowers in water and allowing them to senesce naturally to the stage of "sleepiness" (achieved after approximately six days) (Plate 1.1.).

2.2.2 Preparation of glassware and equipment

All equipment used which comes into contact with the RNA during its extraction, requires special treatment, with either DEPC and/or in some cases siliconization using dimethyldichlorosilane.

A solution of DEPC (0.2 % v/v) was used to rinse all glassware, centrifuge tubes, homogenizing equipment, spatulas and weighing boats and pipette tips, followed by autoclaving to remove the DEPC. All glassware, prior to the DEPC treatment, requires siliconization.

Large pieces of glassware require rinsing in a 6 % solution of dimethyldichlorosilane in chloroform, followed by copious rinsing in water and finally baking for two hours at 180°C. Small items can be siliconised in a vacuum dessiccator, by placing 1 ml of dimethyldichlorosilane in a small beaker alongside the items to be treated, in the vacuum dessiccator. A vacuum is applied for 5 minutes and then quickly released to distribute the vapour evenly. The vacuum is reapplied for a further 30 minutes and then the objects rinsed and baked in a similar manner as mentioned earlier. The purpose of DEPC treatment is, as mentioned previously, to destroy possible ribonuclease contaminants. Siliconisation prevents the loss of RNA through binding to the glass walls.

2.2.3 Total RNA extraction from flower components

Flowers were dissected into petals, receptacles and ovaries using new, sharp blades. Each single, separate flower part, on dissection, was weighed and immediately placed into liquid nitrogen. The material was maintained in this frozen state until ready for grinding. Each frozen flower part was ground separately using a mortar and pestle. Liquid nitrogen was added at regular intervals to prevent thawing of the material. Thorough grinding was necessary at this stage to ensure adequate extraction of RNA. A total of 10 g of petal tissue, 5 g of receptacles or 5 g of ovaries was used in each extraction.

Crystalline phenol was liquified using 92 % phenol with 8 % water (with heating to 60°C) and equilibrated with Tris-lithium-EDTA (TLE) solution (0.2 M Tris, 0.1 M LiCl and 5 mM EDTA in deionised, DEPC treated water, adjusted to pH 8.2 with HCl). Equilibration involved extraction of the phenol with an equal volume of TLE solution, with the addition of 0.5 ml sodium hydroxide (to adjust the pH to 8.0), followed by two additional extractions with the TLE solution. One extraction of each tissue type required 200 ml of liquified phenol.

The frozen tissue homogenate from each of the flower parts was then suspended in a solution containing 100 ml grinding buffer (0.18 M Tris, 0.09 M LiCl, 4.5 mM EDTA and 1 % SDS, made up in DEPC treated deionised water with pH adjusted to 8.2 with HCl) and 33 ml equilibrated phenol.

The suspension was then allowed to thaw, before being further homogenised in the Dounce homogeniser to break down cell walls. The homogeniser consists of a roughened plunger fitting tightly into a glass homogenisation tube. The plunger is inserted into and drawn out of the glass tube containing the cell homogenate, thus forcing the cells past the rough surface of the plunger causing rupture of cells, and release of contents. Excessive homogenisation of the tissue at this stage can cause shearing of the RNA.

Chloroform (33 ml) was then added to the phenol/ grinding buffer/ cell homogenate and mixed. This mixture was poured into a flask and heated in a water bath at 50°C for 20 minutes. This procedure causes further breakdown of cell walls and the release of the cellular contents.

The contents of the flask was then transferred into 50 ml centrifuge tubes and centrifuged in a Sorvall SS34 rotor at 11500 rpm (15256 *x g*).

After centrifugation, the upper aqueous layer was removed without disturbing the interface and placed in additional centrifuge tubes (the non aqueous layer was discarded). A fresh addition of a total of 33 ml phenol was made to the aqueous layer, followed by shaking.

A total of 33 ml of chloroform was then divided equally between the tubes. The tubes were vigorously shaken and balanced before recentrifugation in the SS34 rotor at 11500 rpm (15256 *x g*) for 10 minutes. The large amount of unwanted material (carbohydrate and protein) which occurred particularly in the petal and ovary preparations, necessitated a repetition of this step.

The aqueous layer was again recovered and placed in glass separating funnels. Phenol (33 ml) was added, followed by shaking. Chloroform was then added and the contents of the funnel mixed. The layers were allowed to separate, after which the lower phenol/chloroform layer and interface were removed and discarded. Phenol followed by chloroform (as above) was added to the retained aqueous layer, for two further extractions. A final extraction with 66 ml chloroform was then carried out to remove all remaining traces of phenol.

To remove all traces of chloroform, a final centrifugation at 11500 rpm (15256 *x g*) for 15 minutes was carried out. Droplets of chloroform were then suctioned from the bottom of the centrifuge tubes using pasteur pipettes. It is important that all the chloroform is removed so that it does not interfere in the subsequent RNA precipitation step by preventing the adherance of the RNA pellet to the wall of the tube during centrifugation and thus effecting the loss of RNA material during aspiration of the supernatant.

The selective recovery of RNA from the large volume of liquid, requires the inducement of precipitation of the RNA. The precipitation of RNA is effected by the addition of sufficient lithium chloride (8 M) to the aqueous supernatant, to bring the final concentration to 2 M LiCl, at which concentration precipitation occurs. The RNA was allowed to precipitate overnight at 4°C. The precipitate was then collected by centrifugation at 4°C at 16000 rpm in the SS34 rotor for 30 minutes (29532 *x g*). The pellet was then rinsed with a few millilitres of LiCl (2 M) to remove impurities. The pellets were resuspended in a total of 5 ml water. A volume of LiCl (8 M) equivalent to a third of the volume of redissolved RNA solution, was added to once again bring the concentration of LiCl to 2 M. The RNA was left to precipitate at 4°C for at least 2 hours (in general, overnight to ensure complete precipitation). Centrifugation for 30 minutes at 4°C in the SS34 rotor (29532 *x g*) was once again carried out, and the resulting pellets rinsed as before with LiCl (2 M).

The RNA pellets were then resuspended in 50 μl of water and placed in microcentrifuge tubes. A final precipitation of the RNA was effected by the addition 5 μl of sodium acetate followed by 137 ml of ethanol with mixing. This step was carried out at -20°C/ -70°C overnight. The RNA can be stored in this state at -20°C or -70°C for several weeks.

To recover the RNA for resuspension and subsequent concentration determination (absorbance at 260 *nm*) and for use in the next step of separation of the messenger RNA from the total RNA, the RNA was centrifuged in a microcentrifuge for 30 minutes at 10°C. The RNA was analysed on an agarose gel to determine the extent of degradation of the RNA.

The 1.5% agarose mini-gel was made according to a protocol set out by AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL (1989). Molecular biology grade agarose (0.45 g) was mixed with 29.34 ml DEPC-treated water and 0.66 ml Tris-acetate. The mixture was then boiled in a microwave oven, until all lumps of gel were eliminated, before adding 45 ml of heated distilled water. The gel was allowed to cool to 55°C (to prevent buckling of the casting tray) and then poured into the casting tray with the well comb in position. After setting of the gel, the comb was removed. The gel was placed in a mini-gel electrophoresis unit with running buffer (12.24 ml of 50X Tris-acetate and 600 ml water).

The RNA samples (8 ug) were each made up in DEPC treated water. The tubes were then mixed with 2 μl sterile loading buffer (50 % glycerol, EDTA, 1 mM; 0.4 % bromophenol blue and 0.4 % xylene cyanol). After being put on ice for 20 seconds, the samples were centrifuged to collect the contents in the bottom of the tube and loaded into the wells of the gel.

The samples were subjected to electrophoresis until the bromophenol blue marker almost reached the end of the gel. The gel was then covered with a 0.5 mg per litre solution of ethidium bromide and allowed to stain in the dark overnight. The ethidium bromide intercalates in the helical bonds of the RNA and can be detected by viewing on a UV transilluminator. The gel must not be exposed to light excessively when stained with ethidium bromide, since degradation of the ethidium bromide will occur (along with the stained RNA). Before examining under UV light, the gel was destained in distilled water with shaking and frequent water changes for approximately 2 hours to remove background stain.

2.2.4 Extraction of messenger RNA from total RNA

A column for the selection of mRNA from total RNA was constructed, making use of a sterile 2 ml plastic syringe (with plunger removed). The column and glasswool plug were vacuum siliconised and treated with DEPC, followed by rinsing with ribonuclease free water, and autoclaving in the case of the glasswool. The column was washed with 10 ml of NaOH (5 M) and then rinsed with water. Dry oligo (dT) cellulose powder was then mixed with 1 ml of 0.1 M NaOH to make a slurry. The slurry was poured into the column and rinsed with 10 ml water. The column was then equilibrated with 10 to 20 ml loading buffer (0.5 M LiCl, 1 mM EDTA, 0.1 % SDS, 10 mM Tris-Cl, pH 7.5). The pH output of the column effluent was tested to ensure that it was pH 7.5.

The RNA pellet after being recovered by centrifugation in the microcentrifuge for half an hour at 10°C, was resuspended in 1 ml of ribonuclease free water. This was heated to 70°C for 10 minutes. After heating, 10 M LiCl (50 μl) was added to the RNA solution (to make a final concentration of 0.5 M LiCl).

The RNA solution was placed on ice, then passed through the oligo (dT) column. The column was then washed with 1 ml of poly (A) loading buffer. The eluant from this loading step was saved. This eluant was passed through the column two additional times. The column was then rinsed with 2 ml of middle wash buffer (0.15 M LiCl, 1 mM EDTA, 0.1 % SDS, 10 mM Tris-Cl, pH 7.5). The RNA was eluted into a fresh tube with 2 ml of 2 mM EDTA / 0.1 % SDS solution.

The process can be repeated, to ensure complete purity of the mRNA, however, this was found to be unnecessary judging by the apparent purity as determined (see results) after the first elution through the column, while also decreasing the possibility of loss of the mRNA.

The mRNA was precipitated from the solution by adjusting the salt concentration to 0.3 M sodium acetate using the appropriate quantity of 3 M sodium acetate stock solution, and adding 2.5 times the RNA solution volume of ethanol. The mRNA was then allowed to precipitate overnight at -20°C, or for 30 minutes at -70°C.

The precipitate was collected by centrifuging for 30 minutes at 240 000 *x g*. The ethanol was poured off and the pellets allowed to air dry. The pellets were resuspended in a small amount of water and the concentration of the mRNA determined at an absorbance of 260 *nm*.

2.3 RESULTS AND DISCUSSION

The electrophoresis of the total RNA on the non-denaturing gel, indicated that the RNA was indeed intact and present in sufficient quantities, thus making it suitable for use in further steps in the isolation of senescence related genes of the carnation (Plate 2.1. and Plate 2.2.).

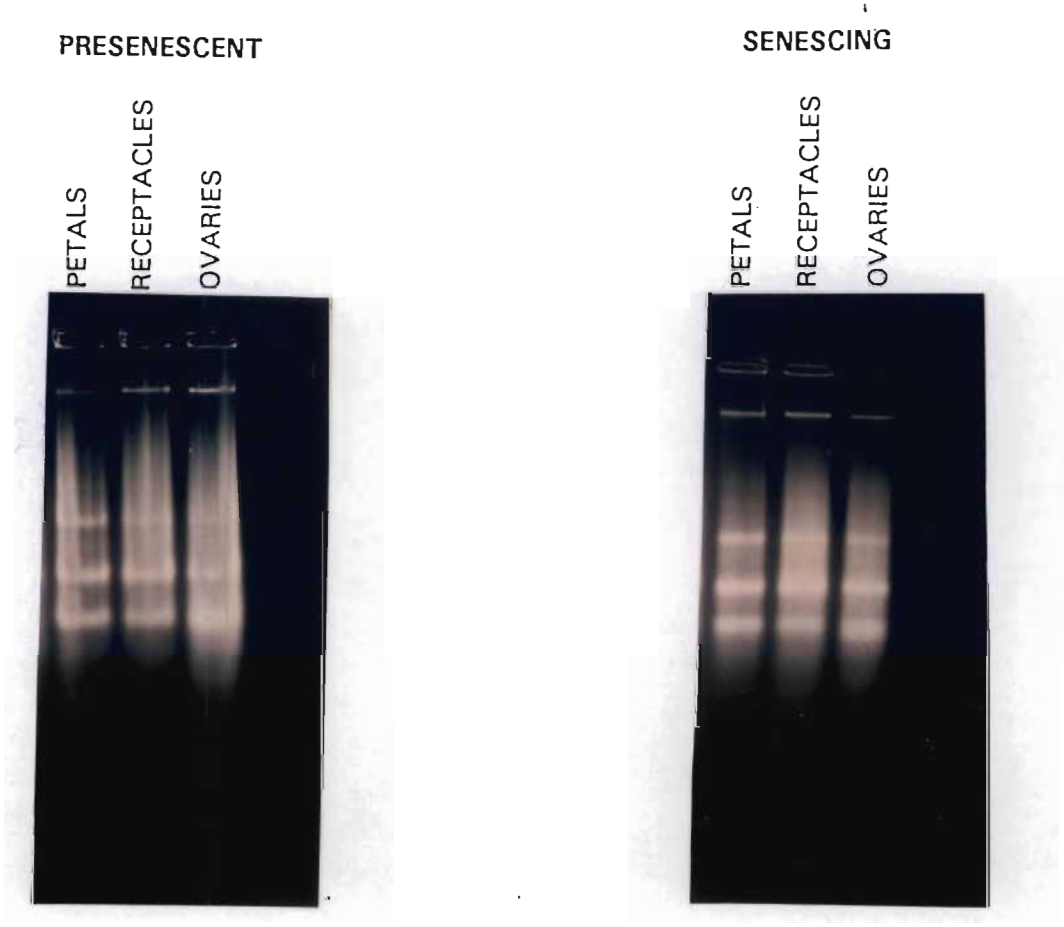


Plate 2.1. and Plate 2.2. Electrophoretic separations of RNA isolated from petals, receptacles and ovaries of carnation flowers before the onset of (2.1.) and during senescence (2.2.) on non-denaturing gels. Intact ribosomal units (25S, 18S and 16S, respectively from top to bottom of the gel) are present as bright bands indicating the undegraded nature of the RNA material.

Each streak of RNA resulting from the electrophoresis of RNA from presenescent (Plate 2.1.) and senescent (Plate 2.2.) ovary, receptacles and petals, exhibited three, brighter stained regions (at approximately 2, 2.5 and 3 cm from the wells) which were the 25S, 18S and 16S ribosomal subunits. These subunits would not have been intact if the RNA had undergone any degradation by ribonucleases or mechanical shearing during its isolation. Other lighter stained bands closer to the wells may have contained small quantities of contaminating DNA. If the RNA had been extensively degraded, a heavy band consisting of small pieces of RNA and ribosomes would have been observed at the end of the gel, with none of the characteristic ribosomal subunit bands.

There appeared to be a greater abundance of RNA isolated from the presenescent carnation material as opposed to the senescing material. This may have been the result of fewer genes being active during senescence of the floral parts, or because of natural degradation of the RNA during senescence owing to the action of degradative enzymes. It would possibly be expected, however, that the ovary, which may become increasingly active during senescence, would have a greater abundance of RNA during the senescence of the rest of the flower, but this did not appear to be the case. Variations in the amount of protein and polysaccharides in the different floral parts at different times in the life cycle of the flower, may influence the success of the extraction of RNA, thus making it difficult to compare isolations quantitatively.

Determination of final RNA concentrations by absorbance, was inhibited by the presence of contaminating polysaccharides / proteins which often formed a gel-like substance which absorbed all the liquid containing the RNA. To release some of the RNA, it was necessary to heat this mass at about 70°C in a water bath, followed by dilution with DEPC-treated water and microcentrifugation to draw off most of the remaining gel-like substance as a pellet, which was subsequently discarded after aspiration of the liquid containing the RNA. There may have been significant losses of RNA through its entrapment in the contaminating material.

To decrease the amount of contaminating protein, 8-hydroxyquinoline (0.1 %), as suggested by KIRBY (1968), was added to the phenol, but it did not appear to significantly reduce the levels of contamination in the final RNA solution. It was observed, however, that use of a bucket-type centrifuge during the extraction, as opposed to a non-vertical fixed-angle rotor, appeared to improve the separation of the phenol/chloroform layer during the extraction, possibly because of decreased mixing of the easily dispersed layers when the tubes were removed from the rotor. A vertical fixed-angle rotor would therefore probably give better separation of the RNA from the contaminating polysaccharides and / or proteins. The contaminating material may also absorb in the 260 *nm* range at which the RNA was detected. Typical quantities of total RNA obtained during the extractions ranged from 30 μg to a maximum of 250 μg.

Isolation of mRNA from total RNA exploits the fact that mRNA is differentiated from the rest of the RNA by the prescence of a poly-A tail. This poly-A tail binds to the complementary oligo-dT residues on the isolation column. The rest of the RNA, including the ribosomal subunits were washed out of the column by rinsing. The final elution buffer containing a stronger concentration of EDTA, released the poly-A tail of the mRNA. An electrophoretic gel of the mRNA would reveal a streak of mRNA over the various size ranges, with no ribosomal subunit bands. A gel of the mRNA was not run, however, because of the small quantity of mRNA isolated. Yields of mRNA were in the range of 30 to 70 % of the original concentration of total RNA.

The mRNA obtained was representative of the genes active before and during senescence of the floral parts of the carnation. The next step in the analysis of the expression of these active genes is the transcription of the mRNA into complementary DNA. This will allow the cloning and multiplication of gene copies for analysis of the source genes and their products. This transcription should take place fairly rapidly after the isolation of the mRNA, since the mRNA is very unstable and readily sheared by any RNAases present.

CHAPTER 3

COPY DNA SYNTHESIS

3.1 INTRODUCTION

The availability of complementary (cDNA) DNA copies of mRNA provides a very powerful tool for analyzing expression as well as structure and organization of eukaryotic genes (OKAYAMA AND BERG, 1982). In addition, the use of cDNA is an absolute prerequisite for the expression of eukaryotic proteins in bacteria since bacteria do not possess the characteristic exon-intron structure of eukaryotic genes and would not be able to process genetic information containing such structures (WINNACKER, 1987). The mRNA, as a product of the expression of genes active in the carnation flower parts isolated before and during senesence, will therefore need to be synthesised into cDNA. This will enable in-depth study of the process of senescence and the genes involved by comparing the genes active before senescence with those activated or deactivated during senescence.

VERMA, TEMPLE, FAN AND BALTIMORE (1972), KACIEN, SPIEGELMAN, BANK, TERADA, METAFORA, DOW AND MARKS (1972) and ROSS, AVIV, SCOLNICK AND LEDER (1972), were all able to achieve success in synthesis of the first strand of globin DNA using a mRNA template. The enzyme used for the syntheses was in each case the DNA polymerase obtained from the RNA tumour virus, avian myeloblastosis virus (AMV) (similar to that of other RNA tumour viruses). AMV DNA polymerase copies RNA or DNA templates by the elongation of hydrogen bonded "primer-initiator" molecules (HURWITZ AND LEIS, 1972). The *in vitro* product of the endogenous reaction, is composed of single and double stranded molecules (JUNGHANS, DUESBERG AND KNIGHT, 1975). Actinomycin D was used to restrict the synthesis by the enzyme to a single strand only. The synthesis was found to be dependent on the prescence of a primer, the most active of which was oligo (dT). The mRNA molecule was found to be particularly suited to serve as a template for DNA synthesis using AMV polymerase (also known as reverse transcriptase) since it possesses a poly A tail and allows hydrogen bonding of a primer (KACIEN, SPIEGELMAN, BANK, TERADA, METAFORA, DOW AND MARKS, 1972). The complementarity of the synthesised DNA to the mRNA template was confirmed by each of the research teams through hybridisation analyses of the cDNA products.

Several laboratories in the late 1970's, were able to synthesise, from a mRNA template, double stranded cDNA which they were subsequently able to clone. Among these were EFSTRATIADIS, MANIATIS, KAFATOS, JEFFREY AND VOURNAKIS (1975) (USA); ROUGEON, KOURILSKY AND MACH (1975) (Switzerland); RABBITTS (1976) (UK); HIGUCHI, PADDOCK, WALL AND SALSER (1976) (USA). The methods, though different, generally involved the use of a series of three enzymatic reactions to synthesise variable lengths and quality of double stranded DNA copies of the globin structural gene (the methods being applicable in principle to any mRNA) (WICKENS, BUELL AND SCHIMKE, 1978). EFSTRATIADIS, MANIATIS, KAFATOS, JEFFREY AND VOURNAKIS (1975) showed that in the prescence of exogenous or foreign mRNA, transcription product of AMV reverse transcriptase is primarily single stranded DNA, even without the prescence of Actinomycin D, which is reported to interfere with the formation of the second strand.

EFSTRATIADIS, KAFATOS AND MANIATIS (1977) confirmed by sequencing both mRNA and cDNA components involved in the *in vitro* synthesis and comparing them, that cloned cDNA was a faithful representation of the original mRNA template.

ROUGEON, KOURILSKY AND MACH (1975) and ROUGEON AND MACH (1976), also used AMV DNA polymerase, in the prescence of dinucleotidetriphosphates (dNTP s) and the appropriate reaction conditions for the enzyme, to synthesise the first strand of the DNA onto the RNA template.

The mRNA was denatured by adding NaOH to a concentration of 0.3 M and centrifuging the mixture in an alkaline sucrose gradient, followed by ethanol precipitation to separate out the cDNA strands from the mRNA. Two approaches were used for the second strand synthesis, the first being, that the first strand of cDNA, now serving as a template was elongated with a homopolymeric tract. This was replicated using a primer (which anneals to the 3'OH end of the cDNA) with *Escherichia coli* (Klenow fragment) or avian myeloblastosis virus (AMV) DNA polymerase (without Actinomycin D). The *Escherichia coli* polymerase (in contrast to the AMV DNA polymerase) acted most efficiently with oligo (dA) primer (on cDNA-dT).

The second approach involved the exclusion of the primer thus examining the efficiency of the self-priming of both elongated and non-elongated single stranded DNA which was to serve as the primer template. Considerable synthesis was found to take place with both the elongated and non-elongated cDNA with both polymerases. The synthesis occurring with the unelongated strand was attributed to the existance of a well matched 3'OH loop on the first strand cDNA (known as a hairpin loop) which snaps back on denaturation, thus serving as a primer. In both approaches S1 nuclease, which degrades single-stranded DNA as opposed to double-stranded, was used to determine the extent of double-strandedness achieved. The loop was found to be accessible to cleavage by this nuclease.

