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DISSERTATION

CLONING AND EXPRESSION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE cDNA FROM ROSA (*Rosa X hybrida*)

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Phiolosophy

Colorado State University

Fort Collins, Colorado

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DONG WANG ENTITLED CLONING AND EXPRESSION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE cDNA FROM ROSA (*Rosa x hybrida*) BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Adviser

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ABSTRACT OF DISSERTATION

CLONING AND EXPRESSION OF

1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE cDNA FROM

ROSA (*ROSA X HYBRIDA*)

Phytohormone ethylene regulates a variety of physiological processes in plant growth and development including fruit ripening, seed germination, leaf and flower senescence. Three enzymes, namely S-adenosyl-methionine synthase, 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase are involved in the catalysis of methionine via S-adenosyl-methionine, ACC into ethylene. Previous studies suggest that ACC synthase plays a key regulatory role in ethylene biosynthesis.

In the present study, the role of ACC synthase in flower petal senescence was investigated. A cDNA library from senescing petals of *Rosa hybrid* cv. Kardinal prepared in λcDNA ZAP Express Vector was probed with a rose-specific 400bp probe developed using degenerate primers by RT-PCR, and eight putative positive clones were isolated. Inserts in these clones varied from 1200 to 1800bp. Except for difference in length, the sequences of these clones were identical. Full-length clone, RKacc7 of 1750bp contains an open reading frame of 480 amino acids (58 KDa) which is preceeded by an untranslated leader of 269bp and ends with a 38bp sequence at the 3'-end. The deduced amino acid sequence of the polypeptide contains the eleven conserved amino

acid residues, the substrate and pyridoxal phosphate binding sites that are characteristic of all ACC synthases.

The *in vitro* transcripts from the full-length clones when translated in rabbit reticulocyte lysates exhibited a 55 KDa polypeptide which comigrated with a polypeptide synthesized from mRNA fraction isolated from senescing petals and were both immunoselected by anti-ACC synthase antibodies. RKacc7 was cloned into pET plasmid for over expression in *E.coli.* Fusion polypeptides with a His-Tag at the amino terminus and a S-Tag at the carboxyl terminus were purified by affinity chromatography. *In vivo* synthesized polypeptides were detected either by S-Tag based detection system or with anti-ACC antibodies in western blot. RT-PCR based studies showed that *in planta* RKacc7 is specifically expressed in rose petals, ovary and sepals. Genomic southern blots probed with RKacc7 showed multiple bands beyond those expected from restriction sites in cDNA or even after taking into account intron lengths. The number and sizes of these DNA fragments are consistent with a pattern expected from a multigene family.

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Chapter 1. Ethylene synthesis, perception and role in flower senescence

The development and survival of any organisms depends on their ability to perceive and respond to their environment. Responses to internal and external signals are frequently elicited by hormones, which promote changes in morphology to accommodate the changes in habitat. In plants ethylene is the simplest hormone that governs plant development and stress-related processes. Almost a century ago, Neljubow identified ethylene as an active component of illuminating gas, which caused the horizontal growth of etiolated pea seedlings (Abeles *et al.*,1992). This 2-carbon olefin is a powerful elicitor of morphological changes during all stages of the plant life cycle. Ethylene can promote seed germination, elongation of stems, petioles, roots, fruit peduncles, and flower fading (Abeles *et al.*, 1992). In seed germination, ethylene causes the hypocotyl to swell and broaden. Treatment of *Arabidopsis* seedlings with ethylene results in a short and thick hypocotyl and root, as well as an exaggerated apical hook. This is known as the triple response, and has been used to isolate ethylene mutants (Guzman and Ecker, 1990). As the plant matures, ethylene also serves as a regulator of a variety of developmental and stress responses, which include

sex determination, fruit ripening, flower senescence, leaf abscission, defense against pathogens, and responses to mechanical trauma. A critical role for ethylene has also been established in the determination of cell fate in the root epidermis (Tanimoto *et al.*, 1995), in pathogen-induced systemic activation of defense genes (Penninckx *et al.*, 1996), as a signal in mediating the wound response of tomato plants (O'Donnell *et al.*, 1996), and in the formation of nitrogen-fixing nodules (Penmetsa and Cook, 1997). The commercial implications of ethylene, particularly in fruit ripening and flower aging, have made this hormone a topic of investigation. The remarkable progresses made in genetics, biochemical, and molecular analyses of ethylene biosynthesis and signaling transduction have enhanced earlier studies in the physiology of the hormone.

I. Ethylene biosynthesis

Ethylene production can be regulated by internal, environmental, and stress factors. The rates of ethylene production vary during stages of growth and development. High rates of ethylene synthesis are identified in actively dividing cells, fruit ripening, and senescing cells. The rate of ethylene production increases with aging of leaves and flowers, and with ripening of climacteric fruits. Plant hormones, such as auxin, can induce ethylene production. There are many examples of increased ethylene production following various disturbances caused by stress. These disturbances can be induced by abiotic or biological agents. Examples of abiotic stress include chemicals, temperature extremes, and mechanical wounding. Biological stress can result from disease and insect damage (Abeles *et al.*, 1992).

1. Ethylene biosynthesis

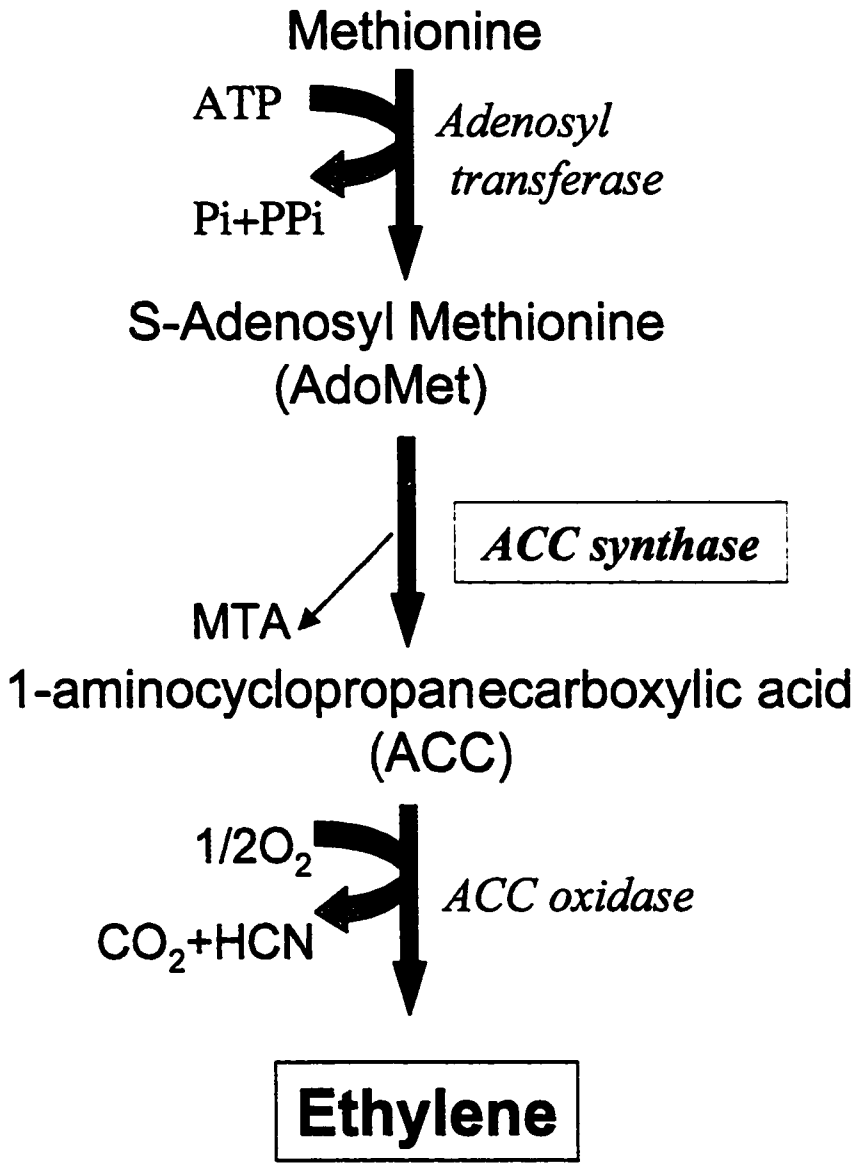
The pathway of ethylene biosynthesis was elucidated by Shang Fa Yang and his collaborators in the late 1970s (Adams and Yang, 1979) and has provided the basis for all subsequent biochemical and genetic analysis of the pathway [Fig.1.1]. All vascular plants analyzed to date synthesize ethylene via the Yang cycle, wherein methionine is the biological precursor of ethylene; it is converted to S-adenosyl-methionine (AdoMet) by the enzyme methionine adenosyl transferase (Zarembinski and Theologis, 1994). AdoMet is transformed into the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. Expression of ACC synthase is induced by stimuli that lead to increased ethylene production, suggesting that ACC synthase activity is rate-limiting for synthesis of ethylene. ACC is then converted into either ethylene, CO_2, and HCN by ACC oxidase or N-malonyl-ACC (MACC) by malonyl transferase. The latter reaction constitutes a possible regulatory step by inactivating ACC. The methionine is recycled through the pathway by converting methylthioadenosine to methionine (Yang and Hoffman., 1984).

2. ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14)

ACC synthase is a cytosolic enzyme which catalyzes the first committed step in the ethylene biosynthetic pathway. Based on the observation that ACC synthase activity is inhibited by pyridoxal 5'-phosphate (PLP)-utilizing enzyme inhibitors and requires addition of exogenous PLP for maximal activity, ACC synthase is believed to

Fig.1.1 *The ethylene biosynthesis pathway*. Ethylene is formed from methionine via AdoMet and the cyclic, nonprotein amino acid ACC. The enzymes catalyzing the individual steps of this pathway are Adenosyl transferase, ACC synthase and ACC oxidase. The MTA (5'-methylthioadenosine is utilized for the synthesis of new methionine via a modified Methionine cycle (Miyazaki and Yang,1987).

Ethylene Biosynthesis



be a PLP-requiring enzyme (Yu *et al.*, 1979). ACC synthase belongs to the α-family of PLP-dependent enzymes and shares a modest level of sequence similarity with other members of this family, like aspartate aminotransferase (AATase) and tyrosine aminotransferase (TATase) (Alexander et al., 1994; Christen & Metzler, 1985). The several isoforms of ACC synthase are present in relatively low abundance and its high lability made it difficult to purify this protein by classic chromatography procedures, and to study its biochemical properties. A cell-free preparation of ACC synthase was first obtained from ripening tomato fruit (Boller *et al.*, 1979). Until 1986, ACC synthase was first purified 6000 fold from the wounded, lithium-treated tomato pericarp by conventional and high-performance liquid chromatography column (Bleecker *et al.*, 1986, 1988). A partially purified preparation of 1-aminocyclopropane-1-carboxylate (ACC) synthase from tomato fruit tissue was used to generate monoclonal antibodies against the enzyme. A monoclonal IgG immunoaffinity gel was used to isolate a single protein from a relatively crude enzyme preparation. Immunoaffinity purification with the monoclonal antibody yielded a 50 kDa polypeptide (Bleecker *et al.*, 1988). *In vivo* labeling of wounded tissue with [^{35}S] methionine followed by extraction and immunopurification in the presence of various protease inhibitors yielded one major radioactive band of 50 kDa molecular mass (Bleecker *et al.*, 1988). Van Der Straeten (1989) purified ACC synthase from LiCl-induced tomato fruit slices. Two-dimensional gel electrophoresis indicated that ACC synthase activity is associated with a 45-kDa polypeptide, with a pI value of 5.8. The

molecular weight of native enzyme purified from etiolated mung bean hypocotyl segments was 125 kDa, which indicated that the enzyme probably existed as a dimer of identical 65 kDa subunits (Tsai *et al.*, 1988). Sato *et al.* (1991) purified ACC synthase from zucchini (*Cucurbita pepo*) fruit tissue. The molecular mass of the native enzyme was found to be 86 kDa by gel filtration, that of the denatured enzyme 46 kDa by SDS-PAGE. This indicates that ACC synthase of zucchini is a dimer consisting of two identical subunits, too. Yip *et al.* (1991) identified apple ACC synthase to be a 48-kDa protein. Antibodies against ACC synthase from wound-induced mesocarp tissue of winter squash recognized ACC synthase from wounded tomato pericarp tissue and wounded winter squash hypocotyls but not from auxin-induced winter squash, tomato or mung bean hypocotyls (Nakagawa *et al.*, 1988). These results indicated that there are two isoforms of ACC synthase, one wound-induced and another auxin-induced, and that the two forms are sufficiently different to be distinguished immunologically. It appears that several isoforms of ACC synthase exist. Wounded tomato fruit tissue contains at least three isoenzymes with pI values of 5.3, 7, and 9 (Mehta *et al* ., 1988). The deduced amino acid sequences of ACC synthase cloned from different plant tissues can be divided into different pI groups: Group I is acidic, pI in the range of 5.61 to 5.74; Group II is near neutral , the pI range is from 6.21 to 7.03; Group III is neutral, pI value is from 7.22 to 7.86; and Group IV is alkaline, the pI is from 8.00 to 8.79 (Fluhr and Mattoo, 1996).