A similar self-priming reaction was reported by ENGLUND (1971) and GOULIAN, LUCAS AND KORNBERG (1968), using T4 DNA polymerase. In this case, however, the loop was partly mismatched, with the pairing of two internal sequences: one near the 3' terminus and the other further back. The T4 DNA polymerase has exonucleic activity which degrades the unmatched regions before beginning synthesis to form the hairpin loop.

EFSTRATIADIS, KAFATOS, MAXAM AND MANIATIS (1976) were able to synthesise fully representative double stranded cDNA. This was done by the sequential actions of reverse transcriptase, DNA polymerase 1 and S1 nuclease. The mRNA was freed from the first strand by incubation with 0.3 M sodium hydroxide at 37°C for 12 to 16 hours. The material was further purified by extraction in phenol and chloroform followed by passage through a G150 Sephadex column. RNAses H, A and T1 were also used successfully to degrade completely both free and bound RNA. The first strand once purified, was precipitated in 0.1 M sodium acetate and ethanol. The hairpin loop was exploited as a primer and was subsequently cut successfully with the S1 nuclease, thus creating an "open gene". The double stranded cDNA was purified and precipitated in a similar manner to the single-stranded cDNA.

HIGUCHI, PADDOCK, WALL AND SALSER (1976) applied a similar method *in vitro* using AMV DNA polymerase for the first strand synthesis followed by 0.3 M NaOH treatment at 90°C, thus removing the cDNA from the RNA.

The cDNA was then isolated using phenol extraction and a Sephadex G-100 column. Second strand synthesis involved no primer and made use of DNA Polymerase I (Pol I). The double stranded molecule synthesised *in vitro* was successfully cloned.

RABBITTS (1976) used a very different method of DNA synthesis while achieving cloning into the plasmid at the same time. This was achieved by cleaving a plasmid vector (mColE1) enzymatically and tailing this with poly (dT) with terminal transferase, to produce a tail of greatly varying length. This tail was used as a primer for first strand synthesis using the mRNA as a template. A further poly (dT) extension was made on the cDNA joined to the plasmid. These were mixed with plasmids which had been tailed with poly (dA) and the mixture denatured and annealed. The second strand of the cDNA was produced *in vivo* by a repair mechanism of the plasmid.

These techniques for double-stranded cDNA synthesis, all have the difficulty of producing few full-length cDNA s corresponding to the complete mRNA sequence. Incomplete cDNA sequences can still be useful as hybridisation probes but cannot direct the synthesis of complete proteins after being cloned into expression vectors (OKAYAMA AND BERG, 1982).

OKAYAMA AND BERG (1982) proposed a method, similar to that of RABBITTS (1976) for the production of full length DNA, while simultaneously achieving cloning. The step causing the greatest degree of loss of clones per microgram of mRNA, and also often loss of important sequences from the 5' end of the clone, is the S1 mediated cleavage of the hairpin loop. OKAYAMA AND BERG (1982) were able to circumvent this step by using a specially designed plasmid-vector primer. The mRNA was transcribed into the first strand of cDNA using a T-tailed cloning vector as a primer. A linker fragment (excised from another plasmid) was then annealed to the recombinant molecule followed by ligation to effect circularization of the molecule. The synthesis of the second strand of DNA was achieved by replacing the RNA in the hybrids with DNA using RNase H, DNA polymerase I and DNA ligase, before introducing into the *Escherichia coli* host.

Other methods which achieved improved yields of full length cDNA, or at least cDNA s with intact 5'-proximal ends, include enriching for a particular mRNA sequence before cDNA synthesis, or fractionation of cDNA to enrich for particular size classes (CHANG, NUNBERG, KAUFMAN, EHRLICH, SCHIMKE AND COHEN, 1978), or alternative priming procedures to eliminate S1 nuclease digestion (LAND, GREY, HANSER, LINDERMAIER AND SCHUTZ, 1981).

GUBLER AND HOFFMAN (1983) made a modification to the method of OKAYAMA AND BERG (1982). This modified method combines the classical oligo(dT)-primed first-strand synthesis with the novel RNase H/DNA polymerase I-mediated second strand synthesis, while excluding the elaborate vector primer system and also eliminating the need for S1 nuclease digestion, or sizing (GUBLER AND HOFFMAN, 1983). Reverse transcriptase is used for the oligo(dT)-primed synthesis of the first strand of cDNA from mRNA, with the reaction being stopped with EDTA. As described by OKAYAMA AND BERG (1982) the product was extracted with phenol and precipitated with ethanol out of 2 M ammonium acetate. RNase H was then used to remove the RNA from the RNA / DNA strand. Second strand synthesis was achieved with DNA polymerase I and *Escherichia coli* DNA ligase (to seal any nicks). The products were then extracted twice more with phenol before precipitation out of 2 M ammonium acetate. The cDNA at this stage was ready for tailing and cloning. The use of DNA ligase in the second strand synthesis was, however, found to be unnecessary by GUBLER AND HOFFMAN (1983).

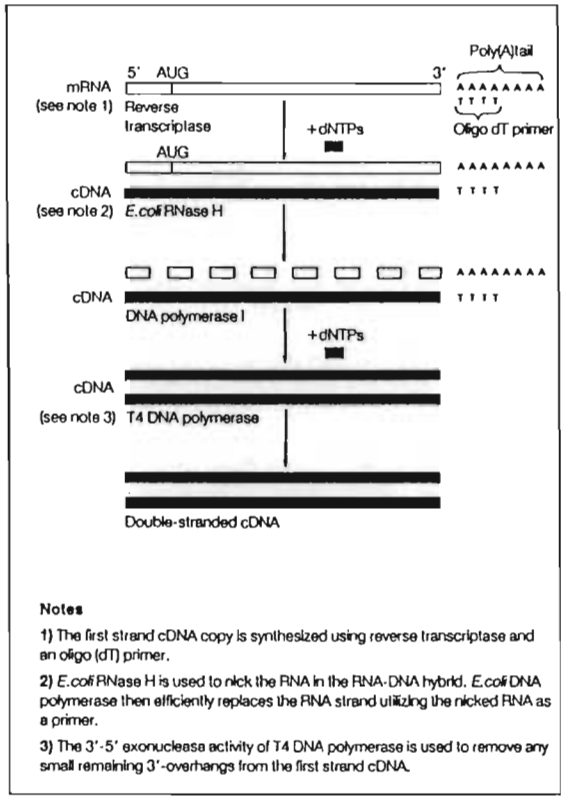
The procedure of GUBLER AND HOFFMAN (1983) was further modified by SARTORIS, COHEN AND LEE (1987) to obtain better yields. This was achieved by using Moloney Murine Leukaemia Virus (Mo-MuLV) reverse transcriptase (as opposed to AMV reverse transcriptase) in the absence of Actinomycin D for synthesis of the first strand. Actinomycin D, though it inhibits the formation of the hairpin loop at the 3' end, also inhibits RNA dependent DNA polymerase activity to a large extent, decreasing cDNA yields.

The second strand was then synthesised directly without any preceding purification step, followed by size fractionation. To insert the cDNA into the vector, the procedure made use of oligodeoxynucleotide adaptors (Eco RI adaptors with one blunt end and one "sticky" end), thus avoiding the need for methylation and subsequent digestion with restriction endonucleases.

One of the more recent methods of generating libraries from mRNA makes use of a technique known as the polymerase chain reaction (PCR), incorporating the Mo-MuLV reverse transcriptase for cDNA synthesis (EHRLICH, 1987). The PCR technique will be further discussed in Chapter 4.

3.2 MATERIALS AND METHODS

Synthesis of cDNA was achieved using the optimised, and guaranteed ribonuclease free "cDNA Synthesis System Plus" developed by and obtained from Amersham [a modification of the methods described by OKAYAMA AND BERG (1982) and GUBLER AND HOFFMAN (1983)]. A schematic representation of the synthesis procedure follows (Figure 3.1.).



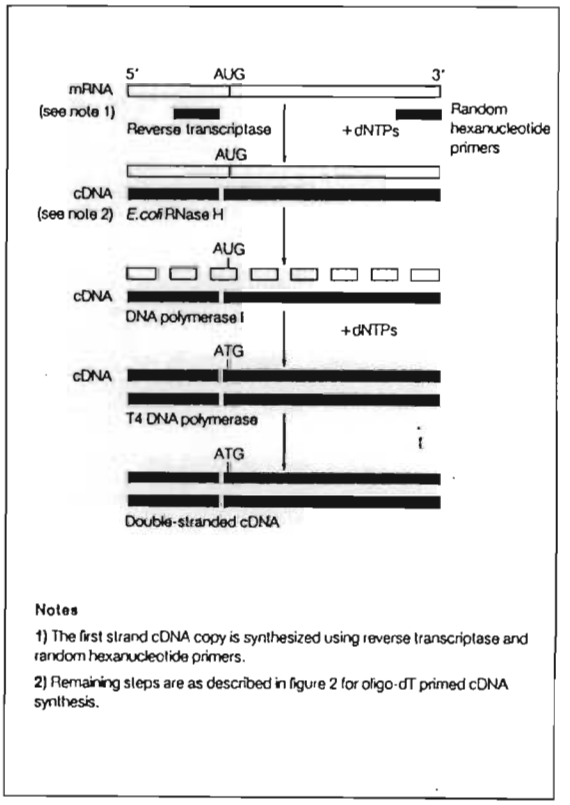


Figure 3.1. Schematic representation of the synthesis of cDNA from mRNA in the "cDNA Synthesis System Plus" developed by Amersham, showing synthesis using oligonucleotide primers or alternatively random hexanucleotide primers respectively (AMERSHAM, 1989).

A separate synthesis reaction was set up initially to monitor the efficiency of the cDNA synthesis reaction with the carnation mRNA as compared to a standard of high quality rabbit globin poly $(A)^+$ mRNA and to determine if any modifications (as recommended by Amersham) in the technique needed to be made. Such modifications might include heat denaturation of mRNA to destroy secondary structure formation within the mRNA which would hinder oligo dT priming of first strand synthesis, using random primers in combination with or in preference to oligo dT primer, or optimising the protocol further for specificity for the carnation mRNA used as substrate for the reverse transcriptase, or reisolating new mRNA in the case of the synthesis failing due to poor quality mRNA. The separate control reactions involved the inclusion of labelled [α-^{32}P]dCTP. This isotope has a short half-life of 14 days and since decay of the isotope would cause disintergration of the cDNA, this necessitates the use of a separate labelling reaction distinct from the actual synthesis.

A duplicate first strand synthesis of mRNA arising from the six carnation component extracts and the control, was set up. One set of samples was terminated and analysed after synthesis of the first strand, while the other set was subjected to second strand synthesis before analysis for [α-^{32}P]dCTP incorporation into the synthesised nucleic acid. All work involving the radioactivity was done behind a 2 cm thick perspex screen, with tubes containing radioactive material confined in a perspex box while being transported.

The first step of the synthesis involved the priming of the first strand using oligo dT or random primers and then initiation of synthesis using reverse transcriptase. The oligo dT primer was used in preference to the random primers since full length cDNA was the most desirable criterion for the purposes of the present study. This is another reason why it is essential to ensure that no degradation of the mRNA occurred, which would possibly result in the loss of the poly A tail to which the oligo dT primer binds.

For the reaction to synthesise cDNA optimally, it is essential that the mRNA, enzymes and buffers are in the correct ratios. The concentration of mRNA was determined spectrophotometrically at 260 *nm* (Chapter 2) thus allowing the withdrawal of a volume containing 3 μg for use in a final first strand reaction volume (including reagents and enzymes) of 30 μl. A globin mRNA standard was used in a separate reaction for comparison of cDNA synthesis efficiency. Water baths were set at 42°C and 12°C. The non-enzymic components were removed from the freezer, allowed to thaw and placed on ice. The enzymes were allowed to remain at 20°C until just before use, to retain their full activity. The substrate mRNA was then thawed and also placed on ice. A microcentrifuge tube for each of the different mRNA samples including the standard was set on ice. The synthesis components were then added with a micropipette in the following order: first strand synthesis buffer (5X), (6 μl) [250 mM Tris HCl, pH 8.3, 250 mM KCl, 40 mM $MgCl_2$ and 25 mM dithiothreitol (DTT)], sodium pyrophosphate solution (1.5 μl), human placental ribonuclease inhibitor (1.5 μl), deoxynucleoside triphosphate (each at a concentration of 20 mM) mix (3 μl), oligo dT primer, [α-^{32}P] dCTP, mRNA (3 μg) and sufficient water to make the reaction mix up to a final volume of 30 μl.

The contents of the tubes were mixed gently and then centrifuged for a few seconds in the microcentrifuge before adding 60 units (3 μl) of reverse transcriptase enzyme to each of the tubes. Incubation at 42°C for one hour (a minimum of 40 minutes) was followed by placing the samples on ice. The synthesis of the first strand was now complete. The duplicate set of tubes was retained at this stage for radioactive analysis of the efficiency of synthesis of the first strand.

The remaining set of first strand synthesis mix was left on ice and the second strand synthesis components were then added to it. The second strand synthesis reaction buffer (56.1 μl) (200 mM Tris HCl, pH 7.5, 50 mM $MgCl_2$, 1 M KCl and 10 mM BSA), was added first, followed by the [α-^{32}P]dCTP, 2.4 units ribonuclease H (3 μl), 69 units *Escherichia coli* DNA polymerase 1 (20 μl), in that specific order. Enough water was then added to give a final reaction volume of 150 μl.

The second strand reaction mix was gently shaken and then incubated sequentially at 12°C for 60 minutes and then at 22°C for 60 minutes. This was followed by incubation at 70°C for 10 minutes. The tubes were then centrifuged in the microcentrifuge for a few minutes and placed on ice. T4 DNA polymerase (6 units, 1.5 μl) was added to each of the tubes. The tube contents were then mixed gently and incubated at 37°C for 10 minutes. The reaction was stopped by adding 6 μl of 0.25 M EDTA pH 8.

The method used for analysis of percentage radioactivity incorporation was carried out as follows: An aliquot (2 μl) of each reaction mixture (from the first and second strand syntheses) was transferred to a tube containing 20 μl of water and mixed thoroughly. A known volume (2 μl) of this dilution was spotted onto the centre of each of two 2.4 cm discs of Whatman DE 81 paper, one designated A and the other B. Filter disc A served to measure total radioactivity in the sample, while filter disc B retained only the radioactivity incorporated into the nucleic acid. This was achieved by washing filter disc B extensively. This involved six washes in 0.5 M Na_2HPO_4, 5 minutes per wash, followed by 2 washes in water and then two washes in 95 % ethanol (each of these being one minute long). Filter disc A was left unwashed.

The filters were dried thoroughly and then placed in plastic scintillation vials with 4 ml aqueous scintillation fluid. Counts were measured in a 3800 Liquid Scintillation Counter. The percentage of input radioactivity incorporated into the DNA and thus mass of cDNA synthesised in the reactions was calculated as follows:

Percentage

incorporation = (cpm on filter B/ cpm on filter A) x 100

Yield of cDNA was calculated as follows:

Percentage of labelled [-^{32}P]dCTP incorporated = X %

Amount of unlabelled dCTP in (30 ul) first strand and (150 μl) second strand reaction mix = 15 *n*moles

Therefore assume amount of unlabelled dCTP incorporated = (X % of 15 *n*moles)

Residue molecular weight dNMP (1 M) = 350 g

Total amount of dNTP s incorporated = 4(X % x 15)

Molecular weight dCTP = 350

Therefore the % weight of cDNA synthesised = 4 x 350(X % x 15)ng

= 210Xng

weight of input RNA = 3 μg

Therefore yield of cDNA (% mRNA transcribed) = 210X/3 x10^3 x 100%

= 7X

3.3 RESULTS AND DISCUSSION

The efficiency of first strand synthesis of cDNA was determined through measurement of the counts per minute by a scintillation count of the filter papers A and B. These values were used for the calculation of percentage incorporation of radioactive dCTP and thus yield of cDNA.

Table 3.1. Percentage incorporation of dNTP s, amount of cDNA synthesised and percentage yield of cDNA generated during first strand synthesis from 3μg of mRNA from each floral organ of the carnation as compared to a control of globin mRNA.

ORGAN Presenescent = 1 Senescent = 2	% INCORPORATION	cDNA SYNTHESISED (ng)	% YIELD cDNA
Petal 1	1.88	394.80	13.16
Receptacle 1	0.83	173.04	5.77
Ovary 1	0.40	83.58	2.79
Petal 2	0.54	114.24	3.81
Receptacle 2	1.88	395.43	13.18
Ovary 2	0.35	73.92	2.46
CONTROL	3.94	827.40	27.58

It would be expected that a similar percentage of cDNA would have been transcribed from the source mRNA used from each of the floral parts. It can be seen from the yield (27.6 %) of first strand cDNA obtained for the control (rabbit globin poly(A)$^+$ mRNA) that the reaction was occurring at optimal efficiency, since the predicted percentage of mRNA transcribed into first strand cDNA was 15 to 30 %. The transcriptions involving mRNA from the floral organs, however, were not as efficient, with only the presenescent petal mRNA and senescing receptacle mRNA producing nearly the minimum desired 15 % first strand transcription, the other organs falling far short of this efficiency. According to MANIATIS, FRITSCH AND SAMBROOK (1982), individual species of mRNA may be reverse transcribed with differing efficiencies.

Factors, according to guidelines set out in the "cDNA Synthesis Plus" system, which may influence this efficiency are firstly the purity and quality of the mRNA used. The RNA isolated, particularly in the case of the ovaries and petals was heavily contaminated with a glutinous protein or carbohydrate. It was to be expected, however, that this contamination would have been removed by the column isolation of the mRNA. If this contamination had remained, it may have caused an overestimation of the amount of mRNA used in the reaction or it may have interfered with the mechanism of the reaction. The level of secondary structure in the mRNA may also have an effect on the efficiency of transcription . The length of incubation and enzyme to mRNA ratio used for the first strand synthesis, though recommended for most mRNA s, may not have been optimal for the mRNA isolated from the carnation.

To optimize the reaction incubation time, a set of time course reactions could have been executed, while different enzyme : substrate ratios may have been experimented with.This however would have required the use of further mRNA and enzymes, both of which were in limited quantities.

The amount of second strand cDNA was determined in a similar fashion to the determination amount of first strand cDNA synthesised determination. The scintillation counts on filter papers C and D (filter C being unwashed ie. total radioactivity and D being washed ie. incorporated radioactivity) were measured and percentage incorporation measured by (cpm on filter C/ cpm on filter D) x 100.

The yield percentage of second strand cDNA transcribed from the first strand cDNA is calculated by (amount of second strand cDNA/ amount of first strand cDNA) x 100. The results of the second strand reaction are depicted in Table 3.2.

TABLE 3.2. Percentage yield of second strand synthesis from the product of first strand synthesis.

ORGAN Presenescent = 1 Senescent = 2	% INCORPORATION	cDNA SYNTHESISED (ng)	% YIELD cDNA
Petal 1	1.55	325.92	82.50
Receptacle 1	0.43	89.88	51.94
Ovary 1	0.34	72.15	86.40
Petal 2	0.47	98.36	86.10
Receptacle 2	1.78	374.43	94.69
Ovary 2	0.34	71.82	97.16
CONTROL	3.24	679.56	82.13

The percentage of first strand cDNA transcribed into second strand cDNA is expected to be more than 90 %. As can be seen from Table 3.2, some of the final yield percentages of double strand cDNA were high (above 90 %) such as was achieved from senescing receptacle and ovary first strand cDNA. Second strand synthesis yields from presenescing petal and ovary first strand cDNA, as well as from the control globin and senescing petal first strand cDNA, were not much lower than the expected 90 % level (above 80 %).

The yield percentage of second strand cDNA of presenescing receptacle was however considerably lower than the expected 90 % (50-60 %). This may have been the result of faulty reproduction of the conditions or reagent ratios required for optimal efficiency of the second strand reaction.

The cDNA was subjected to further purification and size fractionation (Chapter 4) in preparation for cloning into a vector, before being examined electrophoretically.

CHAPTER 4

cDNA LIBRARY CONSTRUCTION

4.1 INTRODUCTION

The development of recombinant DNA techniques permitting clonal replication of eucaryotic DNA segments in bacteria brought a revolutionary change in approach to genetic research. This set of techniques allows a DNA fragment containing the gene of interest to be replicated as a clone in a bacterium thus allowing isolation of eucaryotic genes. The first clones (mammalian) to be isolated in this way were complementary copies of mRNA s (ROUGEON AND MACH, 1976; BLATTNER, WILLIAMS, BLECHL, DENNISTON-THOMPSON, FABER, FURLONG, GRUNWALD, KIEFER, MOORE, SCHUMM, SHELDON AND SMITHIES, 1977). Later clones of purified genomic sequences were also made (TILGHMAN, TIEMEIER, POLSKY, EDGELL, SEIDMAN, LEDER, ENQUIST, NORMAN AND LEDER, 1977).

Cloning may be applied also to unfractionated DNA but it is necessary to recognize the DNA fragment of interest after it has been cloned (BLATTNER, WILLIAMS, BLECHL, DENNISTON-THOMPSON, FABER, FURLONG, GRUNWALD, KIEFER, MOORE, SCHUMM, SHELDON AND SMITHIES, 1977).

One of the most important elements in gene cloning, is the vector. The vector, in combination with the foreign molecule to be cloned becomes the recombinant DNA molecule, which can be amplified by replication of the vector in suitable host cells. To perform this task, the vector must possess the following properties :

It must be able to replicate in the host cell (through a replicon interacting with host enzymes engaged in the initiation of DNA replication). There should be a some genetic information that can be used as a genetic marker to allow the development of a selection system for recombinant versus non recombinant molecules eg. antibiotic resistance in plasmids. It should also have unique cleavage sites for as many enzymes as possible into which foreign DNA may be inserted. These sites must lie outside essential genes such as the origin of replication, but should lie within a marker gene involved in the selection of recombinant molecules so that insertion of foreign DNA will lead to marker inactivation. If expression of the cloned DNA is required, vector DNA should also contain controlling elements such as promoters and ribosome binding sites (WINNACKER, 1987).

A vector is thus needed for the purposes of replicating and storing the cDNA generated from the mRNA (whose production is specific for active genes) from the climacteric and preclimacteric carnations (WINNACKER,1987).