The ACC synthase active site was characterized and sequenced in apple (Yip *et al.*, 1990). The active site was probed with NaB^3H_4 or Ado[^{14}C]Met. HPLC

separation of the trypsin digest yielded a single radioactive peptide. Peptide sequencing of both ^{3}H- and ^{14}C-labeled peptides revealed a common dodecapeptide of Ser-Leu-Ser-Xaa-Asp-Leu-Gly-Leu-Pro-Gly-Phe-Arg, where Xaa was a modified radioactive residue. Acid hydrolysis of the ^{3}H-labeled enzyme released radioactive N-pyrioxyllysine, indicating that the active-site peptide contained lysine at position 4. And their results indicated that it is the same lysine residue at the active site that binds the PLP and covalently links to the 2-aminobutyrate portion of AdoMet during the inactivation and that the active site sequences of the apple and tomato enzymes are highly conserved (Yip *et al.*, 1990). To determine the amino acid residues critical for the structure and function of this enzyme, the tomato Le-ACS2 isoenzyme has been subjected to both site-directed and PCR random mutagenesis (Tarun *et al.*, 1998). Mutant ACC synthases with reduced enzyme activity were selected by a genetic screening. The inactive mutations are clustered in regions that are highly conserved among various ACC synthase (Tarun *et al.*, 1998) suggesting that ACC synthase active sites are conserved. The highest level of homology between different ACC synthases is in the interior portion of the polypeptide, while the carboxyl termini are most divergent (Theologis, 1992; Park *et al.*, 1992; Fluhr and Mattoo, 1996). There are seven regions of high homology among ACC synthases and all known ACC synthases contain, at comparable positions, 11 of 12 invariant amino acids that are involved in the binding of pyridoxal phosphate and substrate in various aminotransferases (Huang *et al.*, 1991 and Rottmann *et al.*, 1991).

The length and primary sequence at the C-terminal region of various ACC synthases sequenced thus far are hypervariable despite a high degree of similarity in the rest of the protein. The expression of an enzymatically active, wound-inducible tomato ACC synthase in a heterologous *E.coli* system provided the opportunity to study the importance of hypervariable C-terminus in enzymatic activity and protein conformation (Li and Mattoo, 1994). Li and Mattoo (1994) constructed several deletion mutants of the ACC synthase gene, expressed them in *E. coli*, purified the protein products to apparent homogeneity, and analyzed both conformation and enzyme kinetic parameters of the wild-type and truncated ACC synthases. Deletion of the C-terminus through Arg429 results in complete inactivation of the enzyme. Deletion of 46-52 amino acids from the C-terminus results in an enzyme that has nine times higher affinity for the substrate S-adenosylmethionine than the wild-type enzyme. The highly efficient, truncated ACC synthase was found to be a monomer of 52 kDa as determined by gel filtration, whereas the wild-type ACC synthase, analyzed under similar conditions, is a dimer. These results demonstrate that the nonconserved C-terminus of ACC synthase affects its enzymatic function as well as dimerization.

Li *et al.* (1996) observed the effects of N-terminal deletions on ACC synthase activity. A series of nested N-terminal deletions were prepared on the full-length and C-terminal deleted ACC synthase cDNAs. These wild-type and mutant ACC synthase cDNAs were over-expressed in a heterologous *E. coli* expression system. It was found that removal of residues 2-12 from the non-conserved N-termini of wildtype and C-terminal deleted ACC synthases led to a slight increase in both *in vivo*

ACC production and *in vitro* ACC synthase activity. Further deletion of 11 amino acids through Glu-23 from the N-termini of both wildtype and C-terminal deleted ACC synthases resulted in a substantial reduction in both *in vivo* ACC production and *in vitro* enzyme activity. Deletion of the amino acid residues 3 through 27, from the N-terminus of ACC synthase abolished enzyme activity.

Kinetic analysis of a highly purified double-deletion (C-terminal and N-terminal) mutant of ACC synthase demonstrated that the K_m of this mutant is 42 μM (Tarun *et al.*, 1998), which is much smaller than that of the corresponding C-terminal deletion mutant, 280 μM, and closer to that of wild type, 22 μM. This suggested a clear effect of the non-conserved N-terminal region on ACC synthase function.

Recent preliminary analysis of the apple ACC synthase crystal structure (Hohenester *et al.*,1994; Capitani et al., 1999) and characterization of the apple enzyme expressed in *E coli* (White *et al.*, 1994) have confirmed the dimeric nature of ACC synthase. So, Fluhr and Mattoo (1996) hypothesized that endogenous or exogenous stimuli that cause activation of ACC synthase might utilize post-translational cleavage at the C-terminus to form a monomeric enzyme with better catalytic efficiency than the unprocessed full-length enzyme.

Another important characteristic of ACC synthase is that its substrate, AdoMet, serves as a suicide inactivator (Satoh and Yang, 1988). At least a portion of AdoMet binds covalently to ACC synthase during irreversible inhibition of the enzyme by the substrate, S-adenosyl-L-methionine (SAM), during its catalytic action (Satoh and Yang, 1988). A functional tomato ACC synthase gene was isolated, sequenced and

expressed in *E. coli* (Li *et al.*, 1992). The expression of this ACC synthase gene in *E. coli* was inactivated by incubation with SAM, the half-time of which was SAM concentration dependent.

The complementary DNA for ACC synthase was cloned first from zucchini fruit (Sato and Theologis, 1989). A cDNA expression library was prepared and screened with antibodies produced against partially purified enzyme from auxin- and lithium-treated tissue. The crude antiserum was purified by affinity chromatography with total proteins from uninduced tissue and the antibodies against uninduced proteins were removed. The purified antibodies were highly enriched for those recognizing ACC synthase. The cloned sequence was expressed in *E. coli* and yeast, and the ACC synthase activity was confirmed. The polypeptide is encoded by a mRNA 1900 nucleotides long. *In vivo* studies using the ACC synthase cDNA as probe showed that the ACC synthase gene is induced by a diverse group of inducers, including wounding, Li^+ ions, and the plant hormone auxin in fruit (Sato and Theologis, 1989).

Subsequently, Van der Straeten and his colleagues(1989,1990a) cloned two ACC synthase cDNAs from ripe tomato fruit. The degenerate oligonucleotides from peptide sequences of the purified enzyme were used to screen a tomato fruit cDNA library. Two partial cDNA clones were obtained corresponding to two genes now known as *LE-ACS2* and *LE-ACS4*.