The bacteriophage lambda, a double stranded, DNA virus of *Escherichia coli* has long been the favourite subject for geneticists and has been extensively studied with its structure and characteristics clearly elucidated. The linear genome of lambda is packaged in an icosahedral head with a tail fibre. The tip of this tail fibre adsorbs to receptor sites on the outer membrane of the host during infection. The receptors coded by the *Escherichia coli lam B* gene, are also required, for maltose uptake. A growth medium with maltose and lacking glucose will thus stimulate their production (WINNACKER, 1987). The chromosome of phage λ is 48.6 kb and is arranged in a linear duplex with complementary stranded ends 12 nucleotides in length (known as cohesive ends or *cos* sites) (SCHULER AND ZIELINSKI, 1989). In the early stages of infection, the phage can select one of two paths of replication and as such is known as a temperate phage (SILHAVY, BERMAN AND ENQUIST, 1984). One path is that of lytic growth in which the circular phage DNA is extensively replicated and translated to form phage proteins. These proteins are used by the newly replicated phage DNA to form encapsidated progeny phage. The host bacterium lyses, releasing a large number of infectious phage particles. The alternate path is that of lysogen replication. In this case, the phage DNA becomes integrated into the host DNA, forming a so-called prophage. The phage DNA is replicated along with the host DNA and as such can be transmitted to other bacteria. The host cell which is called a lysogen, is not damaged (SCHULER AND ZIELINSKY, 1989).

If the prophage is not stably integrated, ie. if a functional *int* gene is absent, it will be lost after a few replications. The lysogenic state is retained through the continual production by the *cI* gene of the prophage of λ repressor which binds to the left and right λ operators. The repressor may be inactivated by the process of induction causing the uncovering of the operators and the start of the lytic cycle by the transcription of *Escherichia coli* RNA polymerase. Other phage genes involved in the establishment of the lysogenic state include *cII and cIII. The cII* protein activates the transcription of *cI* while repressing other lytic genes, while *cIII protein* works with *cII* to inhibit a host protease (coded by *HfI* or high frequency of lysogenation gene) that normally destroys the *cII* protein (SILHAVY, BERMAN AND ENQUIST, 1984). Phage λ thus produces turbid plaques in a lawn of host bacteria, with the cells growing in the centre of the plaques (causing the turbidity) being lysogenic. There are also clear plaque mutants of the λ, with mutations affecting functions involved in the maintenance or establishment of the lysogenic state. One example of a mutation in λ that is useful, is a *cI* mutation called *cIts857.* This mutation creates a temperature sensitive repressor. At a low temperature (30°C) this mutant produces a turbid plaque (ie. lysogenic state) while at 37°C to 42°C induction occurs to produce clear plaques ie. lytic state.

Not all *Escherichia coli* strains can support λ growth. Some are unable to adsorb the phage and are called λ resistant. These are distinguished from strains that are unable to both adsorb and support λ development. Phage λ requires the *lam B* gene (*lam B* protein) produced for adsorption (SILHAVY, BERMAN AND ENQUIST, 1984). Mutant bacteria that do not express the *Hfl* protein force the phage into the lysogenic pathway (WINNACKER, 1987). Cell lysis and plaque formation in *Hfl*⁻ strains of bacteria is only observed when the *cI* repressor is inactivated eg. by mutation or insertion of recombinant DNA (WINNACKER, 1987).

Manipulations of the λ phage *in vitro* and *in vivo,* have resulted in the production of a large variety of different phage vectors for use in cloning (WINNACKER, 1987). The usefulness of the λ phage as a cloning vector arises from the fact that the central portion or "stuffer fragment" (20 to 25 kb, approximately 40% of the genome) is unessential for replication and lytic propagation (SCHULER AND ZIELINSKY, 1989). The "stuffer fragment" is bordered by right and left phage arms which are essential for replication and head and tail assembly of proteins (SCHULER AND ZIELINSKY, 1989). The "stuffer fragment" can therefore be replaced by foreign DNA , causing these vectors to be known as replacement vectors (WINNACKER, 1987). To facilitate cloning, several phage vectors have been constructed by standard mutant selection techniques, which have the "stuffer" region bordered by restriction enzyme sites. Restriction sites on the phage arms have also been removed by mutation to simplify cloning (SCHULER AND ZIELINSKY, 1989).

Two lambda vectors that are suitable for cloning cDNA s are λgt10 and λgt11 and λZAP. Phage libraries cloned in λgt10 are useful for screening with nucleic acid probes. λgt11 is capable of translation of the cloned DNA fragment to produce a polypeptide, and thus is known as an expression vector. The polypeptide may be screened with antibody probes (YOUNG AND DAVIS, 1983). For the purposes of the present study the use of an expression vector would not be necessary since all the proteins involved in senescence are not known or isolated for the purposes of antibody production. The phage λZAP has an extra feature in that it provides colour selection for desired clones.

The phage λgt10 (*imm434 b527)* (Figure 4.1.) is a *cI+* phage and as such forms a turbid plaque. It contains a single *Eco* RI cleavage site within the phage repressor gene. It can accept DNA insert fragments of up to 7.6 kb pairs. When a DNA fragment is inserted into the repressor site gene (*cI)* at the *Eco* RI site a recombinant *cI-* phage is formed which as such produces plaques with a clear centre. Phage λgt10 is thus a member of a family of immunity insertion vectors developed by MURRAY, BRAMMAR AND MURRAY (1977). These were developed to use the turbid / clear plaque differentiation as a method of detecting recombinant phage (MURRAY, BRAMMAR AND MURRAY, 1977). The phage λgt10 was developed to provide a vigorously growing vector which was able to take insert DNA a few base pairs shorter than previous immunity insertion vectors and is thus itself a bit longer than other vectors. This is required since the popular method of *in vitro* packaging often favours the packaging of wild type length molecules rather than shorter molecules. Using λgt10 rather than the other immunity insertion vectors thus may increase the recovery of short molecules 10 fold (HUYNH, YOUNG AND DAVIS, 1985).

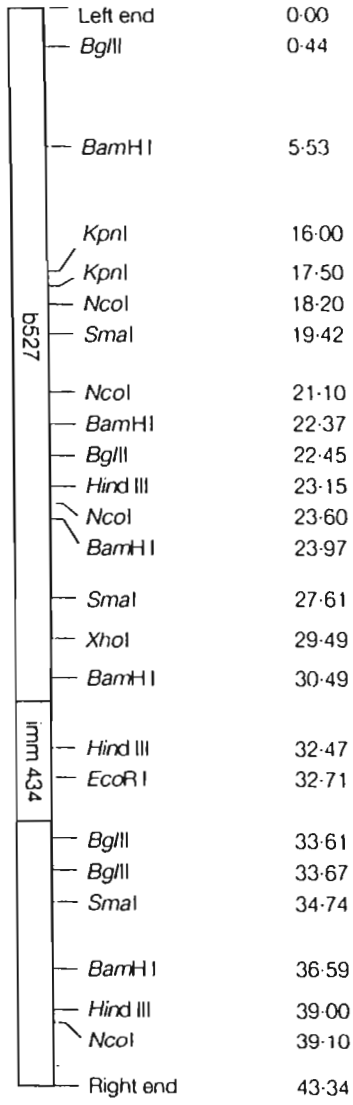


Figure 4.1. Map of λgt 10 showing restriction sites marked with distances in kilobase pairs from the left end. The *imm 434* substitution replaces DNA sequences between 72.9 and 79.3 % of the phage, while the *b527* deletion removes DNA sequences between 49.1 and 57.4 % of the phage (AMERSHAM cDNA CLONING SYSTEM, 1989)

One disadvantage of λgt10 is that it does not require an insert to be packaged. Selection against non-recombinant phage (*cI*+) can be done during amplification of the phage library. This is done by using the *Escherichia coli* strain carrying the high frequency of mutation (*Hfl*+) as a host for phage infection. This effectively represses cI^{+} phage and plaque formation by producing a totally lysogenic bacterium. The cI^{-} phage form plaques on this strain with normal efficiency. Similar results may be obtained by using an *Escherichia coli* host carrying the mutation *lyc 7* which is probably an allele of the *hfl* locus (LATHE AND LECOCQ, 1977; SCHERER, TELFORD, BALDARI AND PIROTTA, 1981). A current method of cloning (Amersham cDNA cloning system) thus provides two *Escherichia coli* strains ie. L87 and NM514. Strain NM514 carries the *lyc B7* mutation or Hfl^{+} genotype responsible for the selection process against non-recombinant phage. The L87 strain is a wild type *Escherichia coli* strain on which recombinant phage will produce clear plaques and non- recombinant, turbid plaques, giving an estimate of non-recombinant background levels.

The use of phage arms lacking 5' phosphate groups reduces this non-recombinant background, since self ligation of the genes is prevented in cases where no insert DNA is present (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989)

Cloning into λgt10 requires the cleavage of the vector at the *Eco* RI site and preparation of cDNA by blunt-ending and the addition of *Eco* RI linkers for insertion into the phage vector arms. Certain methods require the treatment of double-stranded cDNA s with *Eco* RI methylase and S-adenosyl-methionine to methylate and protect *Eco* RI cleavage sites within the cDNA s from subsequent digestion with the *Eco* RI enzyme. A brief DNA Pol I treatment is used to increase the number of flush-ended ds cDNA molecules (SEEBURG, SHINE, MARTIAL, BAXTER AND GOODMAN 1977). *Eco*RI kinased linkers are then ligated onto the ends and the long concatamers of excess linkers that are formed, are removed from the ends by digestion with *Eco*RI (HAYMERLE, HERZ, BRESSAN, FRANK AND STANLEY, 1986; HUYNH, YOUNG AND DAVIS, 1985). A modification which eliminates the use of *Eco*RI methylase, phosphorylated linkers or *Eco*RI unphosphorylated oligonucleotides as adaptors (HAYMERLE, HERZ, BRESSAN, FRANK AND STANLEY, 1986; STOVER, VODKIN AND OAKS, 1987).

During the ligation reaction of the adaptor to the cDNA, only one strand of the adaptor forms a covalent bond using the 5' phosphate from the cDNA or vector, the other strand of the adaptor remains attached only by non-covalent Watson-Crick base pairing. After removing the non-covalently bonded pieces, the vector and cDNA are left with long, complementary single-stranded extensions which anneal readily. The adapted cDNA is then kinased and ligated to the vector. Efficiency of the ligation of the adapted cDNA into the vector can be monitored by taking samples before and after the ligation and electrophoresing them on a 1.0 % agarose gel to detect the apparent molecular weight changes that should occur during ligation (HAYMERLE, HERZ, BRESSAN, FRANK AND STANLEY, 1986).

Problems in ligation usually occur during ligation of the adaptor onto the cDNA, through insufficiently polished ends or bad preparation of adaptors (HAYMERLE, HERZ, BRESSAN, FRANK AND STANLEY, 1986).

Before ligation into the vector, it is necessary to remove excess linkers or adaptors which would otherwise interfere with the ligations, and to achieve size selection fractionation (HUYNH, YOUNG AND DAVIS, 1985). This is done on a molecular exchange column containing e.g. Bio-Gel A-50m. Fractions of the desired size range for cloning (e.g. > 500 bp) are then chosen, pooled and ligated to the vector using T4 DNA ligase. This enzyme, in the presence of ATP is the only ligation enzyme able to join blunt-ended duplex restriction fragments. It is also able to ligate cohesive ends and can catalyse the repair of single-stranded nicks in duplex DNA (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989). This is followed by *in vitro* packaging of the phage DNA.

In vitro packaging is a method allowing an efficient reintroduction of the phage DNA containing the cloned fragments into the host bacteria by packaging the naked DNA into empty phage heads. The phage DNA may be reintroduced into the host bacteria without packaging using the standard $CaCl_2$ technique which causes the host bacterium to become permeable to entry of the phage, however this method yields at most 10^4 to 10^8 plaques per microgram. The packaging method can yield more than 3×10^8 plaque forming units per microgram of DNA in the presence of suitable maturation factors, this infectivity being hardly distinguishable from that of normal phage particles (AMERSHAM cDNA CLONING SYSTEM, 1989).

The phage capsid is composed of structural proteins encoded by genes. Empty phage heads, packaging factors and phage tails required for the packaging process can be derived from two lysates, each of which is obtained using a phage strain with a genetic defect affecting a different step of the morphogenesis. The strains used carry the temperature sensitive repressor *imm434cIts* so producing temperature sensitive lysogens. A stable lysogenic state can thus be maintained at 32°C, while induction of lytic growth involving the synthesis of the desired structural proteins occurs by a temperature increase to 40°C. To allow accumulation of the packaging proteins by preventing their early release, both strains are mutated in the S gene (amber mutation *Sam 7*) whose products are required for the lysis of bacterial cell walls. One of the lysogens contains an amber mutation in gene D (*Dam*). Heat induction of this lysogen will thus lead to an accumulation of empty prehead particles, since the D protein (decoration protein) is located on the outside of mature phage particles and participates in the maturation of head structures and the threading of DNA into the phage heads. The other lysogen provides for the protein D for the packaging reaction but instead carries a mutation in its E gene. The E protein is a major structural protein and is required for an early phase of phage assembly. Lack of this gene product allows the accumulation of all the components of the head structure in a free form without assembly. When DNA is required to be packaged, the two complementary lysates are mixed in the prescence of exogenous ATP, biogenic amines and the DNA, resulting in the generation of mature phage heads containing the DNA (HOHN, 1979).

Lambda DNA containing no inserts, may yield up to 10^7 to 10^8 plaques per microgram of DNA. Hybridized DNA is less efficient, yielding 1 to 5 x 10^8 plaques per microgram of DNA. Exogenous DNA from the phage lysates, however, may also yield a background of 10^2 to 10^3 plaques. This may be avoided by pretreating the packaging lysates with UV light while still retaining enzymic packaging activity.

This level of background is tested by using appropriate controls to check the actual level of successful packaging of hybridized DNA (WINNACKER, 1989). A small sample of the resulting packaged phage particles is plated out on the appropriate *E. coli* hosts and plaques assessed and screened.

To circumvent many of the problems encountered during the isolation of desired gene sequences which are often in rare abundance, the cDNA from each of the libraries can be amplified very efficiently, starting with far less cDNA, without using a vector, by a more recent technique known as the polymerase chain reaction (PCR) (Figure 4.2.). The amplification is carried out using an *in vitro* enzymatic reaction by a DNA polymerase, appropriate deoxyribonucleotide triphosphates (dNTP s), single-stranded oligonucleotide primers (amplimers), a buffer and salts. The primers (20 to 30 bases in length) are added in great excess, relative to the DNA to be amplified. They hybridize to the single stranded DNA with their 3′ ends facing each other. The polymerase is then able to synthesise new strands between the primers. These new strands will in turn, on denaturation and annealing, hybridize to the primers. After the second cycle of denaturation and annealing, the amplification of the the products becomes exponential, since each strand which is synthesised between the primers is complementary to the primer and thus can serve as a template for successive cycles of the PCR.

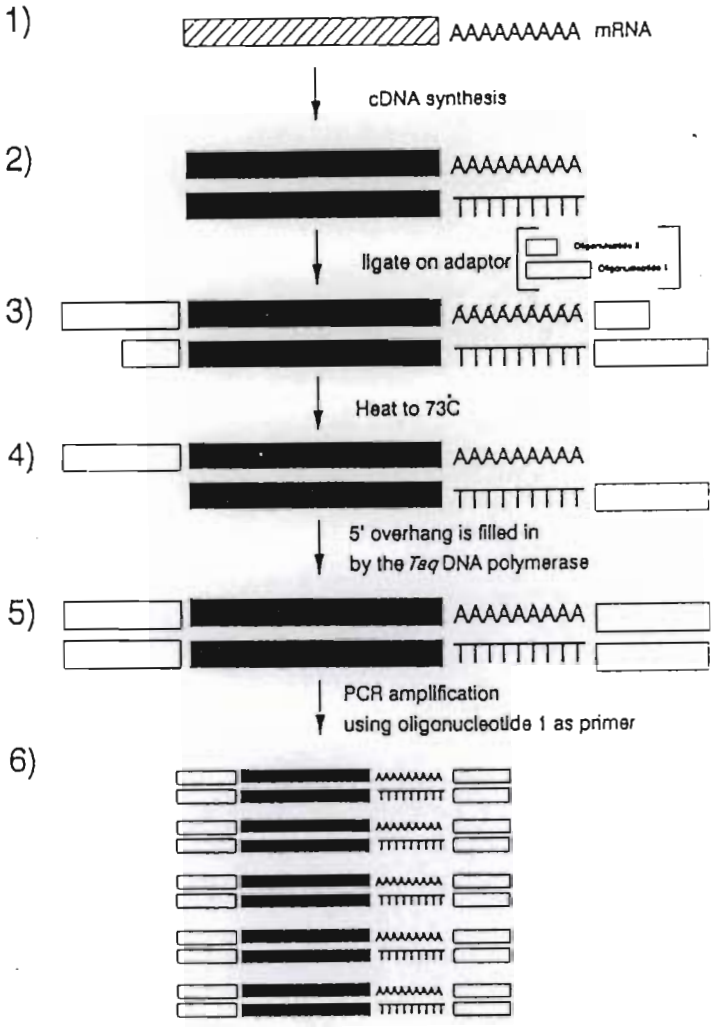


Figure 4.2. Diagram of the main steps in the amplification of cDNA using the PCR protocol (JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS, 1991)

The technique of single-copy gene amplification was developed by MULLIS, FALOONA, SCHARF, SAIKI, HORN AND EHRLICH (1986), using the theory of polymerase chain reactions expounded by KLEPPE, OHTSUKA, KLEPPE, MOLINEUX AND KHORANA (1971). The procedure was tedious, however, since it involved adding a fresh aliquot of the Klenow fragment of *Escherichia coli* since this enzyme becomes inactivated during the denaturation step, as it is unstable at high temperatures. SAIKI, GELFAND, STOFFEL, SCHARF, HIGUCHI, HORN, MULLIS AND EHRLICH (1988) were able to eliminate this problem by utilising the DNA polymerase isolated from the thermostable bacterium *Thermus aquaticus (Taq* DNA polymerase). This polymerase also increased the efficiency of primer-template hybridisation during the reaction, by allowing the use of higher temperatures during annealing and synthesis, thus eliminating mismatched primers and secondary structures and increasing the yield of the desired products.

The selection of the correct primer is an important criterion. It should be able to hybridize to the desired sequence with little hybridization to other sequences present. In many cases the primer is designed to be exactly complementary to the template when the sequence of this is known.

When the sequence of the template is not known, mismatches, which would be unavoidable should preferably occur as far from the 3' end as possible so that extension of the DNA will not be prevented.

The primers should not contain unusual stretches of nucleotide combinations (such as poly-purine/pyrimidine stretches), since this could result in the formation of secondary structures. They should also not be complementary to each other, especially at the 3′ end, to prevent self-annealing of the primers to one another (forming so-called primer-dimers). This would compete with the formation of the desired product (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH and STRUHL, 1989) Examples of amplimers (primers) that would be used in the amplification of a phage λgt 10 library such as was constructed in this study, are as follows:

λ gt 10 5′ primer 5′AGCAAGTTCAGCCTGGTTAAG

λ gt 10 3′ primer 5′TTATGAGTATTTCTTCCAGGG

The sequence on λgt 10 located by the primers would be as follows:

AGC--- Eco RI

I---------><--8BP-->GAATTC

5′ _____ 3′

3′ _____ 5′

<---12BP---><------I

--ATT

(AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989)

Important considerations for the DNA to be amplified is that it should be very pure, containing, in particular, no SDS, sodium acetate, agarose gel components or traces of DEPC (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989).

The PCR technique has been used very successfully by JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS (1991), for the amplification of rare sequences isolated from extremely small amounts of plant tissue. The method involves a total RNA extraction from 50 mg or less of plant tissue. The mRNA is not extracted from total RNA using the poly A tail (as was done in this study) thus preventing the potential loss of material. Instead, cDNA was synthesised from total RNA, relying on the cDNA priming by oligo dT to be specific to mRNA sequences with a poly A tail, thus excluding ribosomal RNA. The resulting cDNA was size selected and then blunt-ended using a Klenow fill-in reaction. The blunt-ended cDNA was ligated to annealed amplification adaptors, oligonucleotides one and two, in preparation for PCR. The sequences of the oligonucleotides used (1 and then 2) are as follows:

5'-ATGCTTAGG**AATTC**CGATTTAGCCTCATA-3'

Eco RI

5'-TATGAGGCTAAA-3'

The PCR system used included a modification suggested by DOMEC, GARBAY, FOURNIER AND BONNET (1990), which involved an increase in the extension time, from one to three minutes.

This was designed to promote the amplification of pieces of DNA longer than 500 base pairs. To further enrich for longer pieces of cDNA, after PCR, the cDNA was again size-fractionated on 1.5 % agarose and electroeluted, with the pools of larger cDNA being submitted to further cycles of PCR (JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS, 1991).

The amplification adaptors were removed from the cDNA by digestion with Eco RI and size-fractionation. The cDNA was then ligated into phage λZAPII arms and packaged (JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS, 1991). A preliminary attempt was made to amplify cDNA, using the PCR method described by these authors.

4.2 MATERIALS AND METHODS

Optimised components, including various control DNA s (as described by HAYMERLE, HERZ, BRESSAN, FRANK AND STANLEY (1986) to monitor the performance of the cloning step were obtained from Amersham, to amplify the cDNA obtained in the previous step. The system is effective for any blunt-ended double stranded cDNA molecule up to 7.6 kb.

The first step of the cloning procedure involves ligation of Eco R1 adaptors to the cDNA to ready it for ligation into a prepared vector (λgt10).

The Eco R1 adaptors were supplied at a concentration of 100 pmoles/μl, and consisted of an annealed mixture of two oligonucleotide species (24 and 20 bases in length).

The adaptor molecules were blunt at one end and carried a four-base overhang at the other. Both adaptor molecules lacked 5' phosphates, a modification which prevents adaptor self ligation. To minimise cDNA self ligation, a high molar ratio of adaptor : cDNA was used. The adaptors were ligated with their blunt ends each joined to the blunt end of the cDNA substrate, resulting in a cDNA molecule with cohesive Eco R1 termini.

To begin the adaptor ligation, the ligase / kinase (L/K) buffer (provided by the supplier along with the enzyme) and sterile water was removed from the freezer. The L/K buffer was warmed to 37°C (in the hand) with periodic shaking for 2-3 minutes to dissolve particulate matter before it was placed on ice, along with the thawed sterile water. The cDNA samples, adaptor mix and the blunt ended control DNA were then removed from the freezer and allowed to thaw on ice. At this stage a 1 μl aliquot of cDNA was removed from each of the samples, diluted to 20 μl with TE buffer(10 mM Tris base, 1 mM EDTA) and then divided into two sub-samples and retained for later analysis.The adaptor ligation reaction was performed in microcentrifuge tubes, one for each sample and one for the control. The cDNA, including the control (1 μg in 10 μl) was transferred to the tube followed by 2 μl of L/K buffer in each reaction.

The Eco-R1 adaptors (2.5 μl) were then added later, to 20 μl. The tube contents were mixed gently and centrifuged for a few seconds to collect the whole reaction in the bottom of the tube. The T4 DNA ligase (5 units, 2 μl) was then added. After shaking the tubes gently, they were placed at 15°C for 20 hours. After this incubation, another 1 μl sample for analysis (sample 3) was removed, and diluted with 10 μl of TE buffer and stored at -20°C at this stage before proceeding with the next step.

The next step in the cloning procedure, is the column purification/ size fractionation of the adapted cDNA. The column purification is performed with a molecular exchange column and achieves the removal of unreacted adaptor molecules which would interfere with the subsequent cloning steps. The column simultaneously achieves size fractionation thus enabling selection of adapted cDNA s of greater than 500 base pairs in length the most desirable size for cloning into λgt10. The fractionation technique was done in the following way. The columns (supplied by Amersham) were removed from storage at 4°C and set up vertically. The top and outlet stoppers were removed and the upper reservoir allowed to empty completely. Each column was then equilibrated by passing 5 ml of TE buffer through it. For the collection of fractions from the column, 25 sterile 1.5 ml microcentrifuge tubes were mumbered and placed in a tube rack below the column. When the reservoir had just become empty, 20 μl of ligated sample was loaded onto the column and allowed to sink in. TE buffer (200 μl) was then loaded and a series of 3 drop (120 μl) fractions were immediately collected in the labelled microcentrifuge tubes. Once the 200 μl volume had soaked into the column completely, the column reservoir was filled with TE buffer and further fractions up to a total of 25 were collected.

It is important that the fractions be collected accurately, since fractions 10 to 17 are pooled as the selected pool while tubes 18 to 25 for the "unselected" pool containing the unwanted adaptors. Aliquots (2.5 %) of the selected pool and unselected pool were taken at this stage as control samples 4 and 5 for gel analysis. The concentration of DNA was determined spectrophotometrically at 260 nm.

The cDNA from the selected and non selected fractions was analysed for size distribution of the DNA on a 1.4 % agarose gel under denaturing conditions (loading buffer: 50 mM NaOH, 1 mM EDTA; running buffer: 300 mM NaOH, 1mM EDTA) and to ensure that the correct fractions had been included in the selected fraction. The DNA was detected by staining with ethidium bromide and viewing on a UV trans illuminator (Plate 4.1., Plate 4.2. and Plate 4.3.).

It is necessary to kinase the adapted, size fractionated cDNA before ligation into dephosphorylated arms to allow covalent insertion. Kinasing involves the conversion of the 5′ hydroxyl groups on the Eco R1 overhangs on the adapted cDNA molecules to the 5′ phosphate groups using the T4 polynucleotide kinase.

The procedure was carried out for each of the samples by mixing, in a sterile microfuge tube 900 μl of the selected pool sample, 100 μl of L/K buffer (thawed as mentioned previously) and 80 units of T4 polynucleotide kinase (10 μl). The tubes were incubated at 37^{o}C for 30 minutes.

It is essential that any remaining kinase be removed once the incubation is complete. This is done by dividing each kinased sample between two sterile microcentrifuge tubes and extracting each twice with an equal volume of phenol:chloroform 1:1, then twice with chloroform:isoamyl alcohol. A butanol extraction step was then used to reduce the volume of the aqueous phase by extracting it into the butanol phase (one volume of butanol removes approximately 0.2 volumes of water). To do this, the aqueous phase was estimated and then 2.2 volumes of butan-1-ol was added to each tube and the contents shaken vigorously. The phases were allowed to separate and the upper butanol layer was removed. The remaining aqueous phase was then pooled and redivided between two 1.5 ml microcentrifuge tubes. The butanol extraction was then repeated as described previously.

The remaining lower aqueous phases were once again combined into one tube. A precipitation of the kinased "adapted" cDNA was effected by adding 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. The tube contents were mixed well (to prevent freezing of the aqueous component and to ensure complete precipitation) and allowed to precipitate at -20°C overnight (minimum of 2 hours). After the precipitation, the tubes were spun in a microcentrifuge for 30 minutes. The supernatant was carefully removed, with care taken not to disturb the pellet of cDNA. To rinse the pellet, 0.5 ml of ice cold 70 % ethanol was added to each sample pellet and gently vortexed. The cDNA pellet was dried under vacuum in a vacuum dessicator to remove all droplets of ethanol. Care was taken not to overdry since this would make the pellet difficult to redissolve. The cDNA pellets were each redissolved in 10 μl of water. The concentration was determined spectrophotometrically and the volume adjusted to achieve a final concentration of 20 ng/ μl.

At this stage in the procedure, the cDNA molecules have Eco R1 ends ready to ligate with the λgt10 vector arms.

The optimal amount of cDNA to ligate into the λgt10 arms for most mRNA species lies between 25 and 120 ng. A test amount of 25 ng of each sample of cDNA was used in the ligation reactions, and the reactions set up in tubes on ice. Lambda gt10 arms (2 μl, 1 μg) followed by 1 μl L/K buffer and 6 μl water were then added. The tube contents were mixed gently and the tubes then spun for a few seconds in the microcentifuge to bring any droplets adhering to the sides of the tube down. To ligate the cDNA to the λgt10 arms, 21 μl T4 DNA ligase (2.5 units) was added followed by gentle mixing. The tubes were then incubated at 15°C for 16 to 20 hours in a water bath. The reaction tubes were again spun briefly in a microcentrifuge to collect the ligation mix. The ligation mixes were now ready for packaging into coat proteins which form the λgt10 capsid.

Simultaneously with the ligation of the the cDNA from the carnation flower components to the λgt10 arms, several control reactions were carried out to monitor the progress of the procedure and provide additional information necessary to analyse the results. These controls include firstly the substitution of the insert cDNA with 5 μl whole λgt10 vector DNA (0.5 μg) with no λgt10 arms. This control is to monitor the overall efficiency of the *in vitro* packaging reactions and biological selection between the two host cell types. The second control involves the omission of insert DNA of any kind. This is to monitor the background plating efficiency on both host cell strains.

The third control involved the use of 2 μl Eco R1 ended control DNA (100 ng) as an insert.This monitors the efficiency of the ligation reaction and the level of stimulation possible above background with the insert present. The performance of the whole cloning system is monitored by the use, as an insert, of the adapted blunt end DNA control fragments originally from the control DNA which had been carried through all the preliminary procedures, along with the carnation cDNA steps.

The plating procedure carried out to determine the titer of the phage is performed in the following manner. The appropriate number of microcentrifuge tubes were set out and water baths prepared at 37°C and 45°C. A 10^2 dilution of the phage was produced by adding 30 μl of the final packaged phage to 270 μl of SM buffer (NaCl, 5.8 g; $MgSO_4.7H_2O$, 2 g; Tris base, 6.05 g; Gelatin, 2 %; made up to $1dm^3$ with H_2O and the pH adjusted with HCl to 7.5).

The dilution was mixed thoroughly. A fresh pipette tip was used to transfer 30 μl of the 10^2 dilution into a tube containing a further 270 μl of SM buffer to create a 10^3 dilution. This process was continued until the 10^7 dilution was attained. A repeat dilution series was produced in the same manner, since accurate phage titres are critical for analysis of results. To avoid excessive amounts of plating, selected dilutions were chosen in the expected ranges. For carnation cDNA and controls 3 and 4 on both hosts L87 and NM514, dilutions 10^4, 10^5, and 10^6 were selected for plating.Dilutions 10^2, 10^3, and 10^4 were used on both hosts for the plating out of control 2. Control 1, consisting of whole λgt 10 DNA, was plated out on L87 using dilutions 10^5, 106 and 107, and on NM514 using dilutions 10^3, 10^4 and 10^5.

Sterile top agar (0.8 % Bacto-agar/L-broth w/v), was melted and held at 45°C in the water bath. The host cells were prepared previously in the following manner. A loopful of each of the glycerol stocks of *Escherichia coli* L87 and *Escherichia coli* NM514 were streaked out onto L-agar plates (1.5 % Bacto-agar/L-broth w/v, autoclaved and poured into sterile plates) and incubated overnight at 37°C. A single colony from each plate was selected and inoculated into tubes containing 10 ml of sterile L-broth (bacto-tryptone, 1 %; bacto-yeast extract, 0.5 %; NaCl, 1 %; / H_2O w/v, pH 7) containing 0.4 % maltose. The tubes were incubated overnight at 37^{oC}, with shaking for maximum aeration. An aliquot (1 ml) of the overnight culture was added to 50 ml of prewarmed L-broth.

Further incubation at 37°C with shaking was carried out for approximately 2 hrs for L87 and 3 hrs for NM514 (OD_{600} of 0.5). The cultures were then cooled on ice, followed by centrifugation at 3000 rpm for 10 minutes at 4°C. The broth was poured off and the cell pellets resuspended in 15 ml of ice-cold 10 mM $MgSO_4$ and mixed thoroughly. The cells were stored at 4°C ready for infection with phage, and should be used as soon as possible for good results. An aliquot (100 μl) of the two host cell types was pipetted into a series of tubes ready to receive 100 μl of each of the previously selected phage dilutions. These plating mixes were incubated at 37°C for 15 minutes before 4 ml of the liquid top agar (at 45°C) was added. After quick mixing, the solutions were poured onto the agar plates (warmed to 37°C). The top agar was allowed to set completely before the plates were inverted (to prevent condensation dripping onto the bacterial growth) and incubated at 37°C overnight.

The plaques were then counted and the phage titre per ml calculated by multiplying the total number of plaques by the dilution power. The most accurate titres are obtained from plates containing between 20 and 500 plaques. The dilution series was inspected for consistency and average titres determined between the dilutions of both duplicates.

These techniques of amplification as performed by the author, proved to be unsuccessful as will be seen by the results in the following section, and a preliminary attempt at amplifying the cDNA by the PCR method with cloning into the λ ZAP phage was carried out. This was done according to the method published by (JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS, 1991). Purified oligonucleotide 1 (5'-ATGCTTAGGAATTCCGATTTAGCCTCATA-3') and oligonucleotide 2 (5'-TATGAGGCTAAA-3') (obtained from Amersham) were used as amplification adaptors. Oligonucleotide 1 (40 μg) and oligonucleotide 2 (100 μg) were dissolved in 50 μl of buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA)(STE buffer). The adapters were heated at 70°C for two minutes. The water bath was turned off and left to cool to 30°C.

The annealed adapter was annealed to the blunt-ended cDNA in the following manner. The blunt-ended cDNA (10 μl) was pipetted into a microcentrifuge tube. Annealed oligonucleotides (2.5 μl), T4 ligase 2.5 units/ml (1 μl), 10 mM ATP (2.0 μl), 10x ligase buffer (supplied with enzyme) (2.0 μl) and H_2O (2.5 μl) were added respectively.

The reaction mix was incubated at 14°C overnight. This was followed by extraction with phenol/chloroform (as was done previously) and applied to a Bio-Gel A-150 column to remove excess adapters. Fractions of 100 μl were collected.

A portion of each of the fractions (5 μl) were amplified using the PCR method. The amplication mix was prepared by pipetting in order 5.0 μl PCR buffer 10x (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM MgCl, 0.1 % (w/v) gelatin), oligonucleotide 1 (6.0 μl), 8.0 μl dNTP stock mixture (1.25 mM of each dNTP), 26 μl of H_2O, cDNA (5.0 μl) and Taq DNA polymerase 5 units/ml (0.5 μl). This mixture was incubated at 73°C for two minutes in a Dri-Block thermal cycler to melt off oligonucleotide 2, followed by 35 cycles of 94°C for 0.8 minutes, 68°C for 1.1 minutes and 73°C for 3 minutes. Selected fractions were cloned into λ ZAP in the same manner as was done using λ gt 10, followed by plating out as described previously (Plate 4.4.).

4.3 RESULTS AND DISCUSSION

The preparation of the cDNA for insertion into the vector was monitored by a sample taken at each stage compared with a series of controls.

The first indication of whether the cDNA will be able to clone well before any cloning preparation begins, is if there is evidence of autoligation, which only occurs if there is satisfactory blunt-ending (which was achieved by T4 DNA POL). This is indicated by shifts to a higher molecular weight relative to the unligated control after electrolysis of the cDNA on a gel. If this did not occur, it would have been necessary to repolish the ends with the T4 DNA polymerase. It is however difficult to observe this effect on gels stained with ethidium bromide (radiolabelled samples would have to be used) with the constraints of sample size used, particularly in the case of the carnation cDNA.

The first step in the cloning process involves the addition of adaptors with a blunt end and an EcoR1 (4 bases) overhang onto blunt-ended cDNA, so resulting in cDNA with cohesive EcoR1 termini. Self-ligation of the adaptors should not occur since they lack 5' phosphates while the high molar ratio of adaptor : cDNA used, minimized self-ligation of the cDNA. The autoligation phenomonen observed with the original cDNA would therefore no longer have occurred. It would be expected that the cDNA would have a slightly slower migration (increased molecular weight with bands of unligated cDNA and unligated adaptor molecules (which migrated the farthest).

To have observed these phenomena, however, it would have been necessary to have radiolabelled the samples, which was not done since the ethidium bromide staining technique was being used.

Size fractionation through the column should have achieved size selection of the cDNA of greater than 500 base pairs with exclusion of the smaller unreacted adaptor molecules. Comparison of gels of the selected fraction (Plate 4.1. and Plate 4.2.) as compared to that of non-selected fraction (Plate 4.3.), showed a concentration of large molecules with few small molecules, whereas the unselected fraction shows no bands of large molecules but instead has a greater concentration of small molecules (adaptors). The molecular weight marker indicates that the size of the selected cDNA is greater than 200 base pairs.

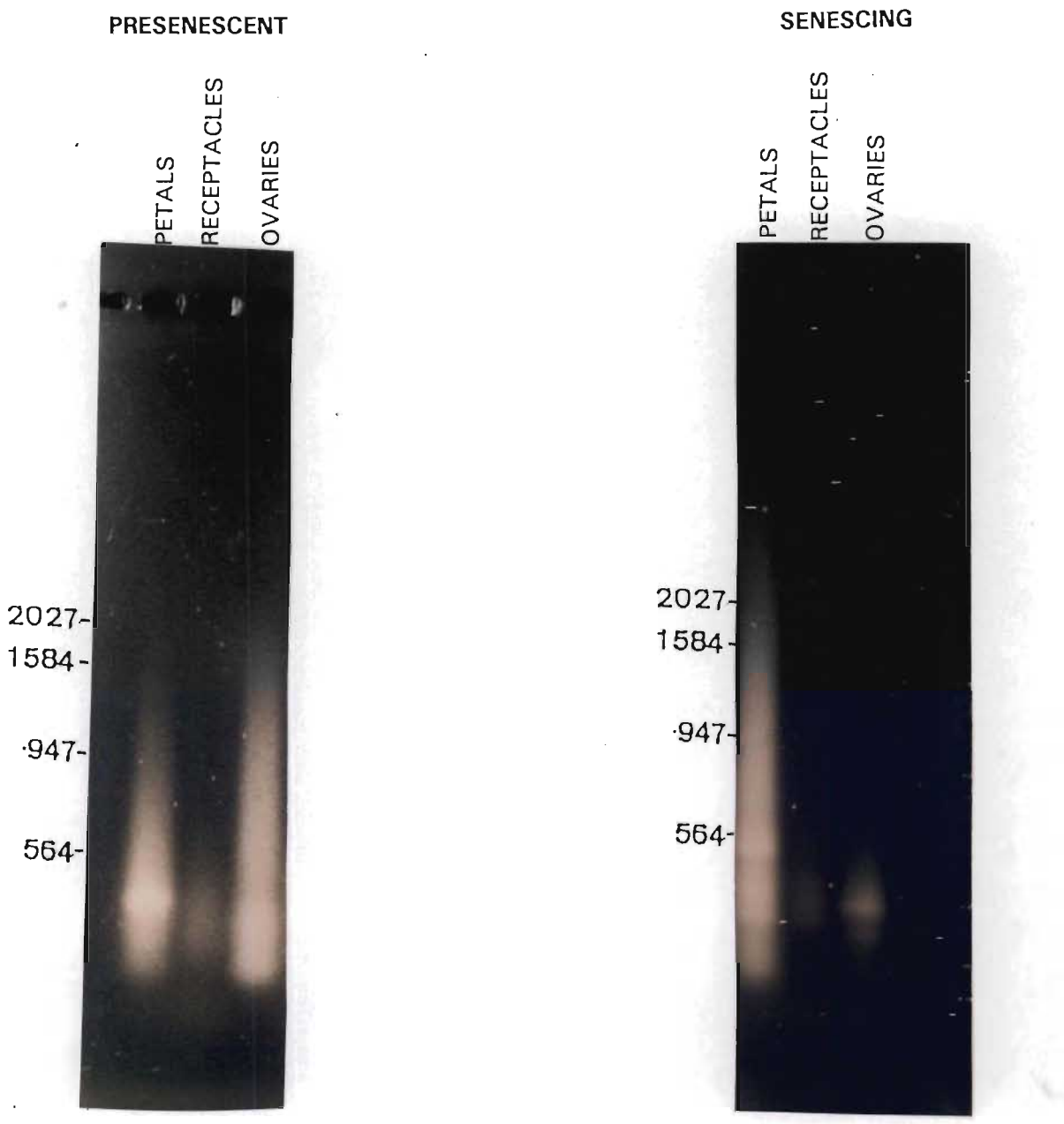


Plate 4.1. and Plate 4.2. Electrophoretic separations of the selected fraction of cDNA from carnation floral tissues before (4.1.) and during (4.2.) senescence, indicating that cDNA larger than 200 base pairs has been selected.

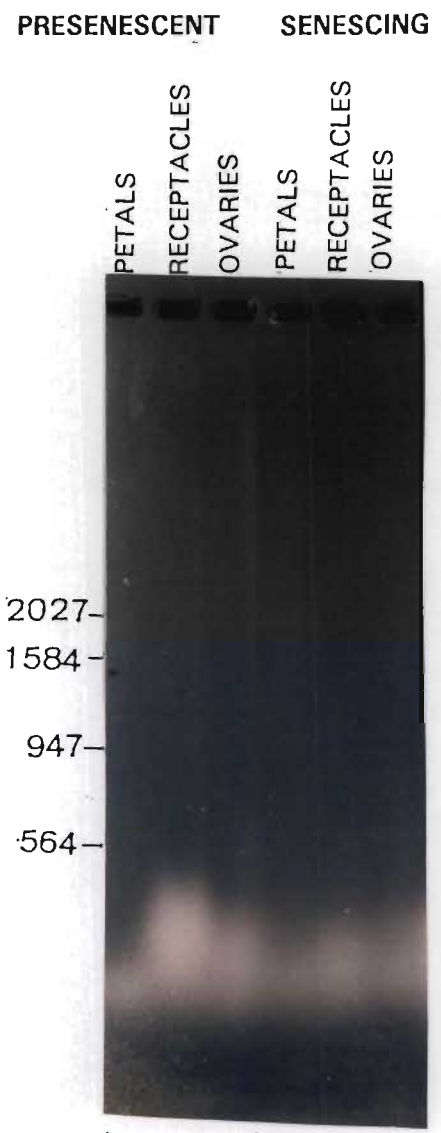


Plate 4.3. Electrophoretic separation of the unselected fraction of cDNA from carnation floral tissues before and during senescence, indicating that cDNA smaller than 200 base pairs has been excluded.

After kinasing, the ends of the cDNA were once more phosphorylated in preparation for insertion into the phage arms. It would therefore be expected that autoligation would occur resulting in the formation of bands of different multiples of cDNA size ranges. This evidence of autoligation would thus be an indication of a successful kinase reaction. To have detected this on the gel, it would have been necessary to have used radiolabelling, which was not performed. Staining with ethidium bromide would not have revealed this evidence due to the small quantities of sample used.

The insertion of the adapted cDNA into phage arms and subsequent packaging steps were monitored using various controls. The controls were all plated out in a similar fashion to the plating out of recombinant λgt 10 containing the carnation cDNA, to pinpoint any steps occurring with decreased efficiency duing the cloning. The controls will be discussed first.

The first control involves the use of whole λgt10 vector DNA, ie. with no insert DNA which monitors the overall efficiency of the *in vitro* packaging reactions and the selection by the two host cell types, L87 (nonselective host, with non recombinant phage producing turbid plaques, and recombinant phage, clear plaques) and NM514 (selective host with nonrecombinant phage inducing complete lysogeny and recombinant phage producing clear plaques). The results of this control are as follows:

Table 4.1. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non selective host L87 as compared to expected numbers using whole lambda gt10 DNA.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS
HOST NM514	3×10^6	4×10^5
HOST L87	$> 3 \times 10^8$	1×10^8
L87:NM514	> 100	> 100

As mentioned previously, L87, being the wild-type host, exhibits turbid plaques when infected with parental λgt 10 producing active CI repressor protein. NM514, becomes completely lysogenic when infected with non-recombinant parental λgt10 producing active CI repressor. There should therefore be more plaques occurring in the L87 wild-type host than NM514 through production of turbid plaques. The fact that infection did occur successfully demonstrates that the phage packaging reaction took place efficiently.

The second control reaction involves the use in the cloning reactions of λgt10 arms containing no insert DNA. Since the arms of the phage were dephosphorylated, no self-ligation should have occurred. The control thus gives a direct measure of background. The results were as follows:

Table 4.2. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing no insert DNA to determine an estimate of background.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS
HOST NM514	$< 2 \times 10^4$	1×10^3
HOST L87	$< 5 \times 10^4$	4×10^4

A very low titre of phage plaques was expected, as was obtained, indicating the prescence of very little uncut or unphosphatased vector molecules.

The third control examines the efficiency of the ligation reaction and measures the stimulation over background obtainable using an EcoR1-ended control insert with phosphatased λgt10 arms. This reaction should produce a recombinant phage DNA. The number of recombinants (pfu/μg) was calculated by subtracting the background titre obtained in control 2, from the titre obtained in later controls, divided by the amount of cDNA used. The results obtained were as follows:

Table 4.3. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing control EcoR1-ended inserts (100 ng).

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 2 \times 10^6$	6×10^6	6×10^7
HOST L87	$> 3 \times 10^6$	3×10^7	
NM514 CTRL3:CTRL4	> 100	> 1000	

These results indicated that the ligation reaction occurred efficiently the number of plaque forming units falling within the expected range. Fewer plaques were obtained on NM514 than L87 as expected, since any non recombinant phage present should not have produced any plaques on NM514 while non-recombinant phage would have produced turbid plaques on L87. The high ratio of plaque forming units obtained for the phage arms containing the control insert as compared to that of the phage arms with no insert indicates that the ligation and other cloning reactions were working efficiently, far above background levels.

The performance of the whole cloning system is monitored by the fourth control. This control involves the use of λgt10 EcoR1 arms with the adapted blunt-ended control DNA fragments which was processed alongside the carnation cDNA through all the cloning procedures. The results obtained are shown in the following Table 4.4.