Since these initial reports, ACC synthase cDNAs and genomic sequences have been cloned from numerous plant species such as apple, zucchini, tomato, Arabidopsis, mung bean, rice, wheat, carnation, geranium, orchid, etc. (Table.1.1). The

Table. 1.1 ACC synthase genes from various hosts

Host	ACC synthase gene	References
tomato	LE-ACS1A LE-ACS1B	Tatsuki et al., 1999
	LE-ACS2 LE-ACS5	Lincoln et al., 1993
	LE-ACS3 LE-ACS4	Nakatsuka et al., 1998
	LE-ACS6 LE-ACS7	Shiu et al., 1998
		Oetiker et al., 1997
		Lincoln et al., 1993
potato	ST-ACS1A ST-ACS1B	Destefano-Beltran et al.,1995
	ST-ACS2	Schlagnhaufer et al., 1995
	ST-ACS4 ST-ACS5 pOIP-1	Schlagnhaufer et al., 1997
mung bean	VR-ACS1 VR-ACS2	Destefano-Beltran et al.,1995
	VR-ACS4 VR-ACS5	Yu et al., 1998
	VR-ACS-7 VR-ACS3	Yi et al., 1999
	VR-ACS-6	
zucchini	CP-ACS1A CP-ACS1B	Huang et al., 1991
	ACCcDNA	Sato and Theologis, 1989
arabidopsis	AT-ACC1 AT-ACC2	van der Straeten et al.,1992
	AT-ACC4 AT-ACC5	Liang et al., 1992
	AT-ACC3	Liang et al., 1993
		Liang et al., 1995
		Abel et al., 1995
wheat	TA-ACS1, TA-ACS2	Subramaniam et al., 1996
carnation	DCACS1, DCACS2	Jones and Woodson, 1999
	CARACC3, CARAS1	Park et al., 1992
	DCACS3	ten Have and Woltering,1997
rice	OS-ACS1 OS-ACS2	Zarembinski,Theologis 1993
	OS-ACS3	Zarembinski,Theologis 1997
geranium	GACS1, GACS2	Clark et al., 1997
	GAC1, GAC2	Wang and Arteca, 1995
apple	Md-ACS1	Sunako et al., 1999
	ACC	Lay-Yee and Knighton, 1995

emerging picture is that ACC synthase is encoded by a divergent multigene family where each gene is differentially regulated by a different subset of inducers. The primary structure of ACC synthase, as deduced from the nucleotide sequence of cDNA clones, shows that the molecular mass of the enzyme from different sources is quite similar. Rapid progress is being made in characterizing the various gene family members encoding ACC synthase.

On the basis of the introns present, ACC synthase genes fall into three classes: four intron genes (zucchini ACC synthase genes: *CP-ACS1A*, *CP-ACS1B*) (Huang et al., 1991), three intron genes (tomato *LE-ACS2* and the rice gene *OS-ACS1*) (Zarembinski and Theologis, 1993), and two intron genes (*LE-ACS3*, *LE-ACS4*, potato gene *ST-ACS1A/1B* and mungbean gene *VR-ACS4* , *VR-ACS5*) (Lincoln *et al*, 1993; Destefano-Beltran *et al.*, 1995).

Two questions concerning the regulation of ACC synthase have been studied using cDNA clones: At what level is ACC synthase regulated? And do developmental, environmental, and chemical factors control the expressions of the same or of different ACC synthase genes?

Many studies have revealed increases in ACC synthase activity during fruit ripening (Itai et al., 1999; Mason and Botella, 1997; Dong and Yang, 1991; Olson *et al.*, 1991; Rottmann *et al.*, 1991 and Yip *et al.*, 1992), flower senescence (Jones and Woodson, 1999; Rottmann *et al.*, 1991; Woodson *et al.*, 1992 and Park *et al.*, 1992), pollination (Llop-Tous *et al.*, 2000; Lindstrom *et al.*, 1999; Bui and O'Neill 1998; Clark *et al.*, 1997) and in response to exogenous signals, such as: wounding (Peck and Kende, 1998; Huang *et al.*, 1991; Olson *et al.*, 1991; and Sato *et al.*, 1989), touch (Arteca and

Arteca, 1999) ; flooding (Olson *et al.*, 1995); IAA (Yi *et al.*, 1999; Peck and Kende, 1998a; Abel *et al.*, 1995), auxin, cytokinin (Botella *et al.*, 1992; Kim *et al.*, 1992; Yip *et al.*, 1992; Huang *et al.*, 1991; Vogel *et al.*, 1998) and ethylene (Peck and Kende, 1998; Woodson *et al.*, 1992 and Rottmann *et al.*, 1991), appear to be based on increased levels of ACC synthase mRNA as shown by RNA blotting or RNase protection assays. Expression of two winter squash ACC synthase genes is differently regulated by auxin and wounding (Nakagawa *et al.*, 1991). At least eight ACC synthase genes have been identified in tomato (Rottmann *et al.*, 1991; Yip *et al.*, 1992; Lincoln *et al.*, 1993; Olson *et al.*, 1995; Nakatsuka *et al.*, 1998). Transcripts corresponding to four ACS genes, *LEACS1A*, *LEACS2*, *LEACS4*, and *LEACS6*, were detected in tomato fruit, and expression analysis using the *ripening inhibitor (rin)* mutant in combination with ethylene treatments and the *Never-ripe (Nr)* mutant has demonstrated that each is regulated in a unique way (Barry *et al.*, 2000).

Two systems of ethylene regulation have been proposed to operate in higher plants (Lelievre *et al.*, 1997). System 1 is functional during normal vegetative growth, is ethylene auto-inhibitory, and is responsible for producing the basal levels of ethylene detectable in all of the tissues including nonripening fruit. System 2 operates during the ripening of climacteric fruit and during petal senescence when ethylene is autostimulatory and requires the induction of both ACC synthase (*ACS*) and ACC oxidase (*ACO*). In tomato, a proposed model suggests that in system1, ethylene is regulated by the expression of *LEACS1A* and *LEACS6*. In fruit a transition period occurs in which the *RIN* gene plays a pivotal role leading to increased expression of *LEACS1A* and induction of *LEACS4*. As a result of increased ethylene synthesis due to *LEACS1A* and *LEACS4* activation, *LEACS2* expression is induced and autocatalysis

is initiated. High ethylene production occurs, resulting in negative feedback on the system-1 developmental pathway, resulting in reduced *LEACA1A* and *LEACS6* expression (Barry *et al., 2000).*

VR-ACS1, VR-ACS6 and *VR-ACS7* are induced by IAA in mungbean (Yi *et al.*, 1999). Results from nuclear run-on transcription assay and RNA gel blot studies revealed that all three genes were transcriptionally active displaying unique patterns of induction by IAA and various hormones in etiolated hypocotyls. In light-grown plants, *VR-ACS1* was induced by IAA in roots, and *VR-ACS6* in epicotyls. IAA was not able to increase the *VR-ACS7* transcript in the light-grown tissues. These results indicate that the expression of the ACC synthase multigene family is regulated by complex hormonal and developmental networks in a gene- and tissue-specific manner in mungbean plants.