Table 4.4. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (50 ng) of control DNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	5×10^5	1×10^7
HOST L87	$>1.5 \times 10^6$	3×10^6	

A decreased level of efficiency was noted in this control. Contrasted against the success of the previous control where the EcoR1-ended inserts resulted in satisfactory levels of plaque formation, this would indicate that the ligation procedure in which the adaptors were added onto the blunt-ended cDNA may not have functioned as efficiently as expected. The plate containing phage of the highest dilution in one of the duplicates contained 1.5×10^7 pfu which would have indicated a more successful ligation, however, the other dilutions did not reflect the same high levels and this high number of plaques was probably due to a pipetting error or insufficient mixing of the solutions during the generation of the dilution series.

Reasons why this reaction may not produced more successful results, may have included errors occurring during plating, such as an incorrect buffer pH, or fluctuating / incorrect incubation temperature. An excessively high concentration of top agar may have been used or the plates may have been overdried which would have resulted in the reduced formation and size of plaques. The use of agar that was too hot (greater than 45°C)would also have damaged phage particles and bacterial cells. Since a repeat plating did not produce improved results, these possibilities are less likely than the possibilty that the kinase reaction did not function as efficiently as desired. This may have resulted from pipetting or incubation errors during the reaction, or that the T4 DNA ligase had deteriorated in activity. Sufficient plaques, however, were formed to establish that the reaction had been moderately successful, indicating that similar results could be expected using carnation cDNA. The carnation cDNA insert ligation results were as follows in Table 4.5.

Table 4.5. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (25 ng) of presenescent petal carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	2×10^3	4×10^4
HOST L87	$>1.5 \times 10^6$	5×10^3	

Table 4.6. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (25 ng) of presenescent receptacle carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	1×10^3	0
HOST L87	$>1.5 \times 10^6$	3×10^3	

Table 4.7. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (25 ng) of presenescent ovary carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	1×10^3	0
HOST L87	$>1.5 \times 10^6$	3×10^3	

Table 4.8. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (25 ng) of senescing petal carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	1×10^3	0
HOST L87	$>1.5 \times 10^6$	3×10^3	

Table 4.9. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (25 ng) of senescing receptacle carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	1×10^3	0
HOST L87	$>1.5 \times 10^6$	1×10^3	

Table 4.10. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda gt10 arms containing adapted blunt-ended inserts (25 ng) of senescing ovary carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	1×10^3	0
HOST L87	$>1.5 \times 10^6$	1×10^3	

These results indicate that the ligation reactions using the carnation cDNA occurred with little success, with results achieved being scarcely above the levels of background (1×10^3). Since the ligation reaction using control cDNA had occurred with much more success, in a range close to expected values, there is a strong implication that the carnation cDNA was of inferior quality for several possible reasons. The first possible reason is that the cDNA was too small. This did not appear to be the case since the gel of the cDNA after fractionation indicated that a reasonable percentage of the cDNA used for the substrate insert was greater than 500 base pairs.

Possibly, if more of the smaller cDNA had been excluded during size fractionation, a greater concentration of the larger pieces may have improved the success of the reaction. The petal cDNA from both the presenescent and senescing material appeared to contain a greater concentration of the larger cDNA, which may account for the greater success of the petal libraries as compared to those of the ovaries and receptacles. Another possibility is that the cDNA was not properly blunt-ended due to the failure of the reaction components or incorrect reaction conditions during the incubation with T4 DNA polymerase. The control cDNA was supplied in a blunt-ended form already, which would thus account for the success of the control reactions when compared to the failure of the carnation cDNA reactions. A further possibility is that the carnation cDNA was not in optimal concentrations for the ligation reaction to occur efficiently. The optimal ratio should theoretically be two vector arms to one cDNA molecule, however since not every cDNA molecule is fully ligatable the optimum may require up to 120 ng of cDNA. Since the minimum of carnation cDNA was used (25 ng) due to sample size constraints, this may have reduced the efficiency of the reaction. The slightly higher numbers of plaques occurring on the L87 host than on the NM514 host possibly indicates that several non-recombinant phage caused lysis. This may suggest that residual kinase activity (due to insufficient purification after that reaction), may have rephosphorylated arms leading to self-ligation of the arms which may also have reduced the success of the ligation of the cDNA inserts. This, however, is unlikely, since the number of plaques occurring on L87 are not significantly higher than those occurring on NM514. Difficulty was also experienced in differentiating between non-turbid and turbid plaques.

The lack of success in the production of the library using the selected fraction prompted a preliminary attempt at expanding the larger fractions of the cDNA libraries of the petals, before and during senescence, using the PCR technique, with cloning into λZAP. This technique resulted in better results. A number of white plaques were formed (as opposed to blue) indicating that a number of recombinant phage were present as can be seen in Table 4.11., Table 4.12. and Plate 4.4.

Table 4.11. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda ZAP arms containing no insert DNA to determine an estimate of background.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS
HOST NM514	$< 2 \times 10^4$	1×10^3
HOST L87	$< 5 \times 10^4$	4×10^4

Table 4.12. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda ZAP arms containing adapted blunt-ended inserts (25 ng) of presenescent petal carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	4×10^4	3.9×10^5
HOST L87	$>1.5 \times 10^6$	7×10^4	

Table 4.13. The number of plaque forming units (pfu)/μg obtained on selective host NM514 and non-selective host L87 as compared to expected numbers using lambda ZAP arms containing adapted blunt-ended inserts (25 ng) of senescing petal carnation cDNA carried through all procedures.

	EXPECTED NO. PFU/μg ARMS	ACTUAL NO. PFU/μg ARMS	RECOMBINANTS PFU/μg INSERT (NM514)
HOST NM514	$> 1 \times 10^6$	3×10^4	2.9×10^5
HOST L87	$>1.5 \times 10^6$	8×10^4	

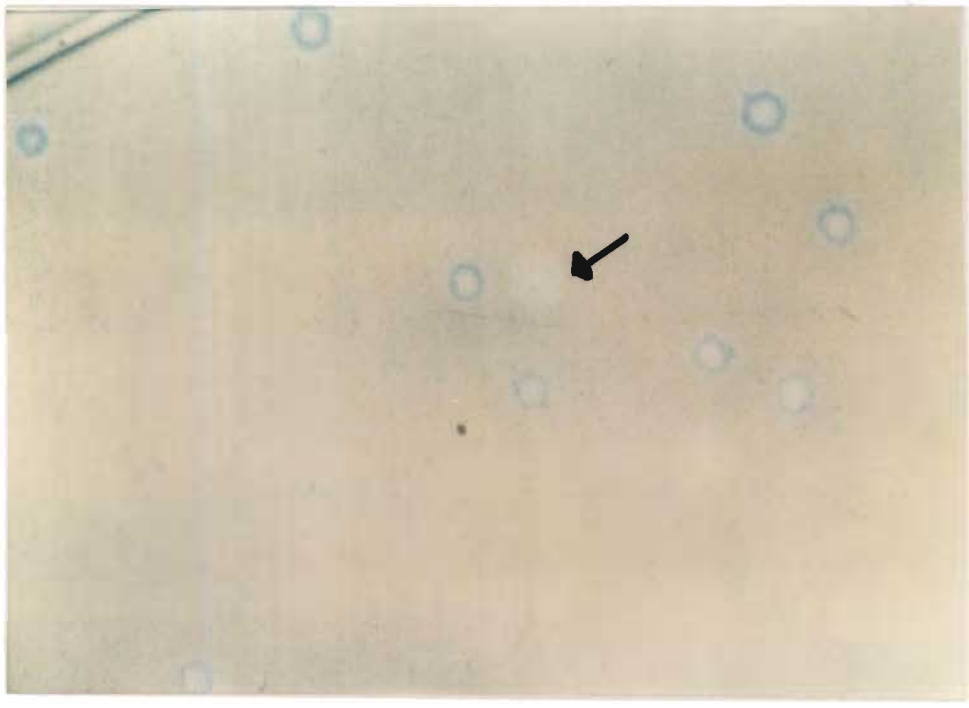


Plate 4.4. Plaques produced by λ ZAP. The white plaque (indicated by arrow) contains recombinant phage containing petal cDNA, while the blue plaques contain normal phage.

CHAPTER 5

cDNA SUBTRACTION LIBRARY CONSTRUCTION

5.1 INTRODUCTION

The purpose of the construction of a subtraction library is to enrich for the presence of target cDNA clones, where no specific hybridization probes are available. A subtraction library strategy was used by HEDRICK, COHEN, NIELSEN AND DAVIS (1984) for the isolation of cDNA clones encoding T cell-specific membrane-associated proteins. The method exploited the fact that the receptor sequence required was expressed only by membrane-bound polysomes in T-cells, and not in B cells which otherwise are very closely related. Single-stranded cDNA from membrane-bound polysomal RNA was therefore hybridized repeatedly with B cell-derived mRNA, with selection for single-stranded unhybridized cDNA, using hydroxylapatite column chromatography. To enrich this cDNA, it was hybridized with T-cell mRNA, the double-stranded RNA-DNA hybrids selected and cloned (HEDRICK, COHEN, NIELSEN AND DAVIS, 1984; WINNACKER, 1987; AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989).

Disadvantages of this technique is that the method involves the use of much complicated manipulation using very small quantities of cDNA material, which is technically difficult (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989).

Another approach to the selection of target clones was used by LAMAR AND PALMER (1984) to create a library enriched for Y chromosome-specific sequences. DNA from one source containing the desired sequence was hybridised with an excess of DNA fragments from the the source lacking the sequence. Sequences found in both sources and thus not the desired sequences, would hybridize to each other, resulting in pieces of DNA hybridized to fragments. The DNA with no complementary sequences to be found among the fragments would hybridize with full-length DNA from the same source, resulting in full-length double stranded molecules. These desired DNA sequences were selected by a cloning step. Selection and library construction were thus accomplished in one step. This method was termed deletion enrichment.

The method used for the construction of the subtraction library is based on that of deletion enrichment and is outlined by AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL (1989). The method is designed to isolate unique sequences of the cDNA from senescing tissue using a selection procedure which allows only such sequences and no other to become cloned and amplified. The selection strategy is arranged in the following way. The cDNA isolated from senescing material which is likely to contain the desired unique sequences being actively expressed (and as such is designated the [+] library) is prepared with Eco RI ends.

The cDNA from the presenescent material ([-]), lacking the unique sequences being expressed, is digested to give small blunt-ended fragments. The [+] cDNA inserts are then mixed with a fifty-fold excess of [-] cDNA fragments.

After dissociating the mixture of the two, single stranded DNA is allowed to hybridize and anneal. This step is followed by ligation into λ gt10 arms, packaging and transfection. Only those fragments which are double-stranded and having an Eco RI site at both ends will be likely to be cloned. Such fragments would probably only result from self-hybridization of unique sequences of DNA from [+] cDNA for which no complementary fragments form the [-] cDNA was present. The blunt-ending and linking of Eco RI ends can be reversed between the two libraries to obtain sequences which become inactivated during senescence.

An alternative to the subtraction method, is that of differential screening, which was used by LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989) to isolate senescence related genes from carnation petals. Duplicate filter lifts of the library containing the desired sequences were taken. The libraries from before and during senescence were then each radiolabelled and one hybridized to each of the filter lifts. Plaques on both filter lifts would become radiolabelled, but only the plaques that are radiolabelled on the one filter and not on the other, will contain the desired sequences.

A major disadvantage of this method, particularly when the sequences required are in relatively low abundance, is that the hybridization is done in a fairly large volume resulting in the failure of some DNA, particularly rare sequences, to hybridize in a reasonable period of time.

The modified deletion enrichment method which utilises a much smaller volume during hybridization (0.05 versus 10 ml) and greater DNA concentration (4 versus 0.01 μg ml^{-1}) was thus used in preference to the differential method to ensure maximum capture of rare sequences (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989).

5.2 MATERIALS AND METHODS

Large scale DNA extractions of [+] and [-] cDNA phage libraries were prepared to obtain more than 1 mg of phage lambda library. This was done as follows:

Clear as opposed to turbid plaques from each of the cDNA libraries (white as against blue in the case of λZAP) were located to select for the recombinant DNA. These were individually reinoculated onto a fresh lawn of host bacteria. After incubation at 37°C for 4 to 6 hours, the phage was harvested by flooding the entire lysed area with SM buffer (NaCl, 5.8 g; $MgSO_4.7H_2O$, 2 g; Tris base, 6.05 g, Gelatin, 2 %; made up to 1dm^3 with H_2O, final pH 7.5).

It is assumed that one plaque is formed by the presence of one phage. To obtain the titer of the phage (ie the number of plaque forming units ml^{-1}), the lysate was diluted in a series dilution of 10^{-3} to 10^{-6} in SM buffer. A sterile 125 ml flask containing 40 ml of lambda broth (LB) (consisting of 1% w/v tryptone, 0.1 % w/v yeast extract, 0.5 % w/v NaCl made up to volume with water and pH adjusted to 7.5 using 1 M Tris before autoclaving) containing 10 mM MgSO4 was inoculated with one single colony of the host *Escherichia coli*. The colony was grown overnight at 30°C in a shaking water bath. A quantity of the cells (0.1 ml for each plate) was then microcentrifuged and resuspended in SM buffer with the addition of 0.1 ml of each phage dilution and mixed well. Before plating, each mixture was incubated at 37°C for 30 minutes and then 3 ml of soft lambda agar was added [LB containing 0.5 % w/v agar before autoclaving and filter sterilized thymine (0.2 %) w/v, maltose (10 %) and $MgCl_2$ or $MgSO_4$ (1 M)]. After swift vortexing, the mixture was poured onto prewarmed (50°C) lambda plates (LB containing 1 % w/v agar) and the plates incubated at 37°C overnight.

In a similar manner to the preparation of host cells for phage titering, 20 ml of the fresh overnight cells were cultured and then inoculated with 5×10^8 λ gt10 phage in a 4 litre flask. To obtain a lysate, the cells must be in the log phase (stationary phase cells are used to obtain lysogens). The culture was incubated at room temperature for 5 minutes.

A litre of LB containing 10 mM $MgSO_4$ prewarmed to 37°C was then added to clear the lysate. The culture was shaken vigorously at 37°C since the greater the level of aeration, the more successful the lysis. Lysis should occur after 5 to 8 hours (not longer than 10 hours). After lysis 1 ml of chloroform was added and sufficient NaCl to result in a final concentration of 0.5 mM, to stabilize the phage, followed by 5 minutes of shaking.

The lysate was then divided into two JA-10 centrifuge bottles and centrifuged for 10 minutes at 10 000 rpm (17700 *x g*), at 4°C, to remove cell debris. To concentrate the phage, the supernatant was transferred to a 1 litre measuring cylinder and a 5X polyethylene glycol (PEG) solution (PEG 50 % w/v H_2O) was added to give a 1X final concentration of PEG. The mixture was mixed gently using a magnetic stirring bar, and allowed to stand overnight at 4°C. The PEG solution induces the phage to precipitate. The supernatant was poured off and the phage precipitate transferred to centrifuge tubes, with a portion of the discarded supernatant being used to rinse out the measuring cylinder. The precipitate was centrifuged for 10 minutes at 4°C (3000 *x g*). The centrifuge tubes were placed on ice. The clear top layer was pipetted off leaving the thick white phase consisting of the PEG precipitated phage. This white phase was resuspended in a minimum of suspension medium (which should not exceed three times the volume of phage precipitate).

The next step involved the removal of the PEG solution leaving the phage behind. This was done using KCl to precipitate the PEG. Sufficient solid KCl was added to result in a final solution concentration of 1 M. The KCl was added in four approximately equal sized aliquots, with thorough mixing after each addition. The mixture was allowed to stand on ice for 30 minutes. After transfer to centrifuge tubes, the solution was centrifuged for 10 minutes (12 100 *x g*) at 4°C (10 000 rpm in a JA-20 rotor). The phage remained in solution while the PEG precipitate was pelleted. The phage supernatant was retained in a glass tube and the phage titre was determined.

The purification of the phage particles was achieved using a CsCl gradient. Three solutions of CsCl were prepared with densities 1.7 g ml^{-1}, 1.5 g ml^{-1} and 1.3 g ml^{-1} respectively. The first layer consisted of 3.5 ml of the first solution (i.e. 1.7 density) covered by 2.5 ml of the second solution to form the second layer, with the third layer being formed by 2.5 ml of the third solution. The layers were pipetted carefully to avoid mixing of the different layers. The phage supernatant was carefully layered onto this gradient. The centrifuge tube was then filled almost to the top with suspension medium. The centrifuge tube was then centrifuged for 2 hours in an SW-28 rotor at 24 000 rpm (104 000 *x g*) at 4°C.

The phage layer is usually located in the lowest gradient. Two other bands, one blue band containing empty phage heads and one containing white cell debris, may also be present.

If a blue band (formed by added ethidium bromide stain and viewed under long wave ultraviolet) does not occur, the gradient should be abandoned as it is unlikely that sufficient phage will be present. Bands having a density of approximately 1.5 g ml^{-1} probably contain the desired phage.

The blue band containing the phage was removed using a 3 ml syringe. The needle (25-G) was pushed carefully through one wall of the tube at the appropriate position and the phage withdrawn. The phage was then transferred into Beckman Ti50 quick-seal tubes. The tubes were filled with CsCl solution of density 1.5 g ml^{-1}, sealed and centrifuged in Ti50 rotor at 30 000 rpm (81 500 *x g*) at 4°C for 24 hours. The only visible blue band was removed as before.

The phage DNA was purified using formamide extraction. To achieve this, the volume of the phage suspension was first determined. A volume of 2 M Tris-Cl (pH 8.5)/ 0.2 M EDTA equivalent to one tenth of the phage volume, was then added to the phage in a microcentrifuge tube, and then mixed by inversion. An equivalent volume (to the phage band) of formamide was added followed by mixing, and the resulting solution was allowed to stand for 30 min at room temperature. A volume of room temperature ethanol (100 %) equivalent to double the volume of the phage band, was added and mixed in gently. This was followed by micro-centrifugation for 2 minutes. The supernatant was discarded and the remaining pellet rinsed with 70 % ethanol. All droplets of the ethanol were removed using a pipette and the still moist pellet dissolved in TE buffer (pH 8.0). The final DNA concentration was then determined by reading the absorbance at 260 nm.

Digestion of the cDNA to produce Eco RI ends was done as follows: An equal quantity of DNA (1 mg), from each library was resuspended in 1 ml of TE buffer, placed in separate 1.5 ml microcentrifuge tubes and mixed with 0.117 ml 10X Eco RI buffer and 0.05 ml Eco RI enzyme (1000 units). This was followed by incubation at 37°C for 5 hours. The reaction was stopped by the addition of 0.5 M EDTA (pH 8.0), and incubated for 10 minutes at 65°C. This digestion may cut some cDNA inserts at an internal Eco RI site. If this occurs, a probe can be generated from these pieces to reselect for the full-length clone from the [+] cDNA library. Alternatively, a vector which utilises cloning sites other than the Eco RI site, such as the Not I site (utilised by the λZAP vector) can be used instead of λgt10, which was in fact included later as a control.

During the digestion, four 10 % to 40 % sucrose gradients were prepared in 38 ml SW-28 centrifuge tubes. Each digest was mixed with an equal volume of 10 % sucrose solution and each split evenly between two tubes ie. two tubes containing the [+] library and two containing the [-] library. The gradients were centrifuged in an SW-28 rotor overnight at 26 000 rpm (130 000 *x g*) at 20°C. The DNA inserts are very small and as such remain near the top of the gradient while the larger phage arms are found in a fraction towards the middle of the tube (Figure 5.1.). Twenty fractions of 0.2 ml were removed from the top of the undisturbed tube using a pipette and each placed in a separate microcentrifuge tube at 4°C.

The remaining fraction was retained until the inserts had been located in the removed fraction. This location of insert can be done by analysing 20 μl of every other fraction on a 1.5 % agarose gel.

The insert DNA (in the sucrose gradient) was then precipitated. This was done by diluting the sucrose with 300 μl of TE buffer and adding 1 ml of 95 % ethanol (there is insufficient salt in the sucrose gradient buffer to allow precipitation) and incubating at -20°C for 2 hours. The precipitated DNA was then collected by centrifuging the tube for 15 minutes before aspirating the supernatant. The pellets were then washed with 70 % ethanol, recentrifuged, the ethanol aspirated and the pellets dried. The pellets of the [+] library (senescing material) was then resuspended and pooled in TE buffer (10 mM Tris base, 1 mM EDTA) to a final concentration of 0.2 mg ml^{-1} and stored at -20°C. The DNA from the [-] library was resuspended and pooled in a final volume of 100 μl and placed on ice.

The next step involved the blunt-ending of the [-] library by the removal of the Eco RI ends. For this, 100 μl of [-] insert DNA (10 to 15 μg), 11 μg 10X S1 nuclease buffer (supplied by the manufacturer with the enzyme) and 1 μl 1:500 S1 nuclease (2 units) were mixed by vortexing briefly. This was followed by brief microcentrifugation and subsequent incubation for 30 minutes. The reaction was then stopped by the addition of 5 μl of 0.5 M EDTA pH 8.0, 200 μl TE buffer and 300 μl phenol/chloroform/iso-amyl alcohol (24:24:1) followed by brief vortexing.

To separate the phases, the mixture was micro-centrifuged for 1 minute. The upper aqueous phase, containing the DNA, was transferred to a new tube. To precipitate the blunt-ended DNA, 30 μl of 3 M sodium acetate (pH 5.2), and 700 μl of ethanol added, followed by mixing and then freezing at -20°C for 2 hours (or on dry ice for 15 minutes). After thawing, the DNA precipitate was collected by microcentrifugation for 15 minutes. The pellet was then washed with 70 % ethanol, recentrifuged and the ethanol aspirated. The pellet was dried under vacuum, and resuspended in 100 μl TE buffer.

After this treatment, the blunt-ended DNA was fragmented into pieces of between 20 to 50 base pairs by digestion with enzymes AluI and RsaI. This was achieved by pipetting into a tube, 100 μl of [-] insert DNA (10 to 15 ug), 12 μl 10X AluI buffer (supplied by the manufacturer with the enzyme), 5 μl AluI (50 units), 4 μl RsaI (60 units) in that order. After vortexing, the reaction mixture was briefly microcentrifuged to pool the tube contents to the bottom of the tube. The mixture was incubated for 3 hours at 37°C. To stop the reaction, 5 μl of 0.5 M EDTA, pH 8.0, was added and incubated for 10 minutes at 65°C. In a similar manner as was done previously, the DNA was extracted with 200 μl of TE buffer, 300 μl of phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation and pelleting. The washed and dried pellet was resuspended in TE buffer to a final concentration of 1.0 $\mu g\ \mu l^{-1}$.