Analysis of transgenic tobacco plants with 2.4 kb 5'-upstream region revealed the *VR-ACS7* promoter-driven GUS activity at a highly localized region of the hypocotyl-root junction of control seedlings, while a marked induction of GUS activity was detected only in the hypocotyl region of the IAA-treated transgenic seedlings where rapid cell elongation occurs. In addition to auxin, brassinosteroid, a growth-promoting natural compound with similar structure to animal steroid hormones, has been shown to induce ethylene production and to act synergistically with auxin to stimulate ethylene synthesis in etiolated mung bean seedlings (Yopp *et al.*, 1979). The brassinosteroid alone failed to increase the GUS activity, suggesting that induction of

VR-ACS7 occurs via separate signaling pathways in response to IAA and brassinosteroid. *VR-ACS6* is also induced by auxin (Yoon *et al.*, 1999).

The promoter contained DNA sequences homologous to various functionally identified auxin-responsive elements. Transgenic tobacco plants carrying the 1719 bp *VR-ACS 6* promoter/ GUS fusion gene were created. Strong GUS expression occurred by auxin treatment of leaves of T0 transformants and hypocotyls of T1 etiolated seedlings. Cytokinin enhanced the IAA-induced expression of GUS reporter gene, whereas ABA and ethylene suppressed the expression.

In tomato, *LEACS7* (Shiu *et al.*, 1998) and *LEACS3* (Olson *et al.*, 1995) promoters have been studied in water stressed plants. *LEACS7* is induced when exposed to excess water in root and in the early wound response of leaves, but *LEACS3* is expressed in flooded roots only. Comparison of these two promoters reveals that the *LEACS3* promoter contains anaerobiosis response elements, while the promoter of *LEACS7* is tagged by a *Sol3* transposon. A *Sol3* transposon is also present in the tomato polygalacturonase promoter in which it serves as a regulatory element (Oosumi *et al.*, 1995). Thus, *Sol3* transposons may affect the regulation of *LEACS7* and may be involved in the communication between the root and the shoot of waterlogged tomato plants (Shiu *et al.*, 1998). The promoter of *LEACS7* revealed an exceptionally high degree of similarity (over 1kb) to the promoter of the wound-repressed potato ACC synthase *ST-ACS2* (Destefano-Beltran *et al.*, 1995). *ST-ACS2* is lacking the *Sol3* element of *LEACS7*. Wound-induced *LEACS7* transcript accumulation was transient

and preceded the production of wound ethylene. It is a primary gene in the root-to-shoot communication *of the flooded plant.*

Ps-ACS1 was isolated from a cDNA library prepared from the apical hooks of etiolated pea seedlings that had been treated with 100 μM IAA for 4 hours (Peck *et al.*,1998). The probe for *Ps-ACS1* hybridized to two transcripts on RNA gel blots. Using 5'-RACE to obtain the DNA sequence of the shorter transcript, the short transcript begins within the second exon of the longer one, so the shorter transcript appears to result from the use of an alternative, internal promoter (Peck *et al.*, 1998). In all instances, increased enzyme activity corresponded to increased levels of ACC synthase mRNA which suggests that ACC synthase is primarily regulated at the level of transcription, although there is some evidence that ACC synthase may also be regulated at a post-transcriptional level. Chappell *et al.* (1984) and Felix *et al.*(1991) found that fungal elicitors added to cultured parsley and tomato cells, respectively, induced ethylene synthesis and ACC synthase activity in the presence of the RNA synthesis inhibitors actinomycin D and cordycepin. *LE-ACS3* genomic gene was isolated and sequenced from tomato and expressed during flooding in the roots (Olson *et al.*, 1995). At all time points, the probe from the *LE-ACS3* coding region hybridized to two bands in the RNA blots, which coincide closely with the size of the *LE-ACS3* transcript before and after processing of its intron. This data demonstrates that *LE-ACS3* gene may be regulated at the post-transcriptional level. In addition, cytokinins elevated ethylene biosynthesis in etiolated Arabidopsis seedlings via a post-

transcriptional modification of one isoform of the key biosynthetic enzyme ACC synthase (Vogel *et al.*, 1998).

An interesting aspect of ACC synthase gene expression is its inducibility by protein synthesis inhibitors such as cycloheximide. This result suggests that the expression of the ACC synthase gene may be under the control of a labile repressor molecule or that their transcripts are labile and cycloheximide simply stabilizes them by removing a labile nuclease (Thomas *et al.*, 1994).

3. The Ethylene-Forming Enzyme (ACC Oxidase)

1-Aminocyclopropane-1-carboxylate (ACC) oxidase is responsible for the final step in the production of ethylene by higher plants. ACC oxidase was far more difficult to study than ACC synthase because the activity of the enzyme is completely lost when tissues are homogenized (Kende 1989 and Yang *et al.*, 1984). A major obstacle to a better understanding of ethylene biosynthesis was biochemical purification of ACC oxidase. However, molecular biology has offered an alternative strategy to identify genes related to ethylene synthesis and action. The clone *pTOM13* had been isolated from a ripe tomato fruit cDNA library and shown to be homologous to an mRNA which accumulated in leaves after mechanical wounding. The protein encoded by this mRNA may be ethylene related. Hamilton (1990) transformed tomato with antisense *pTOM13*, which inhibited ethylene production in transgenic plants. Evidence has been presented that the *Cucurbita* ACC synthase *in vitro* translation product has a relative molecular mass of 53 kDa, but *pTOM13* encodes a 35 kDa

protein, indicating that pTOM13 does not encode ACC synthase. The predicted amino-acid sequence of the *pTOM13* polypeptide shows 33% identity and 58% similarity to flavone-3-hydroxylase, supporting the suggestion that *pTOM13* product could be part of the ACC-oxidase system, as the first step of one proposed reaction mechanism involves the hydroxylation of ACC. These data also implied that ACC oxidase might be a member of a class of hydroxylases that require Fe(II) and ascorbate as cofactors. Ververidis and John (1991) were the first to demonstrate authentic ACC oxidase activity in soluble extracts from melon fruit by adding Fe(II) and ascorbate as cofactors to the enzyme assays. Since then, ACC oxidase has been purified and biochemically characterized from apple (Dong *et al.*, 1992; Pirrung *et al.*, 1993) and avocado (McGarvey *et al.*, 1992). ACC oxidase catalyses the following reaction:

$$\text{ACC}+O_2+\text{ascorbate} \xrightarrow[CO_2]{\text{Fe(II)}} C_2H_4+2H_2O+HCN+CO_2 + \text{dehydroascorbate}$$

The CO_2 is required by ACC oxidase (Dong *et al.*, 1992). It was found that in the absence of CO_2, the apple ACC oxidase activity was abolished.

The conservation of histidine residues among the ACC oxidases and other dioxygenases has suggested that ACC oxidase contains critical histidine residues at the active site (John 1997). Shaw and colleagues(1996) have shown that substitution in apple ACC oxidase by site-directed mutagenesis of His 177 by either Phe or Asp; Asp 179 by His and Ala; or His 234 by Phe leads to a complete loss of activity in the crude extracts of *E. coli* cells expressing the mutant proteins. They concluded that His

177, Asp 179 and His 234 are essential for enzyme activity, and suggested that these residues act as the Fe(II)-binding sites (John 1997).

It has long been recognized that various lipophilic compounds such as phosphatidylcholine, Tween 20, Triton X-100, and osmotic shock treatment, all of which could modify membrane structure, greatly reduce the rate of ethylene synthesis in plant tissues. Moreover, when tissues producing ethylene actively are homogenized, the ethylene-forming capability is totally lost. These observations lead to the suggestion that the ethylene-forming system is highly structured and requires membrane integrity (Yang and Hoffman, 1984). Immunofluorescence labelling of pink tomato cells shows that ACC oxidase is largely located at the cell wall (Rombald *et al.*, 1992).

On the basis of *in vivo* conversion of ACC to ethylene, ACC oxidase activity was judged to be constitutive in most instances. Because ACC synthase was shown to be rate limiting for ethylene biosynthesis, smaller changes in ACC oxidase activity were thought to constitute a "fine control" of ethylene formation (Yang and Hoffman, 1984).

Preparations of ACC oxidase purified to varying degrees show the enzyme to be a monomer of 36 to 41 kDa (Fluhr and Mattoo, 1996). Solubilized ACC oxidase was purified from apple fruits to apparent homogeneity. Its molecular mass was shown to be 35 kDa by SDS-PAGE and 39 kDa by gel filtration, indicating that the enzyme is monomeric (Dong *et al.*, 1992).

The first ACC oxidase gene was identified in tomato fruit by differential screening (Slater *et al.*, 1985). A cDNA library prepared from ripening tomato fruit was screened by differential hybridization with cDNA from ripe and mature green tomatoes as probes. One of the ripening-related clones, *pTOM13*, coded for a 35 kDa protein, its mRNA increased during fruit ripening and during wounding of green fruit and leaves, and was correlated with enhanced ethylene synthesis (Smith *et al.*, 1986). This putative cDNA clone was identified by inhibiting ethylene synthesis with an antisense gene expressed in transgenic plants. A direct test of its function was made by expression of a *pTOM13* gene in *Saccharomyces cerevisiae* (Hamilton *et al.*, 1991). Cultures of transformed yeast converted ACC to ethylene, whereas control cells did not. Independent identification of a cDNA encoding ACC oxidase came from studies on elicitor-induced ethylene formation in cultured tomato cells. In such cells, promotion of ethylene synthesis is based on induction of ACC synthase and of ACC oxidase (Spanu *et al.*, 1991). Oocytes of *Xenopus laevis* injected with RNA from elicitor-treated tomato cells gained the ability to convert ACC to ethylene by expression of ACC oxidase. *pTOM13* antisense RNA injected together with tomato cell RNA inhibited the expression of ACC oxidase in oocytes. Therefore, the RNA directing the synthesis of ACC oxidase in oocytes must have had homologous regions to *pTOM13*. Several clones with high homologies to *pTOM13* were isolated from a cDNA library prepared from elicitor-treated tomato cells. One of them, *pHTOM5*, was identified as encoding ACC oxidase by functional expression in oocytes. Sequence

comparison of *pTOM13* and *pHTOM5* showed 88% identity at the nucleotide level (Spanu *et al.*, 1991).

ACC oxidase also is encoded by multiple genes. Those identified thus far are all expressed (Table 1.2). Five gene members have been identified in tomato (Bouzayen *et al.*, 1993), four in Petunia hybrida (Tang *et al.*, 1993) and two in mung bean (Kim and Yang, 1994). A comparision of the nucleotide sequences of ACC oxidase genes, found sequence conservation in the internal coding regions but not in the C-terminal regions which are divergent. The members of ACC oxidase gene family in tomato (Barry *et al.*, 1996) and in broccoli (Pogson *et al.*, 1995) express differentially in response to developmental and environmental cues.

ACC oxidase is constitutively present in most tissues at a low level of activity (Ecker, 1995) and is induced by ACC, which is a product of ACC synthase, the rate-limiting enzyme in the biosynthetic pathway of ethylene, during fruit ripening (McGarvey and Christoffersen, 1992), senescence (Wang and Woodson, 1992), wounding (Callahan *et al.*, 1992) and by fungal elicitors (Spanu *et al.*, 1993). Xylanase from *Tricoderma viride* elicits ethylene biosynthesis in tobacco leaf tissue which is accompanied by an accumulation of ACC, an increase in extractable ACC synthase activity, and increases in ACC synthase and ACC oxidase transcripts (Avni *et al.*, 1994). And ACC oxidase increases in some plants in response to internal and external factors such as auxin, and ethylene that influences its own formation.