The next step involved the actual hybridization of the DNA's from [+] Eco RI-ended libraries and [-] blunt-ended fragments. To achieve this, 25 μl deionized formamide (50 % v/v final), followed by 10 μl containing 10 μg of [-] DNA fragments (ie. 50 times in excess of [+] insert DNA), and 1 μl [+] insert DNA were placed in a 0.4 ml tube. To this was added 12.5 μl of 20X SSC (5X final), 0.5 μl 1 M $NaPO_4$ pH 7.0, (10 mM final concentration), 0.5 μl 0.1 M EDTA pH 8.0 (1 mM final) 0.5 μl 10 % SDS (0.1 % final concentration) and finally 1 μl 10 mg ml^{-1} yeast tRNA (0.2 mg ml^{-1} final concentration). The tube contents were vortexed to mix, and briefly microcentrifuged to repool them. The tube was then placed in a bath of boiling water for 5 minutes to melt the strands. After brief microcentrifugation, the tube contents were incubated for 18 to 24 hours at 37°C to allow reannealing of complementary strands. It is at this stage (ie. after reannealing) that only unique sequences of [+] DNA will be able to regenerate clonable, double stranded fragments with Eco RI ends (as discussed earlier, other non-unique sequences will form partially single-stranded/ double-stranded molecules without clonable ends).

After the incubation step, 200 μl of TE buffer was added to the hybridization mixture and the tube contents transferred to a 1.5 ml tube. The empty tube was washed with 250 μl of TE buffer which was then added to the hybridization mixture, resulting in a final volume of 500 μl. To remove all traces of SDS and formamide, a phenol/chloroform/iso-amyl alcohol (24:24:1) extraction was carried out.

To do this 500 µl of phenol/chloroform/iso-amyl alcohol was added to the tube followed by vortexing and microcentrifugation for 1 minute to separate the phases. The upper aqueous phase was transferred to a new tube. This was then extracted with phenol/chloroform/iso-amyl alcohol. The subraction DNA contained in the aqueous phase separated in this step, was precipitated by the addition of 50 µl 3 M sodium acetate pH 5.2, and 1 ml of ethanol, and incubation at -70°C for 15 minutes followed by washing and drying of the pellet. This pellet was resuspended in 12 µl of TE buffer and represented the subtraction library containing genes expressed during senescence of each of the different components of carnation flowers.

The cDNA resulting from this subtraction may be amplified using PCR and/ or, as was done in this study, inserted directly into a phage vector using methods described in previous chapters. After amplification in the phage vector, the subtraction insert was removed from the phage arms by digestion as described previously in Chapter 4. The digestate was analysed alongside controls of phage arms containing no insert, on 1.5 % agarose gel stained with ethidium bromide.

5.3 RESULTS AND DISCUSSION

The titre of the phage containing insert, precipitated from the lysates produced from the large scale extractions of each the [-] and [+] cDNA phage libraries, was determined to be 1.6 mg (ie. greater than the minimum required amount of 1 mg of phage library).

The CsCl gradient purification of the phage revealed that sufficient phage was present. As expected, a very narrow blue band of phage was observed when the ethidium bromide stain was viewed under UV light, near the bottom of the gradient. Another lighter blue band was also observed, not much higher up the gradient. This probably consisted of empty phage heads and cell debris. Both of these bands were withdrawn to ensure that the phage band had indeed been retained. The second CsCl gradient produced a clearer distinction between the two bands, enabling the phage band to be removed. The volume of the phage band was determined to be 500 μl. After formamide extraction for further purification, the concentration of DNA was determined to be more than 1 mg.

After digestion of the phage to release the inserts from the phage arms (Plate 5.1.), a sucrose gradient (Figure 5.1.) was generated to isolate the insert from the phage arms. The phage, being lighter than the phage arms, separated into the uppermost gradient layer and was carefully withdrawn.

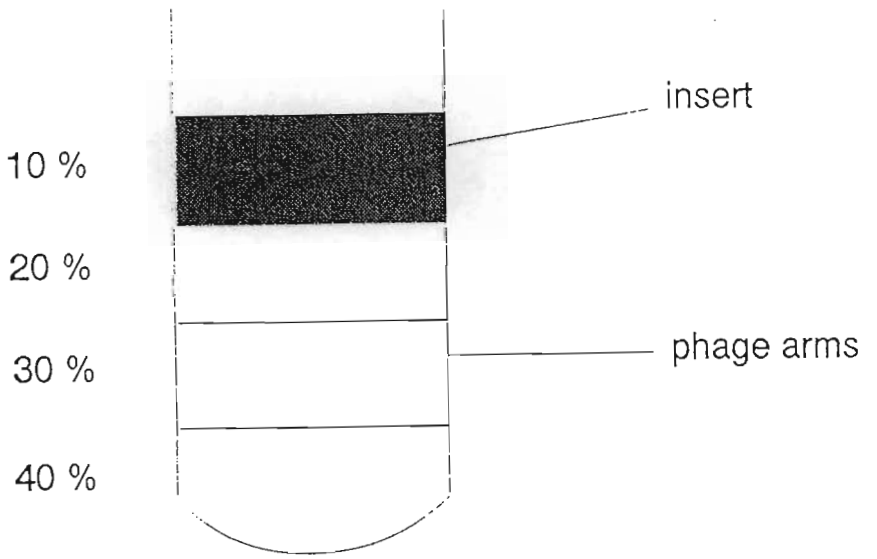


Figure 5.1. Distribution of insert and phage arm molecules as occurs in a 10 to 40 % sucrose gradient.

The cDNA insert, produced as a result of the subtraction strategy, and representing the genes active during senescence within the carnation petal, can be seen in the gel depicted in Plate 5.1., which was run after enzymic digestion to release the insert from the arms. The heavier bands appearing closer to the well contain the phage arms. The approximate length of cDNA isolated during the subtraction process executed on the carnation petal libraries, was determined to be 1 kb.

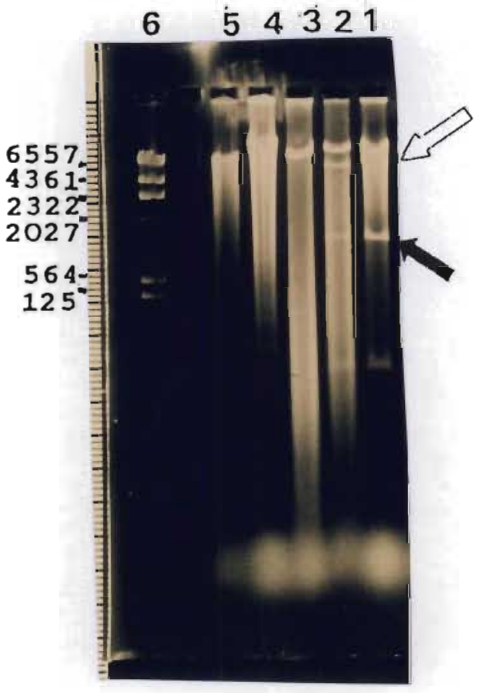


Plate 5.1. Electrophoretic gel showing products of digestion of phage λ ZAP containing subtraction insert (indicated by black arrow) and heavier phage arms (indicated by white arrow) (well 1 and 2), and controls containing purely phage arms (well 3, 4, and 5) run against molecular weight markers (well 6).

CHAPTER 6

GENERAL DISCUSSION

Senescence in cut flowers is a complex phenomenon involving many physiological and molecular events. These result in visible evidence in the case of the carnation, of in-rolling of the petals before final termination of the flower. As mentioned previously, characterization and understanding of these events is of economic importance for the cut-flower trade and research into ageing. Several parallels have also been identified between floral senescence and fruit ripening (GRIERSON, 1984).

There are a considerable number of events occurring during senescence which may be regulated at the gene level and might be induced by the action of a particular gene such as the one isolated. Many of the events occurring during senescence of the carnation have been studied and elucidated. One of the major manifestations of senescence is its effect on water relations, causing water and ion loss (ACOCKS AND NICHOLS, 1979). The loss of turgor is also augmented by changes in cell walls, resulting from the possible enzymic breakdown of large polymers such as pectic substances, hemicelluloses and sugars (DE VETTEN AND HUBER, 1990).

Changes such as loss of fluidity in cell membranes can occur due to enzymic breakdown and changes in the nature of proteins and lipids, assisted by free-radical lipid degradation (MAYAK, LEGGE AND THOMPSON, 1983; WOODSON, 1987). Sugars may be mobilized from the petals to the ovary and receptacle (HALABA AND RUDNICKI, 1989).

Growth regulators are interactively involved in initiating, promoting, preventing and orchestrating the events occurring during senescence. These growth regulators encompass five groups of plant hormones, namely, ethylene, cytokinins, abscisic acid, auxins and gibberellins (COOK AND VAN STADEN, 1987). Ethylene plays a dominant role in promoting senescence. It is synthesized enzymatically from methionine, through the intermediates S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (ADAMS AND YANG, 1979), with alternate conversion of SAM to polyamines (EVEN-CHEN, MATTOO AND GOREN, 1982). Each of these intermediates and derivatives plays a significant role in senescence. Ethylene has been postulated by SISLER AND GOREN (1981) to have a specific binding site in petals, possibly causing the indirect or direct release of a secondary message causing transcription of new mRNA. According to LAWTON, RAGHOTHAMA AND WOODSON (1990) ethylene does not interact with preformed factors, but instead, activates gene expression via mediation by labile protein factors synthesised on cytoplasmic ribosomes.

Auxins appear to be involved in the mobilization of assimilates during senescence (COOK AND VAN STADEN, 1988), while abscisic acid appears to increase the sensitivity of the flower to ethylene (HALEVY AND MAYAK, 1981). The gibberellins, being growth hormones, may play a role in ovary growth during senescence and may cause a decline in ethylene synthesis (COOK AND VAN STADEN, 1988).

The initiation of regulation of synthesis in the petals has been shown to involve an extrapetal site, possibly the ovary, receptacles or styles (SACALIS, 1989), thus requiring a study of these organs in addition to the petals.

Senescence has been shown to be at least partially regulated at the transcriptional level and is not an uncontrolled destructive event. This programmed nature of senescence was suggested to be necessary to allow maintenance of integrity of membranes long enough to allow mobilization of nutrients (MEYER, GOLDSBROUGH AND WOODSON, 1991). Some of the control may be at the translational rather than transcriptional level. A molecular approach to the study of carnation senescence has given new insight into the subject (WULSTER, SACALIS AND JANES, 1982).

The procedures covered in each Chapter require further discussion, when considered in the light of the study as a whole.

The RNA isolation was the first procedure involved in the study. No quantitaive analyses of RNA could be made due to the extensive loss of RNA, depending on the levels of the gelatinous contaminating material. This contaminating material, which proved to be a major problem in the isolation, could have been either of protein or polysaccharide nature, or a mixture of the two. The material, however, appeared not to consist of protein, because addition of hydroxyquinoline for precipitation of proteins did not lead to any significant improvement of RNA purity. Possibly the addition of a polysaccharide precipitant such as Polyclar may have alleviated this. As mentioned earlier, use of the swing-bucket rotor as opposed to the fixed angle rotor, led to improved results through decreased disturbance of the centrifuged layers. The RNA isolation method used was time consuming and tedious, requiring large amounts of plant material, and vulnerable at many stages to possible contamination with RNases. A simpler method, mentioned briefly earlier, described by (JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS, 1991) could possibly alleviate some of these problems. The method requires less than 50 mg of plant tissue which would be particularly useful for studies concerning the styles of the carnation. The method utilises a considerably lower volume of chemicals, namely phenol containing 0.1 % 8-hydroxyquinoline, and a homogenization buffer containing NaCl, TrisHCl, SDS, EDTA, heparin, dithiothreitol and ATT.

Grinding in liquid nitrogen, phenol extractions, and precipitations are all carried out in a 1.5 ml DEPC-treated micro centrifuge tube. The method does, however, require that the RNA, once synthesised into cDNA, be amplified using PCR.

The successful extraction of undegraded RNA was followed by the synthesis of cDNA using the RNA strands as a template. This was to copy the mRNA representing the expression of the genes into more stable DNA once more, to allow amplification. The first step involved the synthesis of an mRNA-DNA hybrid using a reverse transcriptase enzyme using either an oligo (dT) primer, or random hexanucleotide primers. The oligo (dT) primer, usually the more common choice for the synthesis reaction, hybridises with the poly (A) tail of the mRNA. Theoretically, using this primer, it should be be possible to obtain a complete DNA copy of the RNA, however, it is very difficult to prepare intact cDNA copies of long stretches of RNA using a single primer (WINNACKER, 1987). Ideally, if the sequence of the RNA to be copied is known, specific primers of at least 12 bases long, corresponding to known base sequences can be synthesised. Alternatively, random hexanucleotide primers consisting of short lengths of oligonucleotides can be used, which cannot produce an entire clone in the intact form, but will provide a collection of clones which together represent the entire clone (WINNACKER, 1987).

It was not anticipated that the mRNA from the carnation would be very long judging by the length of the clones obtained by LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989), and since more than one clone may have been isolated, it was considered more desirable to use the oligo d(T) primers. The Success of the reaction is dependent as mentioned before, on the quality and species of mRNA used for the synthesis and the optimisation of the reaction conditions. The expected yield of the first strand synthesis, as projected by the suppliers of the reaction components, was 15 to 30 % of the mRNA used. This level of efficiency was indeed obtained with the control globin mRNA, indicating the viability of the reaction components. The carnation mRNA, however, produced yields between approximately only 3 and 13 %, which against the previously mentioned expected yield appears unacceptably low. WINNACKER (1987), however, suggests an expected yield under optimised conditions in the range of 5 to 20 % which is closer to what was obtained using the carnation mRNA.

The aim was to obtain the longest possible copies of cDNA from a limited amount of mRNA. Yields of cDNA can be affected by the quality and nature of the starting mRNA. The quality of the mRNA had initially been determined to be suitable for synthesis (Chapter 1), although degradation may have occurred just prior to or during the synthesis reaction. The purity of the reverse transcriptase used is important since contaminating ribonuclease can interfere with the synthesis, which would require a greater inclusion of dNTP s (WINNACKER, 1987), however, the purity of the reaction components was guaranteed by the supplier.

Ribonuclease contaminants may have been introduced from outside sources such as fingertips and glass/plasticware, although these would have also affected the control reaction. Other factors which would have undermined the optimisation could have been errors in the overestimation of concentration of mRNA used in the reaction due to contamination affecting spectrophotometric absorption assays. The carnation mRNA may not lend itself readily to transcription due to e.g. secondary structure, since as mentioned by MANIATIS, FRITSCH AND SAMBROOK (1982), different species of mRNA are not transcripted with the same efficiency. The yield of first strand synthesis using petal mRNA obtained by LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1988) was unfortunately not mentioned in their report and therefore cannot be used for comparison. It is possible to clone RNA-DNA hybrids directly (WOOD AND LEE, 1976) but the cloning methods most commonly used require the synthesis of a second strand to produce double-stranded cDNA molecules (EMERY, 1984).

The method modified by AMERSHAM (1989) employed in this study utilizes *Escherichia coli* RNase H to nick the RNA in the RNA-DNA hybrid while *Escherichia coli* DNA polymerase (Klenow fragment) replaces the RNA strand using the nicked RNA as a primer. T4 DNA polymerase is used to remove any small remaining 3'-overhangs from the first strand cDNA. This process of "self-priming" is used since it leads to higher yields of cDNA than other methods of priming (WINNACKER, 1987).

As an alternative to the *Escherichia coli* DNA polymerase and T4 DNA polymerase, AMV reverse transcriptase may be used. This enzyme produces more full length copies of the cDNA than the other combination of enzymes, which tend to produce shorter and thus less ideal reaction intermediates. The AMV reverse transcriptase, however, lacks the 3' to 5' activity of the other two enzymes which prevents the AMV transcriptase from transcripting molecules which do not possess base-paired ends (ROUGEON AND MACH, 1976). The yield percentage for second strand synthesis from the first strand, according to the method used was expected to be above 90 %. Yields for second strand synthesis reported in other literature vary between 30 % and 90 % (WINNACKER, 1987). The yields obtained in this study were close to what was expected by the suppliers of the reagents, being mostly in the region of 80 to 90 %, with only the presenescent receptacle percentage yield falling below this (approximately 50 %). The relatively low yield of second strand presenescing receptacle cDNA may have been as a result of low levels of starting material although this was a problem common to most of the other samples of carnation material, without a similar loss in yield. Possibly pipetting or incubation error may have resulted in less optimal conditions of synthesis for this particular sample.

The double stranded cDNA was then ready for the ligation into an appropriate vector molecule for cloning. Three types of vectors are in common use ie. plasmids, cosmids and bacteriophage vectors (EMERY, 1984).

Plasmids consist of a circular duplex of DNA with a limited number of specific restriction sites. They occur in an extrachromasomal state, naturally in various microorganisms, conferring on their host various phenotytypic traits not required for bacterial reproduction. Bacteriophages are viruses which infect bacteria, resulting in the lysis of bacteria causing plaques on a lawn of bacteria. Bacteriophages consist of a central core of DNA surrounded by a protein coat. After the phage DNA is injected into the host, it circularizes by means of the joining of the cohesive end sites (*cos* sites). A cosmid is an artificial construct of plasmid DNA packaged in a phage protein coat. The technology involved in the use of the plasmid vectors is simpler than other vectors. An advantage of the phage vector is that infection by phage into the host is often more successful than the transformation procedure required for the introduction of the plasmid into the host (WINNACKER, 1987). Another advantage of the phage vectors over the plasmid, is that the recombinants are more stable in the phage and therefore easier to store (EMERY, 1984). The cosmids were specifically designed for cloning of large DNA fragments of up to 50 kb, a facility not required by this study (EMERY, 1984). The phage vector was thus chosen for this study because of the limited amount of cDNA available, to ensure maximum infection and stable storage. The lambda phage has been extensively developed for use as a vehicle for recombinant material into the *Escherichia coli* host. Phage lambda gt10 was used in this study, while lambda ZAP which provides a feature of colour selection for desired clones was used for a pilot run using PCR (polymerase chain reaction) technology to boost yields of clones.

The process of introduction of the cDNA into the phage genome, requires initially the blunt-ending of the cDNA. Adaptors must then be joined to the blunt end of the cDNA and provide an overhang to produce cohesive EcoR1 termini for ligation of the cDNA into the phage arms. Unreacted adaptors and very small pieces of DNA were excluded by passage through a fractionation column. Selected cDNA was thus greater than 200 base pairs.

The ends of the cDNA were kinased and phosphorylated and the insertion of the cDNA into the phage arms and packaging were monitored by resultant infectivity into two host *Escherichia coli* strains. These controls revealed that the phage ligation and packaging reactions were functioning efficiently for the control samples, with little background. The control monitoring the efficiency of the recombinant phage containing the blunt-ended control cDNA, however, indicated that the ligation procedure in which the adaptors were added onto the blunt-ended cDNA may not have functioned as efficiently as expected. This may have been as a result of pipetting or incubation errors in the reaction or because the T4 DNA ligase had deteriorated in activity. The ligation reactions using the carnation cDNA occurred with far less success than the control. This appeared to indicate that the carnation cDNA was of inferior quality. This may have been because the blunt-ending of the carnation cDNA may not have been successful since the control cDNA was supplied already blunt ended.

The excessively high percentage of small pieces of cDNA may have reduced the efficiency of the ligation, since petal libraries which had a slightly higher success of insertion than the rest, appeared to have a higher percentage of larger molecules of cDNA. The low concentration of cDNA may also have reduced the success of the reaction.

The polymerase chain reaction technology which was developed fairly recently (1985) has revolutionized approaches to molecular biological techniques, since the ability to synthesize large amounts of a specific DNA fragment from a small amount of a complex template has significantly facilitated subsequent analysis (ERLICH, 1989). A preliminary attempt using PCR technology to boost the amount of cDNA of the petal libraries followed by cloning into lambda ZAP arms produced more success, possibly since the number of larger pieces of petal cDNA had been increased considerably. Protocols have also been developed which make it possible to use PCR to synthesise a cDNA PCR template from an mRNA transcript with reverse transcriptase. Successful results have been achieved in this manner even with the use of unpurified RNA (1 μg total RNA being of sufficient quantity) (ERLICH, 1989). The carnation presenescent and senescing cDNA was now stored and amplified as libraries and could be carried through the procedure of subtraction to isolate those genes active specifically during senescence.

The subtraction procedure is based on the method of deletion enrichment, in which cDNA from the senescing material was prepared with EcoRI ends and cDNA from the presenescent material was digested to give small blunt-ended fragments in fifty-fold excess. After hybridization and annealing of the mixture, the fragments were ligated in λgt10 arms. Only those fragments from the senescing library which had self-hybridized with EcoRI sites on both ends would be able to clone successfully, ie. the sequences unique to the senescing petals (AUSUBEL, BRENT, KINGSTON, MOORE, SEIDMAN, SMITH AND STRUHL, 1989). This method was chosen instead of the alternative differential screening method since the hybridization is done in a smaller volume, so favouring the isolation of sequences that are in low abundance. The cDNA insert finally isolated as a result of the subtraction strategy, and representing the genes expressed during senescence, was determined to be 1 kb.

Previous studies of senescence related genes from carnation, have resulted in the isolation and characterization of a few genes. These have been compared with some of the fruit ripening genes that have been isolated (MEYER AND WOODSON, 1990). As mentioned earlier, the gene that was isolated from the petals in the present study was 1 Kb in size. The three genes initially isolated by (LAWTON, HUANG, GOLDSBROUGH AND WOODSON, 1989), also from the petals, were called pSR5, pSR8 and pSR12 respectively. These three genes were also found to be expressed in the climacteric carnation ovary, and calyx.

The pSR8 gene was found to be 957 base pairs long, a similar length to the gene isolated in this study, and was proposed to be responsible for the initiation of production of glutathione-S-transferase (25 kDa). It was suggested that this gene may be induced possibly by lipid peroxidation occurring during senescence (MEYER, GOLDSBROUGH AND WOODSON, 1991). It is interesting to note that this gene was not isolated in a complete form but was constructed from stretches of cDNA containing overlapping sequences, the bigger piece containing 660 base pairs and the other, the remaining 297 (MEYER, GOLDSBROUGH AND WOODSON, 1991). There is a possibility that the cDNA isolated by subtraction in this study, may also not be a complete gene since it is not genomic. MEYER, GOLDSBROUGH AND WOODSON (1991) included a gene that was constitutively expressed as a probe to serve as a control as well as the senescence related gene in their studies of gene expression induced by growth regulators (cytokinin). This is a concept that would be useful to adopt for the study of expression of the 1 Kb cDNA isolated in this study.

The pSR5 gene isolated by LAWTON, HUANG, GOLDSBROUGH AND WOODSON (1989) was found to produce a synthesis product polypeptide of unknown identity of 81 kD. The pSR12 was found to be a low copy number gene of over 12 Kb, induced by ethylene. It was found that the SR 12 transcript contains an open reading frame of 2193 base pairs sufficient to encode a protein of 82.8 kDa. No homology was found between this gene and other known products at the DNA or protein level.

It is interesting to hypothesise about the identity of synthesis products from isolated senescence related genes. HOBSON AND NICHOLLS (1977) showed that during senescence, a few enzymes such as esterase showed change in activity. Acid phosphatase, RNAase and ATPase increased in activity, while at the onset of wilting, a number of hydrolases and glycosides showed a peak in activity. Carrot roots stimulated by ethylene showed marked increases in levels of phenylalanine ammonia lyase, 4-coumarate Co-A ligase, chalcone synthase and hydroxyproline-rich glycoproteins (ECKER AND DAVIS, 1987).

WOODSON (1987) found that polypeptides of apparent molecular weights of 70, 62, 35.5 and 24 kDa increased, while those of 70.5, 67.5, 46.5, and 31 kDa decreased during senescence. Translation products of mRNA which increased with senescence produced proteins of 81, 58, 42, 38 and 35 kDa. WANG, BRANDT, AND WOODSON (1993) showed by *in vitro* translation, that the pSR132 gene which mRNA that accumulates in response to ethylene in the carnation, encodes a polypeptide of 36 kDa. This protein revealed homology with bacterial carboxyphoshoenolpyruvate mutase and phosphoenolpyruvate mutase, thus possibly functioning in phosphonate biosynthesis during senescence. An unidentified 35 kDa translation product was also obtained from mRNA using cDNA clones obtained from ethylene stimulated tomatoes (SMITH, SLATER AND GRIERSON, 1986).

Other proteins isolated from tomatoes using cDNA clones from tomatoes stimulated by natural ripening and wounding (natural and induced ethylene production) include polygalacto-uranase (a 48 kDa enzyme associated with softening) and ACC synthase (55 kDa) (SLATER, MAUNDERS, EDWARD, SCHUCH AND GRIERSON, 1985). A cDNA clone of 1146 base pairs was isolated from ethylene induced bean leaves which was identified in causing the production of a chitinase (BROGLIE, CORUZZI, LAMPPA, KEITH AND CHUA, 1983). A study on auxin-repressed strawberry receptacles resulted in the isolation of cDNA of 723 base pairs which caused the production of a protein of molecular weight of 12 500 Da of unknown identity (REDDY AND POOVAIAH, 1990). An enzyme ACC synthase gene, was synthesized from oligonucleotides on the basis of the sequence of the enzyme isolated from tomato (PARK, DRORY AND WOODSON, 1992).

The PCR amplification product of this was used to screen a cDNA library of senescing carnation. The isolated clone contained a 1950 base pair insert which encoded a protein 58 kDa. The sequence was found to be 61 %, 61 %, 64 %, and 51 % identical to zucchini squash, winter squash, tomato and apple respectively. The synthase was not induced by wounding and appeared to be involved in the autocatalytic ethylene response (PARK, DRORY AND WOODSON, 1992).

There are many possible avenues for research using the isolated senescence related carnation cDNA. One of the more obvious applications would be to use the isolated clone as a probe to analyse the effects of growth regulators and senescence chemical inhibitors on the gene activity by examining probe binding of nuclear extracts of different stages of floral development. It may be confirmed that a specific step e.g. ethylene biosynthesis is a regulated step. The isolated clone may be used to map sites of external regulation. The gene may be used to synthesise proteins using e.g. the rabbit reticulocyte lysate, or wheatgerm lysate translation techniques (PELHAM AND JACKSON, 1976) and the resultant amplified proteins compared to other sequenced senescence related proteins. This may lead to the determination of dominant enzymes involved in senescence which have possibly not been elucidated before. This will also enable examination of whether hormone binding sites are preformed or labile by examining the effect of cycloheximide on protein synthesis in studying formation of the sites. Sequencing of the gene may reveal conservation of sequence between it and other genes of similar activity, regulated by e.g. ethylene, such as the gene involved in fruit ripening. Such similarities may include areas such as promoters with binding sites for nuclear proteins, such as examined by (RAGOTHAMA, LAWTON, GOLDSBROUGH AND WOODSON, 1991). The technique of deletion analysis could be used to show these areas of importance in e.g. ethylene responsiveness indicating possibly a "ethylene responsive box". Complementarity DNA binding may also be used to examine areas responsible for binding for possible homology.

Interesting information may be gleaned by screening the cDNA libraries obtained in this study, before and during senescence by using probes obtained from *Arabidopsis thaliana* and the results compared.

The expression of the gene may also be examined through promoter fusions of the sequences to a GUS reporter gene in transgenic carnations and transient expression assays carried out as was done using petunias, with effects of the application growth regulators or inhibitors studied (VAN DER KROL, MUR, BELD, MOL AND STUITJE, 1990; RAGOTHAMA, LAWTON, GOLDSBROUGH AND WOODSON, 1991).

One of the goals which research into the senescence of carnations would hope to attain, is the development of carnations in which at least some of the dominant genes involved in senescence have been disabled, leading to increased longevity of the cut flower. One possible technique for accomplishing this is the incorporation of a gene in the genome of the carnation which produces anti-sense RNA to block the formation of the mRNA produced by the senescence promoting gene involved. A technique making use of a similar idea involves the incorporation of a gene into the carnation genome resulting in the production of antibodies to senescence related proteins (CARLSON, 1988).

Other techniques that would render senescence genes inactive are gene disruption (SCHERER AND DAVIS, 1979), dominant negative mutations (HERSKOWITZ, 1987) or the incorporation of a gene causing the production of the "anti-senescence gene" ribozyme (HASELHOFF AND GERLACH, 1988). If the gene product of the senescence-related gene can be identified, a counter enzyme for example could be transferred to the plant, as was done by VAN DER KROL, MUR, BELD, MOL AND STUITJE (1990) in experiments with flavonoid genes of petunia flowers in which an addition of a limited number of gene copies may lead to a suppression of gene expression. Sequencing and identification of the isolated gene and generated gene products could therefore be the initial area towards which future research concerning the isolated gene could be directed.

REFERENCES

ACOCKS, B. AND NICHOLS, R. 1979. Effects of sucrose on water relations of cut, senescing, carnation-flowers. *Annals of Botany* 44:221-230.

ADAM, Z., BOROCHOV, A. MAYAK, S. AND HALEVY, A.H. 1983. Correlative changes in sucrose uptake. ATPase activity and membrane fluidity in carnation petals during senescence. *Physiologia Plantarum* 58:257-262.

ADAMS, D.O. AND YANG S.F. 1979. Ethylene biosynthesis: Identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Science USA* 76:170-174.

AMERSHAM, (ANON.) 1989. *cDNA synthesis plus*. Amersham, UK. 43pp.

AMERSHAM cDNA CLONING SYSTEM, (ANON.) 1989. *cDNA cloning system - λ gt10*. Amersham, UK. 50pp.

ARIE, B. AND FERGUSON, I. 1990. Ethylene production by growing and senescing pear fruit cell suspensions in response to gibberellin. *Plant Physiology* 95:943-947.

AUSUBEL, F.N., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH J.A. AND STRUHL, K.A. 1987. *Current protocols in molecular biology*. Greene, New York.

BAUMGARTNER, B.H., KENDE, H. AND MATILE, P. 1975. Ribonuclease in senescing morning glory. *Plant Physiology* 55:734-737.

BLATTNER, F.R., WILLIAMS, B.G., BLECHL, A.E., DENNISTON-THOMPSON, K., FABERI, H.E., FURLONG, L-A., GRUNWALD, D.J., KIEFER, D.O., MOORE, D.P., SCHUMM, J.W., SHELDON, E.L. AND SMITHIES, O. 1977. Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. *Science* 196:161-164.

BLEECKER, A.B., ROBINSON, G. AND KENDE, H. 1988. Studies on the regulation of 1-aminocyclopropane-1-carboxylate synthase in tomato using monoclonal antibodies. *Planta* 173:385-390.

BOROCHOV, A. DRORI, A., TIROSH, T., BOROCHOV-NEORI H. AND MAYAK, S. 1990. Quantitative and qualitative changes in membrane proteins during petal senescence. *Journal of Plant Physiology* 136:203-207.

BOROCHOV, A. AND ADAM, Z. 1984. On the role of membrane integrity in the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in carntion petals. *Federation of European Biochemical Societies Letters* 173:139-141.

BOROCHOV, A., WALKER, M.A., KENDALL, E.J., PAULS, K.P. AND MCKERSIE, B.D. 1987. Effects of freeze-thaw cycles on properties of microsomal membranes from wheat. *Plant Physiology* 84:131-134.

BOROCHOV, A. AND WOODSON, W.R. 1989. Physiology and biochemistry of flower petal senescence. *Horticultural Reviews* 11:15-43.

BOUZAYEN, M., LATCHE, A, PECH, J-C. AND MARIGO, G. 1989. Carrier mediated-uptake of 1-(malonylamino)cyclopropane-1-carboxylic acid in vacuoles isolated from *Catharanthus roseus* cells. *Plant Physiology* 91:1317-1322.

BOUZAYEN, M., LATCHE, A. AND PECH, J-C. 1990. Subcellular localization of the sites of conversion of 1-aminocyclopropane-1-carboxylic acid into ethylene in plant cells. *Planta* 180:175-180.

BRITTEN, R.J. AND DAVIDSON, E.H. 1969. Gene regulation for higher cells: a theory. *Science* 165:349-357.

BROGLIE, R., CORUZZI, G., LAMPPA, G., KEITH, B. AND CHUA, N-H. 1983. Structural analysis of nuclear genes coding for the precursor to the small subunit of wheat ribulose-1.5-bisphosphate carboxylase. *Biotechnology* 1:55-61.

BROWN, D.D. 1967. *Methods in Developmental Biology*. Wilt, F.H. and Wessells, N.K. (eds.). Thomas Crowell Co., London. pp685.

BROWN, J.H., PALIYATH, G. AND THOMPSON, J.E. 1990. Influence of acyl chain composition on the degradation of phosphatidylcholine by phospholipase D in carnation microsomal membranes. *Journal of Experimental Botany* 41:979-986.

BROWN, J.H., LEGGE, R.L., SISLER, E.C., BAKER, J.F. AND THOMPSON, J.E. 1986. Ethylene binding to senescing carnation petals. *Journal of Experimental Botany* 37:526-534.

BROWN, J.H., CHAMBERS, J.A. AND THOMPSON, J.E. 1991. Acyl chain and head group regulation of phospholipid catabolism in senescing carnation flowers. *Plant Physiology* 95:909-916.

BUFLER, G. AND ROMANI, R.J. 1983. Polysomal population in relation to ethylene production and the senescence of cut carnation flowers and floral parts. *Journal of the American Society of Horticultural Science* 108(4):554-557.

BUFLER, G., MOR, Y., REID, M.S. AND YANG, S.F. 1980. Changes in 1-aminocyclopropane-1-carboxylic acid content of cut carnation flowers in relation to their senescence. *Planta* 150:439-442.

BUFLER, G., ROMANI, R.J. AND REID, M.S. 1983. Polysomal population in relation to ethylene production and the senescence of cut carnation flowers and floral parts. *Journal of the American Society of Horticultural Science* 108:554-557.

CARLSON, J.R. 1988. A new means of inducibly inactivating a cellular protein. *Molecular and Cell Biology* 8:2638-2640.

CHANG, A.C.Y., NUNBERG, J.H., KAUFMAN, R.J., ERLICH, H.A., SCHIMKE, R.T. AND COHEN, S.N. 1978. Phenotypic expression in *Escherichia coli* of a DNA sequence coding for mouse DHFR. *Nature* 275:617-623.

CHING YU SHIH, DUMBROFF, E.B. AND THOMPSON, J.E. 1988. Identification of a naturally occurring inhibitor of the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene by carnation microsomes. *Plant Physiology* 89:1053-1059.

CHIRGWIN, J.M., PRZYBYLA, A.E., MACDONALD, R.J. AND RUTTER,W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.

COOK, E.L. 1985. The senescence of the cut carnation (*Dianthus caryophyllus* L. cv. White Sim) flower. *Ph.D. Thesis, University of Natal, Pietermaritzburg.*

COOK, E.L. AND VAN STADEN, J. 1986. The ovary as a sink in the senescence of cut carnation flowers. *Acta Horticulturae* 181:345-351.

COOK, E.L. AND VAN STADEN, J. 1988. The carnation as a model for hormonal studies in flower senescence. *Plant Physiological Biochemistry* 26:793-807

COOK, E.L., VAN STADEN, J. AND ACKERMANN, C. 1988. Manipulating carnation petal senescence. I. The interaction and transport of benzyladenine and indoleacetic acid. *Scientia Horticulturae* 35:143-156.

DANIEL, V. AND GAFF, D.F. 1980. Sulphydryl and disulphide levels in protein fractions from hydrated and dry leaves from resurrection plants. *Annals of Botany* 45:163-171.

DAVIDSON, E.H. AND BRITTEN, R.J. 1973. Organization, transcription and regulation in the animal genome. *Quarterly Review of Biology* 48:565-613.

DAVIDSON, E.H. AND BRITTEN, R.J. 1979. Regulation of gene expression: possible role of repetitive sequences. *Science* 204:1052-1059.

DE VETTEN, N.C., AND HUBER, D.J. 1990. Cell wall changes during the expansion and senescence of carnation (*Dianthus caryophyllus*). *Physiologia Plantarum* 78:447-454.

DOMEC, C.B., GARBAY, M., FOURNIER, H., AND BONNET, J. 1990. cDNA library construction from small amounts of unfractionated RNA: Association of cDNA synthesis with polymerase chain reaction amplification. *Analytical Biochemistry* 188:422-426.

DROILLARD, M-J., BUREAU, D. AND PAULIN, A. 1989. Changes in activities of superoxide dismutases during aging of petals of cut carnations (*Dianthus caryophyllus)*. *Physiologia Plantarum* 76:149-154.

DYER, T.A. AND LEAVER, C.J. 1981. Proteins and Nucleic Acids. In: *The Biochemistry of Plants: A Comprehensive Treatise*. Vol 6. Stumpf, P.K. and Conn, E.E. (eds. in chief). Academic press, New York. pp112-168.

ECKER J.R., AND DAVIS R.W. 1986. Inhibition of gene expression in plant cells by expression of antisense RNA. *Proceedings of the National Academy of Science USA* 83:5372-5376.

ECKER J.R., AND DAVIS R.W. 1987. Plant defence genes are regulated by ethylene. *Proceedings of the National Academy of Science USA* 84:5202-5206.

EFSTRATIADIS, A., MANIATIS, T., KAFATOS, F.C., JEFFREY, A. AND VOURNAKIS, J.N. 1975. Full length and discrete partial reverse transcripts of globin and chorion mRNA's. *Cell* 4:367-378.

EFSTRATIADIS, A., KAFATOS, F.C., MAXAM, A.M. AND MANIATIS, T. 1976. Enzymatic *in vitro* synthesis of globin genes. *Cell* 7:279-288.

EFSTRATIADIS, A., KAFATOS, F.C. AND MANIATIS, T. 1977. The primary structure of rabbit *B*-globin mRNA as determined from cloned DNA. *Cell* 10:571-585.

EISINGER, W. 1977. Role of cytokinins in carnation flower senescence. *Plant Physiology* 59:707-709.

ELLISTON, K. AND MESSING, J. 1989. The molecular architecture of plant genes and their regulation. *In: Plant Biotechnology* Kung, S.D. and Arntzen, C.J. (eds.). Butterworths, London. pp115.

ERLICH, H.A. (ed.) 1989. *PCR technology. Principles and applications for DNA amplification*. Stockton Press, New York. 246pp.

EMERY, A.E.H. 1984. *An introduction to recombinant DNA*. Wiley, New York. 223pp.

ENGLUND, P.T. 1971. The initial step of *in vitro* synthesis of deoxyribonucleic acid by the T4 deoxyribonucleic acid polymerase. *Journal of Biological Chemistry* 346:5684-5687.

EVEN-CHEN, Z., MATTOO, A.K. AND GOREN, R. 1982. Inhibition of ethylene biosynthesis by AVG and by polyamine shunts label [3,4-^{14}C] from methionine into spermidine in aged orange peel slices. *Plant Physiology* 59:707-709.

FEATONBY-SMITH, B.C., VAN STADEN, J. AND COOK, E.L. 1987. Cytokinins in cut carnation flowers. III. *In vitro* growth of ovaries following IAA and [8-*14*C]benzyladenine application. *Plant Growth Regulation* 5:87-96.

FEDORCKSAK, I., NATARAJAN, A.T. AND EHRENBERG, L. 1969. On the extraction of ribonucleic acid with template activity form barley embryos using diethyl pyrocarbonate as nuclease inhibitor. *European Journal of Biochemistry* 10:450-458.

FRENKEL, C., KLEIN, I. AND DILLEY, D.R. 1968. Methods for the study of ripening and protein synthesis in intact pome fruits. *Plant Physiology* 43:1146-1153.

GARROD, AND HARRIS, 1978. Effects if gibberellic acid on senescence of isolated petals of carnation. *Annals of Applied Biology* 88:309-314

GILBART, D.A. AND SINK, K.C. 1971. The effect of endogenous indole acetic acid and keeping quality of poinsettia. *Journal of the American Society of Horticultural Science* 96:3-7.

GOLDBERG, R.B. 1986. Regulation of plant gene expression. *Philosophical Transcripts of the Royal Society of London,* ser. B 314:367-384.

GOULIAN, M., LUCAS, Z.J. AND KORNBERG, A. 1968. Enzymatic synthesis of DNA. XXV. Purification and properties of DNA polymerase induced by infection with phage $T4^{+}$. *Journal of Biological Chemistry* 243:627-638.

GRIERSON, D. 1984. Nucleic acid and protein synthesis during fruit ripening and senescence. In: *Cell ageing and cell death*. Vol. 25. Davies, I. and Sigel, D.C. (eds.). Cambridge University Press, Cambridge. pp189-202.

GRIERSON, D. AND COVEY, S. 1989. *Plant Molecular Biology*. Blackie, New York. pp100-122.

GRINNAN, E.L. AND MOSHER, W.A. 1951. Highly polymerized ribonucleic acid preparation from liver and depolymerization. *Journal of Biological Chemistry* 191:719-725.

GUBLER, U. AND HOFFMAN, B.J. 1983. A simple but very effective method for generating cDNA libraries. *Gene* 25:263-269.

HALABA, J. AND RUDNICKI, R.M. 1989. Invertase inhibitor in wilting flower petals. *Scientia Horticulturae* 40:83-90.

HALEVY, A.H. AND MAYAK, S. 1981. Senescence of cut flowers. *Horticultural Reviews* 3:59-143.

HALEVY, A.H. AND WHITEHEAD, C.S. 1988. Pollination-induced corolla abscission and senescence and the role of short-chain fatty acids in the process. In: *Cell separation in plants. Physiology, biochemistry and molecular biology. Series H. Cell biology*. Vol. 35. Osborne, D.J. and Jackson, M.B. (eds.). Springer Verlag, Berlin. pp221-232.

HANLEY, K.M., MEIR, S. AND BRAMLAGE, W.J. 1989. Activity of ageing carnation flower parts ant the effects of 1-(malonylamino)cyclopropane-1-carboxylic acid-induced ethylene. *Plant Physiology* 91:1126-1130.

HANLEY, K.M. AND BRAMLAGE, W.J. 1989. Endogenous levels of abscisic acid in aging carnation flower parts. *Journal of Plant Growth Regulation* 8:225-236.

HASELHOFF, J. AND GERLACH, W.L. 1988. Simple ENA enzymes with new and highly specific endoribonuclease activities. *Nature* 334:585-591.

HAYMERLE, H., HERZ, J., BRESSAN, G.M, FRANK, R., STANLEY, K.K. Efficient construction of cDNA libraries in plasmid expression vectors using and adaptor strategy. *Nucleic Acids Research* 14:8615-8624.

HEDRICK, S.M., COHEN, D.I., NIELSEN, E.A. AND DAVIS, M.M. 1984. Isolation of cDNA clones encoding T cell-specific membrane associated proteins. *Nature* 308:149-153.

HEIDECKER, G. AND MESSING, J. 1986. Structural analysis of plant genes. *Annual Review of Plant Physiology* 37:439-466.

HERSKOWITZ, I. 1987. Functional inactivation of genes by dominant negative mutations. *Nature* 329:219-222.

HIGUCHI, R., PADDOCK, G.V., WALL, R. AND SALSER, W. 1976. A general method for cloning eucaryotic structural gene sequences. *Proceedings of the National Academy of Science USA* 73:3146-3150.

HOBSON, G.E. AND NICHOLS, R. 1977. Enzyme changes during petal senescence of carnation. *Proceedings of the Associates of Applied Biology* 85:445-447.

HOFFMAN, N., YANG, S.F. AND MCKEON, T. 1982. Identification of 1-(malonylamino)cyclopropane-1-carboxylic acid as a major conjugate of 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor in higher plants. *Biochemical and Biophysical Research Communications* 104:765-770.

HOHN, B. 1979. *In vitro* packaging of lambda and cosmid DNA. *Methods in Enzymology* 68:299-309.

HUPPERT, J. AND PELMONT, J. 1962. Ultrafuge studies of RNA degradation. *Archives of Biochemistry and Biophysics* 98:214-223.

HUNTER, G.D. AND BUTLER, J.A.V. 1956. Stimulation by ribonucleic acid of induced *B*-galactosidase formation in *Bacillus megaterium*. *Biochimica Biophysica Acta* 20:405-409.

HURWITZ, J. AND LEIS, J.P. 1972. RNA-dependent DNA polymerase activity of RNA tumor viruses: I. Directing influence of DNA in the reaction. *Journal of Virology* 9:116-129.

JACOB, F. AND MONOD, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology* 3:318-356.

JEFFCOAT, B. AND HARRIS, G.P. 1972. Hormonal regualtion 9of the istribution of ^{14}C-labelled assimilates in the flowering shoot of carnation. *Annals of Botany* 36:353-361.

JEPSON, I., BRAY, J. JENKINS, G., SCHUCH, R. AND EDWARDS, K. 1991. A rapid procedure for the construction of PCR cDNA libraries from small amounts of plant tissue. *Plant Molecular Biology Reporter* 9:131-138.

JONA, R. 1988. Cell wall pectic content as an early signal for cell separation. In: *Cell separation in plants. Physiology, biochemistry and molecular biology. Series H. Cell biology.* Vol. 35. Osborne, D.J. and Jackson, M.B. eds. Springer Verlag, Berlin. pp 425-437.