Indole-3-acetic acid (IAA), which stimulates ethylene production by enhancing ACC synthase activity, also causes an increase in ACC oxidase transcript and activity

Table.1.2 ACC oxidase genes from various hosts

Host	ACC oxidase cDNA and gene	Reference
tomato	LE-ACO1 LE-ACO2	Barry et al., 1996
	LE-ACO4 LE-ACO3	Nakatsuka et al., 1998
	pTOM13	Hamilton et al., 1991
mung bean	ACO1 ACO2	Yu et al., 1998
	VR-ACO1 VR-ACO2	Kim and Yang,1994
geranium	GACO1 GACO2	Clark et al., 1997
	ACO GACO3	Wang et al., 1994
carnation	CARAO1	ten Have and Woltering,1997
rice	OS-ACO2 OS-ACO3	Chae et al., 2000
	OS-ACO1	Mekhedov and Kende, 1996

levels. The IAA-induced increase in ACC oxidase mRNA level and enzyme activity was blocked by 2,5-norbornadiene (NBD), a competitive inhibitor of ethylene action. This indicates that IAA induced ACC oxidase through the action of ethylene. In the IAA treated pea seedlings, the level of ACC synthase mRNA and enzyme activity started to increase less than 1 hour after the start of IAA treatment, whereas ACC oxidase activity and transcript levels began to rise after 2 hours of IAA treatment. These results indicate that the enzymes of ethylene biosynthesis are sequentially induced after treatment of intact pea seedlings with IAA (Peck and Kende, 1995).

In vivo incubation of tissues with the ethylene precursor ACC demonstrated that ACC oxidase activity increases after pollination in the stigma in orchid flowers (Nadeau *et al.*, 1993). RNA blot hybridization of floral tissues indicates that the increase in ACC oxidase activity is due to *de novo* synthesis of mRNA and presumably protein, which is induced after pollination. Based on comparison of nucleotide sequence between cDNA clone and genomic clone, ACO1 from petunia is encoded in four exons interrupted by three introns. The other three members of the petunia ACC oxidase gene family shared identical intron numbers and positions with ACO1 and their exons showed greater than 80% homology. The proteins encoded by ACO1, ACO3 and ACO4 share more than 90% identity with one another and more than 70% identity with ACC oxidases from other species (Tang *et al.*, 1993). The ACC oxidase proteins share significant sequence homology with other enzymes that require Fe(II) and ascorbate for catalytic activity (Tang *et al.*, 1993).

The fruit specificity of the apple ACC-oxidase promoter was investigated in transgenic tomato plants using a nested set of promoter fragments fused to the *GUS* reporter gene (Atkinson *et al.*, 1998). The promoter fragments 1966bp and 1159bp were generated from the apple ACC oxidase genomic clone. Both fragments conferred ripening-specific expression in transgenic tomato. The results indicate that fruit ripening specificity is maintained by both promoter fragments.

Two promoters of *CM-ACO1* and *CM-ACO3* in melon were characterized during development and in response to various stimuli (Lasserre *et al.*, 1997). The promoter-β-glucuronidase gene *(GUS)* fusion technique has been used to study the expression of *CM-ACO1* and *CM-ACO3* in transgenic tobacco plants. The results indicated that *CM-ACO3* was strongly expressed during flower development, whereas the *CM-ACO1* promoter was positively regulated during senescence and in response to wounding, heavy metal treatment and pathogen attack. *CM-ACO1* has homology with putative wound elements from other wound-induced genes and with characterized ethylene-responsive elements (EREs) (Bouquin *et al.*, 1997). Two separate regions of the *CM-ACO1* promoter activated *GUS* expression in response to ethylene treatment and wounding. These results suggested that induction of *CM-ACO1* gene expression occurs via two separate signal transduction pathways in response to wounding and ethylene treatment (Bouquin *et al.*, 1997).

The chimeric *LEACO1* promoter-β-glucuronidase fusions were transformed into tomato and tobacco to analyse in detail the transcriptional regulation of *LEACO1*

expression (Blume and Grierson, 1997). The results indicated that there were significant increases in *LEACO1* transcription during ripening, leaf and flower senescence, abscission, response to wounding, infection by pathogens, and treatment with methyl jasmonate and α-amino butyric acid (Blume and Grierson, 1997). The *LEACO1* upstream region contains a 420-bp direct repeat which is present in multiple copies in the tomato genome and is very similar to sequences in the promoters of the tomato *E4* (Cordes et al., 1989) and *2A11* genes (Pear et al., 1989). The region covering the repeats resembles the remnant of a retrotransposon. Two copies of a small transposable element have been found in the 5'-flanking sequence and the third intron of *LEACO3* (Blume *et al.*, 1997).

II. Ethylene signal transduction

Ethylene-dependent processes in plants are determined both by the production of the hormone and by the competence of cells to respond. The highly regulated biosynthesis of ethylene discussed above is only the first step in a complex series of events that enable the plant to initiate ethylene-dependent processes. Study of the mechanisms of ethylene perception particularly with the help of genetic mutants has drawn the most detailed picture of plant hormone interactions at the molecular level. The genes involved in ethylene signal transduction have been identified in Arabidopsis (Ecker 1995) and tomato (Wilkinson *et al.*, 1995; Lashbrook *et al.*, 1998; Tieman *et al.*, 2000).

1. Arabidopsis Mutants to Ethylene:

In the model plant *Arabidopsis thaliana*, ethylene induces the triple response in dark-grown seedlings (Abeles *et al.*, 1992; Ecker 1995). Ethylene-treated seedlings display exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and inhibited elongation of the hypocotyl and root. These highly reproducible changes have made it possible to screen large populations of seedlings for mutants that fail to display the triple response, or mutants that constitutively show the response, even in the absence of ethylene. The seedling triple response phenotype in Arabidopsis has been used to study the genetics of the components of the ethylene response pathway, and several classes of ethylene-related mutants have been identified (Johnosn and Ecker, 1998). The first class of mutants are called ethylene-insensitive or *ein* mutants. The second class of mutants are called constitutive response mutants, which can be further subdivided based on sensitivity or lack of sensitivity to ethylene synthesis inhibitors (Johnson and Ecker, 1998). Constitutive response mutants whose phenotypes are suppressed by ethylene synthesis inhibitors produce 10-100 fold more ethylene than wild-type seedlings and are called ethylene overproduce mutants or *eto* mutants. The mutants that display the triple response regardless of the presence of ethylene or ethylene synthesis inhibitors are termed constitutive triple response (*ctr*) mutants (Guzman and Ecker, 1990; Kieber *et al.*, 1993a).

(1) Ethylene-insensitive mutants:

The first ethylene-insensitive mutant locus identified was *etr1*, which is inherited as a single gene, dominant mutation (Bleecker *et al.*, 1988). In the presence of ethylene,

etr1 mutant seedlings display a complete loss of the triple response, possessing elongated hypocotyls and roots, deficient in ethylene-promoted leaf senescence, seed germination, gene expression, and feedback inhibition of ethylene biosynthesis. Assay of ethylene binding in *etr1-1* leaves demonstrated that the mutant bound 80% less ethylene than wild type seedlings, leading to the proposal that the *ETR1* gene encodes an ethylene receptor (Bleecker *et al.*, 1988).