JUNGHANS, R.P., DUESBERG, P.H. AND KNIGHT, C.A. 1975. *In vitro* synthesis of full-length DNA transcripts of Rous sarcoma virus RNA by viral DNA polymerase. *Proceedings of the National Academy of Science USA* 72:4895-4899.

KACIAN, D.L., SPIEGELMAN, S. BANK, A., TERADA, M., METAFORA, S., DOW, L., MARKS, P.A. 1972. *In vitro* synthesis of DNA components of human genes of globins. *Nature and New Biology* 235:167.

KACPERSKA, A., KUBACKA-ZEBALSKA, M. 1989. Formation of stress ethylene depends both on ACC synthesis and on the activity of free radical-generating system. *Physiologia Plantarum* 77:231-237.

KAY, E.R.M. AND DOUNCE, A.L. 1953. The preparation of sodium ribonucleate with the use of sodium dodecyl sulfate. *Journal American Chemical Society* 75:4041-4046.

KEITH, B. AND CHUA, N. 1986. Monocot and dicot pre mRNAs are processed with different efficiencies in transgenic tobacco. *European Molecular Biology Organisation Journal* 5:2419-2426.

KELLY, J.W., STABY, G.L. AND CHISM, G.W. 1985. Translocation and metabolism of cytokinin in cut carnations. *Journal of the American Society of Horticultural Science* 110:856-859.

KENDE, H. 1989. Enzymes of ethylene biosynthesis. *Plant Physiology* 91:1-4.

KEY, J. 1989. Modulation of gene expression by auxin. *Bioessays* August-September:52-58.

KIRBY, K.S. 1956. A new method for isolation of ribonucleic acids from mammalian tissues. *Biochemical Journal* 64:405-408.

KIRBY, K.S. 1965. Isolation and characterization of ribosomal ribonucleic acid. *Biochemical Journal* 96:266-270.

KIRBY, K.S. 1968. Isolation of nucleic acids with phenolic solvents. *Methods in Enzymology* 12B:87-91.

KLEPPE, K., OHTSUKA, E., KLEPPE, R., MOLINEUX, I. AND KHORANA, H.G. 1971. Studies on polynucleotides: Repair replication of short synthetic DNA's as catalyzed by DNA polymerases. *Journal of Molecular Biology* 56:341-361.

LAMAR E.E. AND PALMER, E. 1984. Y-encoded species-specific DNA in mice. Evidence that the Y-chromosome exists in 2 polymorphic forms in inbred strains. *Cell* 37:171-177.

LAND, H., GREY, M., HANSER, H, LINDERMAIER, W. AND SCHUTZ, G. 1981. 5'-Terminal sequences of eucaryotic mRNA can be cloned with high efficiency. *Nucleic Acids Research* 9:2251-2266.

LARSEN, P.B., RAGHOTHAMA, K.G. AND WOODSON, W. R. 1990. Molecular cloning of a S-adenosyl methionine synthetase cDNA from carnation. *Supplement to Plant Physiology* 93:44, 247.

LASKOV, R., MARGOLIASH, E., LITTAUER, U.Z. AND EISENBERG, H. 1989. High molecular weight ribonucleic acid from rat liver. *Biochimica et Biophysica Acta* 33:247-253.

LATHE, R. AND LECOCQ J.P. 1977. Overproduction of a viral protein during infection of a *lyc*-mutant of Escherichia coli with phage Lambda-*lmm434*. *Virology* 83:204-206.

LAWTON, K.A., HUANG, B., GOLDSBROUGH, P.B. AND WOODSON, W.R. 1989. Molecular cloning and characterization of senescence related genes from carnation flower petals. *Plant Physiology* 90:690-695.

LAWTON, K.A., RAGHOTHAMA, K.G. AND WOODSON, W.R. 1990. Ethylene-induced senescence-related gene expression requires protein synthesis. *Supplement to Plant Physiology* 93:94, 551.

LEWIN, B. (ed.) 1980. *Gene Expression* 2nd ed. Wiley, New York. pp949-957.

LIEBERMAN, M. 1977. Biosynthesis and bioregulation of ethylene in fruit ripening. In: *Proceedings of the International Society of Citriculture.* Vol 3. W. Grierson (ed.). 258pp.

LIEBERMAN, M. 1979. Biosynthesis and action of ethylene. *Annual Review of Plant Physiology* 30:533-541.

LINCOLN, J.E. AND FISCHER, R.L. 1988. Diverse mechanisms for the regulation of ethylene-inducible gene expression. *Molecular and General Genetics* 211:71-75.

LITTAUER, U.Z. AND EISENBERG, H. 1959. Ribonucleic acid from *Escherichia coli*: Preparation, characterization and physical properties. *Biochimica et Biophysica Acta* 32:320-322.

LYNCH, D.V., SRIDHARA, S. AND THOMPSON, J.E. 1985. Lipoxygenase-generated hydroperoxides account for the nonphysiological features of ethylene formation from 1-aminocyclopropane-1-carboxylic acid by microsomal membranes of carnations. *Planta* 164:121-125.

MACLEAN, D.E. AND DEDOLPH, D. 1962. Effects of N6-benzylaminopurine on postharvest respiration of *Chrysanthemum monifolium* and *Dianthus caryophyllus*. *Botanical Gazette* 124:20-21.

MANIATIS, T., FRITSCH, E.F. AND SAMBROOK, J. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor laboratory, New York.

MANNING, K. 1986. Ethylene production and *B*-cyanoalanine synthase activity in carnation flowers. *Planta* 168:61-66.

MANSSON, P.E., HSU, D. AND STALKER, D. 1985. Characterization of fruit specific cDNA's from tomato. *Molecular and General Genetics* 200:356-361.

MAYAK, S. AND HALEVY, A.H. 1972. Interrelationships of ethylene and abscisic acid in the control of rose petal senescence. *Plant Physiology* 50:341-346.

MAYAK, S. AND KOFRANEK, A.M. 1976. Altering the sensitivity of carnation flowers (*Dianthus caryophyllus L.*) to ethylene. *Journal of the American Society of Horticultural Science* 101:583-585.

MAYAK, S. AND DILLEY, D.R. 1976b. Regulation of senescence in carnation (*Dianthus caryophyllus*). Effect of abscisic acid and carbon dioxide on ethylene production. *Plant Physiology* 58:663-665.

MAYAK, S., VAADIA, Y. AND DILLEY, D.R. 1977. Regulation of senescence in carnation (*Dianthus caryophyllus*) by ethylene. *Plant Physiology* 59:591-593.

MAYAK, S., LEGGE, R.L. AND THOMPSON, J.E. 1983. Superoxide radical production by microsomal membranes from senescing carnation flowers. *Planta* 153:49-55.

MEYER, R.C. AND WOODSON, W.R. 1990. Regulation of petal senescence-related gene expression by cytokinin and ethylene. Supplement to Plant Physiology 93:45, 770.

MEYER, R.C., GOLDSBROUGH,P.B. AND WOODSON, W.R. 1991. An ethylene-responsive flower senescence-related gene from carnation encodes a protein homologous to glutathione s-transferases. *Plant Molecular Biology* 17:277-281.

MURRAY, N.E., BRAMMAR, W.J. AND MURRAY, K. 1977. Lambdoid phages that simplify recovery of *in vitro* recombinants. *Molecular and General Genetics* 150:53-61.

MOR, Y. AND REID, M.S. 1980. Isolated petals. A useful system for studying flower senescence. *Acta Horticulturae* 143:19-25.

MOR, Y. HALEVY, A.H., SPIEGELSTEIN, H. AND MAYAK,S. 1985. The site of 1-aminocyclopropane-1-carboxylic acid synthesis in senescing carnation petals. *Physiologia Plantarum* 65:196-202.

MULLIS, K.B., FALOONA, F., SCHARF, S.J., SAIKI, R.K., HORN, G.T. AND ERLICH, H.A. 1986. Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symposium of Quantitative Biology* 51:263-273.

NAPIER, R.M. AND VENIS, M.A. 1990. Receptors for plant growth regulators: recent advances. *Journal of Plant Growth Regulation* 9:113-126.

NICHOLS, R. 1971. Induction of flower senescence and gynoecium development in the carnation (*Dianthus caryophyllus*) by ethylene and 2-chloroethylphosphonic acid. *Journal of Horticultural Science* 46:323-332.

NICHOLS, R., BUFLER, G., MOR, Y., FUJINO, D.W. AND REID, M.S. 1983. Changes in ethylene production and 1-aminocyclopropane-1-carboxylic and content of pollinated carnation flowers. *Journal of Plant Growth Regulation* 2:1-8.

NICHOLS, S.E. AND LATIES, G.G. 1984. Ethylene-regulated gene transcription in carrot roots. *Plant Molecular Biology* 3:393-401.

NICHOLS, R. AND MANNING, K. 1986. Growth substances and post harvest flower senescence. *Acta Horticulturae* 181:161-167.

NOWAK, J. AND VEEN, H. 1982. Effects of silver thiosulfate on abscisic acid content in cut carnations as related to flower senescence. *Journal of Plant Growth Regulation* 1:153-159.

OKAYAMA, H. AND BERG, P. 1982. High efficiency cloning of full-length cDNA. *Molecular and Cellular Biology* 2:161-170.

OSSWALD, W.F., SHUTZ, W. AND ELSTNER, E.F. 1989. Indole-3-acetic acid and p-hydroxyacetophenone driven ethylene formation from 1-aminocyclyopropane-1-carboxylic acid catalyzed by horseradish peroxidase. *Journal of Plant Physiology* 134:510-513.

OVERBEEK, J.H.M. AND VAN WOLTERING, E.J. 1990. Synergistic affect of 1-aminocyclopropane-1-carboxylic acid and ethylene during senescence of isolated carnation petals. *Physiologia Plantarum* 79:368-376.

OWEN, J. AND NAPIER, J.A. 1988. Abscisic acid: new ideas on its role and mode of action. *Plants Today* March-April:55-59.

PALIYATH, G. AND THOMPSON, J.E. 1988. Senescence-related changes in ATP-dependent uptake of calcium into microsomal vesicles from carnation petals. *Plant Physiology* 88:295-302.

PALMITER, R.D. 1974. Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undegraded polysomes and mRNA. *Biochemistry* 14:3606-3612.

PARK, K.Y., DRORY, A. AND WOODSON, W.R. 1992. Molecular cloning of an 1-aminocyclopropane-1-carboxylate synthase from senescing carnation flower petals. *Plant Molecular Biology* 18:377-386

PELHAM, H.R.B. AND JACKSON, R.J. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *European Journal of Biochemistry* 67: 247-256.

PORTER, A.J.R., BORLAKOGLU, J.T. AND JOHN, P. 1986. Activity of the ethylene-forming enzyme in relation to plant cell structure abd organisation. *Journal of Plant Physiology* 125:207-216.

RABBITTS, T.H. 1976. Bacterial cloning of plasmids carrying copies of rabbit globin mRNA. *Nature* 260:221-225.

RAGHOTHAMA, K.G., LAWTON, K.A., GOLDSBROUGH P.B. AND WOODSON, W.R. 1990. Characterisation of trans-acting factors and cis -elements of a flower senescence-related gene from carnation. *Supplement to Plant Physiology* 93:4, 12.

RAGHOTHAMA, K.G., LAWTON, K.A., GOLDSBROUGH P.B. AND WOODSON, W.R. 1991. Characterization of an ethylene-regulated flower senescence-related gene from carnation. *Plant Molecular Biology* 17:61-71.

REDDY, A.S.N AND POOVAIAH, R.S. 1990. Molecular cloning and sequencing of a cDNA for and auxin-repressed mRNA: correlation between fruit growth and repression of the auxin-regulated gene. *Plant Molecular Biology* 14:127-136.

REID, M.S., FUJINO, D.W., HOFFMAN, N.E. AND WHITEHEAD, C.S. 1984. 1-Aminocyclopropane-1-carboxylic acid- the transmitted stimulus in pollinated flowers? *Plant Growth Regulation* 3:189-196.

ROSS, J., AVIV, H., SCOLNICK, E. AND LEDER, P. 1972. *In vitro* synthesis of DNA complementary to purified rabbit globulin mRNA. *Proceedings of the National Academy of Science USA* 69:264-268.

ROUGEON, F. KOURILSKY, P. AND MACH, B. 1975. Insertion of a rabbit *B*-globin gene sequence into an *E. coli* plasmid. *Nucleic Acids Research* 2:2365-2378.

ROUGEON, F. AND MACH, B. 1976. Stepwise biosynthesis in vitro of globin genes from globin mRNA by DNA polymerase of avian myeloblastosis virus. *Proceedings of the National Academy of Science USA* 73:3418-3422.

SAIKI, R.K., GELFAND, D.H., STOFFEL, S., SCHARF, S.J., HIGUCHI,R., HORN, G.T., MULLIS, K.B. AND ERLICH, H.A. 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* 239:487-491.

SACALIS, J.N. 1989. Effect of gynoecium excision on auxin-mediated promotion of petal senescence in cut carnation flowers. *Journal of Plant Physiology* 133:734-737.

SACALIS, J.N., WULSTER, G. AND JANES, H. 1983. Senescence in isolated carnation petals: Differential response of various petal portions to ACC, and effects of uptake of exudate from excised gynoecia. *Zeitschrift fur Pflanzenphysiologie* 112:7-14.

SALUNKHE, D.K., BHAT N.R. AND DESAI, B.B. (eds.) 1990. *Postharvest biotechnology of flowers and ornamental plants*. Springer Verlag, Berlin. 248pp.

SANDERS, I.O., SMITH, A.R. AND HALL, M.A. 1989. Ethylene metabolism in *Pisum sativum* L. *Planta* 179:104-114.

SARTORIS, S., COHEN, E.B. AND LEE, J.S. 1987. A rapid and improved method for generating cDNA libraries in plasmid and phage lambda vectors. *Gene* 56:301-307.

SATOH, S. AND YANG, S.F. 1989. Inactivation of 1-aminocyclopropane-1-carboxylate synthase by L-vinylglycine as related to the mechanism-based inactivation of the enzyme by s-adenosyl-L-methionine. *Plant Physiology* 91:1036-1039.

SCHERER, S. AND DAVIS, R.W. 1979. Replacement pf chromosome segments with altered DNA sequences constructed *in vitro*. *Proceedings of the National Academy of Science USA* 77:4951-4955.

SCHERER, G., TELFORD, J., BALDARI, C. AND PIROTTA, V. 1981. Isolation of cloned genes differentially expressed at early and late stages of *Drosophila* embryonic development. *Developmental Biology* 86:438-447.

SCHULER, M.A. AND ZIELINSKY, R.E. 1989. *Methods in plant molecular biology*. Academic Press, New York. 171pp.

SEEBURG, P.H., SHINE, J., MARTIAL, J.A., BAXTER, J.D. AND GOODMAN, H.M. 1977. Nucleotide sequence and amplification in bacteria of structural genes for rat growth hormone. *Nature* 270:468-494.

SHERER, G.F.E. AND ANDRE, B. 1989. A rapid response to a plant hormone: auxin stimulates phospholipase A_2 *in vivo* and *in vitro*. *Biochemical and Biophysical Research Communications* 163:111-117.

SILHAVY, T.J., BERMAN, M.L. AND ENQUIST, L.W. 1984. *Experiments with gene fusions*. Cold Spring Harbor, New York. 303pp.

SISLER, E.C. AND GOREN, R. 1981. Ethylene binding - the basis for hormone action in plants? *Whats New in Plant Physiology* 12:37-40.

SISLER, E.C., REID, M.S. AND YANG, S.F. 1986. Effect of antagonists of ethylene action on binding of ethylene in cut carnations (*Dianthus caryophyllus)*. *Plant Growth Regulation* 4:213-218.

SLATER, A., MAUNDERS, M.J., EDWARDS, K., SCHUCH, W. AND GRIERSON, D. 1985. Isolation and characterization of cDNA clones for tomato polygalacturanase and other ripening-related proteins. *Plant Molecular Biology* 5:137-147.

SMITH, T.A. 1985. Polyamines. *Annual Review of Plant Physiology* 36:117-143.

SMITH, C.J.S., SLATER, A. AND GRIERSON, D. 1986. Rapid appearance of an mRNA correlated with ethylene synthesis encoding a protein of MW 35000. *Planta* 168:94-100.

STOVER, C.K., VODKIN, M.H., OAKS, E.V. 1987. Use of conversion adaptors to clone antigen genes in lambda gt 11. *Analytical Biochemistry* 163:398-407.

SYLVESTRE, I. AND PAULIN, A. 1987. Accelerated ethylene production as related to changes in lipids and electrolyte leakage during senescence of petals of cut carnations (*Dianthus caryophyllus*). *Physiologia Plantarum* 70:530-536.

THOMPSON, W.F. AND MURRAY, M.G. 1981. Proteins and Nucleic Acids. Marcus, A. (ed.). In: *The Biochemistry of Plants: A Comprehensive Treatise.* Vol 6. Stumpf, P.K. and Conn, E.E., (eds. in chief). Academic press, New York. pp1-74.

TIETZ, A., LUDEWIG, M., DINGKUHN, M. AND DORFFLING, K. 1981. Effect of abscisic acid on the transport of assimilates in barley. *Planta* 152:557-561.

TILGHMAN, S.M., TIEMEYER, D.C., POLSKY, F., EDGELL, M.H., SEIDMAN, J.G., LEDER, A., ENQUIST, L.W., NORMAN, B. AND LEDER, P. (1977). Cloning specific segments of mammalian genome bacteriophage lambda containing mouse globin and surrounding gene sequences. *Proceedings of the National Acadamy of Science USA* 74:4406-4410.

UPFOLD, S.J. AND VAN STADEN, J. 1990. Cytokinins in cut carnation flowers. VII. The effect of zeatin and dihydrozeatin derivatives on flower longevity. *Plant Growth Regulation* 9:77-81.

VAN DER KROL, A.R., MUR, L.A., BELD, M., MOL, J.N. AND STUITJE, A. 1990. Flavonoid genes in Petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *The Plant Cell* 2:291-299.

VAN DER WESTHUIZEN, A.J. AND DE SWARDT, G.E. 1978. Changes in the individual free amino acid concentrations in the petals of carnations during the vase life of the flowers. *Zeitschrift fur Pflanzenphysiologie* 65:125-134.

VAN STADEN, J. 1989. Cytokinins and auxins in carnation senescence as related to chemical treatments. *Acta Horticulturae* 261:69-80.

VAN STADEN, J. AND DIMALLA, G.G. 1980. The effect of silver thiosulphate on the physiology of cut carnations. II. Influence on endogenous cytokinins. *Zeitschrift fur Pflanzenphysiologie* 99:19-26.

VAN STADEN, J., UPFOLD, S.J., BAYLEY, A.D. AND DREWES, F.E. 1990. Cytokinins in cut carnation flowers. IX. Transport and metabolism of *iso*-pentyladenine and the effects of its derivatives on flower longevity. *Plant Growth Regulation* 9:255-261.

VAN STADEN, J., AND BOSSE, C.A. 1989. Cytokinins in cut carnation flowers. VI. The transport and metabolism of zeatin and dihydrozeatin. *Journal of Plant Physiology* 135:160-163.

VAN STADEN,J. 1989. Cytokinins and auxins in carnation senescence as related to chemical treatments. *Acta Horticulturae* 261:69-80.

VAN STADEN, J., BAYLEY, A.D. AND UPFOLD, S.J. 1990. The effect of Benzyladenine derivatives on flower longevity. *Journal of Plant Physiology* 135:703-709.

VEEN, H., HENSTRA, S. AND DE BRUYN,W.C. 1980. Ultrastructural localisation of silver deposits in the receptacle cells of carnation flowers. *Planta* 148:245-250.

VERMA, I.M., TEMPLE, G.F., FAN, H. AND BALTIMORE, D. 1972. *In vitro* synthesis of DNA complementary to rabbit reticulocyte 10S RNA. *Nature and New Biology* 235:163-167.

VON HIPPEL, P., BEAR, D., MORGAN, W. AND MCSWIGGEN, J. 1984. Protein-nucleic acid interactions in transcription: A molecular Analysis.*Annual Review of Biochemistry* 53:389-446.

VOLKIN, E. AND CARTER, C.E. 1951. The preparation and properties of mammalian ribonucleic acids. *Journal American Chemistry Society* 73:1516-1519.

WANG, H, BRANDT, A.S. AND WOODSON, W.R. 1993. A flower senescence-related mRNA from carnationencodes a novel protein related to enzymes involved in phosphonate biosynthesis. *Plant Molecular Biology* 22:719-724.

WANG, T.T., AND YANG, S.F. 1987. The physiological role of lipoxygenase in ethylene formation from 1-aminocyclopropane-1-carboxylic acid in oat leaves. *Planta* 170:190-196.

WANG, H. AND WOODSON, W.R. 1989. Reversible inhibition of ethylene action and interruption of petal senescence in carnation flowers by norbornadiene. *Plant Physiology* 89:434-438.

WESTPHAL, O., LUDERITZ, O. AND BISTER, F. 1952. Uber die ekstraktion von Bakterien mit Phenol/Wasser. *Zeitschrift fur Naturforschung* 7:148-155.

WICKENS, M.P., BUELL, G.N., SCHIMKE, R.T. 1978. Synthesis of double-stranded DNA complementary to lysozyme, ovomucoid and ovalbumin messenger RNA's-optimization for full length second synthesis by *Escherichia coli* DNA-polymerase. *Journal of Biological Chemistry* 253:2483-2495.

WINNACKER, E-L. 1987. *From genes to clones: Introduction to gene technology.* VCH, Weinheim. 634pp.

WOOD, K.O. AND LEE, J.C. 1976. Integration of synthetic globin genes into *Escherichia coli* plasmids. *Nucleic Acids Research* 3:1961-1971.

WOODSON, W.R. 1987. Changes in protein and mRNA populations during the senescence of carnation petals. *Physiologia Plantarum* 71:495-502.

WOODSON, W.R. AND LAWTON, K.A. 1988. Ethylene-induced gene expression in carnation petals. Relationship to autocatalytic ethylene production and senescence. *Plant Physiology* 87:498-503.

WULSTER, G. SACALIS, J. AND JANES, H. 1982. The effect of inhibitors of protein synthesis on ethylene-induced senescence isolated carnation petals. *Journal of the American Society of Horticultural Science* 107:112-115.

YANG, S.F. AND HOFFMAN, N.E. 1984. Ethylene biosynthesis and regulation in higher plants. *Annual Review of Plant Physiology* 35:184-189.

YOUNG, R.A. AND DAVIS, R.W. 1983. Efficient isolation of genes by using antibody probes. *Proceedings of the National Academy of Science USA* 80:1194-1198.