Mutation of the *ein4* locus also results in a dominant ethylene insensitive phenotype, displaying insensitivity to ethylene that is comparable to *etr1* (McGrath and Ecker 1998). Like *etr1* and *ein4*, mutations of the ein2 locus cause virtually complete insensitivity to the effects of ethylene. Ein2 mutations segregate as recessive alleles and display complete insensitivity to ethylene as seedlings, possessing elongated hypocotyls and roots, and a significantly reduced apical hook (Roman *et al.*, 1995). Like *etr1*, the apical hook of *ein2* seedlings is significantly reduced in air without ethylene as well (Roman *et al.*, 1995). In adult plants also, *ein2* mutants are significantly larger than wild type plants, again suggesting that the mutant is resistant to endogenously produced ethylene, levels of which are significantly higher than in their wild-type counterparts (Guzman and Ecker, 1990). These observations suggest that *etr1* and *ein2* mutants are deficient in the negative feedback mechanism that regulates ethylene biosynthesis and completely lacking in ethylene-inducible gene expression (McGrath and Ecker 1998). At least six other ethylene-insensitive loci have been identified in Arabidopsis. Mutations at the *ein3* locus are inherited as single-gene recessive mutations and cause reduced sensitivity to ethylene (McGrath and Ecker

1998). The *ein5*, *ein6* and *ein7* loci were selected as weak ethylene insensitive mutants (van der Straeten *et al.*, 1993; Roman *et al.*, 1995) and recessive mutants (Kieber 1997).

The ers (ethylene response sensor), a gene in the *Arabidopsis thaliana* ethylene hormone-response pathway, was uncovered by cross-hybridization with the *Arabidopsis* ETR1 gene (Hua *et al.*, 1995). The deduced ERS protein has sequence similarity with the amino-terminal domain and putative histidine protein kinase domain of ETR1, but it does not have a receiver domain as found in ETR1 (Chang *et al.*, 1993).

(2) Constitutive response mutants:

The constitutive response mutants are selected as seedlings displaying the triple response when grown in the absence of exogenous ethylene (Zarembinski and Theologis 1994). Plants displaying the constitutive triple response may result from constitutive activation of the ethylene signal transduction pathway or from overproduction of ethylene (Kieber et al., 1993a). Use of inhibitors of ethylene biosynthesis has allowed two types of constitutive response mutants to be identified (Ecker 1995).

The first class of mutants that overproduce ethylene are termed *eto* (ethylene overproducer). Their constitutive triple response phenotype is reverted by treatment with inhibitors of ethylene biosynthesis, or by treatment with ethylene receptor antagonists like silver ion (Guzman and Ecker 1990; Kieber *et al.*, 1993a). These observations suggest that *eto* mutations affect ethylene production. Five different *eto* loci have been identified and they produce from 10-100 fold more ethylene than wild-

type etiolated seedlings (Kieber 1997). The *eto1* is inherited as a recessive trait, *eto2* and *eto3* are inherited as completely dominant traits (McGrath and Ecker 1998).

The second class of constitutive response mutants could not be reverted by inhibitors of ethylene biosynthesis or action and were designated *ctr* mutants (constitutive triple response) (Kieber *et al.*, 1993). As expected, these *CTR* mutants do not make significantly more ethylene than wild-type seedlings. The *ctr* class of mutants is represented by one locus termed *ctr1*, that is inherited as a recessive trait (McGrath and Ecker 1998). The *ctr1* mutation has dramatic effects on the morphology and development of both seedlings and adult plants. Much like *eto* mutants, *ctr1* seedlings display a constitutive triple response in the absence of exogenous ethylene. As adult plants, *ctr1* mutants are severely stunted, and measurement of cell density in the leaf epidermis indicates that the smaller growth habit is due to reduced cell expansion (Kieber *et al.*, 1993a).

A novel mutant, *responsive-to-antagonist1* (*ran1*), is described that displays agonist-like responses to the potent ethylene receptor antagonist *trans*-cyclooctene (TCO) (Hirayama *et al.*, 1999). TCO was chosen for screening because this cyclic olefin acts as a potent competitive inhibitor of ethylene binding to its receptor *in vitro* and *in vivo* (Schaller and Bleecher 1995a). Screening of M2, mutagenized seed populations yielded two independent *ran1* mutants that displayed a characteristic "ethylene" triple response phenotype in response to treatment with TCO. Positional cloning of *RAN1* revealed that this gene encodes a protein with significant similarity to the intracellular copper transporters, human Menkes and Wilson disease proteins and

yeast Ccc2p. In yeast, Ccc2p transports copper from the cytosol into the lumen by the secretory pathway (Yuan *et al.*, 1995). CCC2 gene encodes a P-type ATPase belonging to the Cu^{2+}-ATPase subfamily. Expression of RAN1 in yeast complemented the defect of a ccc2 deletion mutation, indicating that RAN1 possesses copper-transporting activity (Takashi Hirayama *et al.*, 1999).

In addition to triple response, both the hypocotyl elongation and leaf emergence response were used in screening populations of mutant Arabidopsis seedlings, which resulted in a large collection of candidate ethylene mutants. Some of the mutants were allelic to known ethylene mutants, whereas others were identified as novel components of the ethylene response pathway. Three lines have been characterized genetically (Van der Straeten *et al.*, 1999). One of the mutants *mar1* (mimics ACC response) was of special interest, because its phenotype combines both an ethylene insensitive and a partially constitutive response. In the light, the hypocotyl of *mar1* mutant is slightly more elongated, and leaf blades are epinastic and reduced in size. Dark-grown *mar1* seedlings have a partially constitutive triple response that is characterized by a thicker hypocotyl and an exaggerated apical hook. The *mar1* mutation is dominant, and *MAR1* is in the ethylene signal transduction pathway. The *ain2* mutant is strongly insensitive to both ACC and ethylene, with a dramatic delay in flowering time and leaf senescence (Van der Straeten *et al.*, 1999). Besides mar1 and ain2 that were isolated by monitoring hypocotyl elongation of light-grown seedlings, another new ethylene-related locus was identified by screening for leaf emergence mutants. The *slo* mutant showed a delay at several developmental stages, including leaf emergence. The *slo*

mutation enhanced the ACC resistance of *etr1* and *ein2* mutants, indicating that *SLO* acts in an ACC response pathway separate from the one defined by the *ETR1* and *EIN2* genes (Van der Straeten *et al.*, 1999). These new loci may also be involved in the ethylene signal transduction pathway (Table.1.3).

2. Ethylene signal transduction pathway:

The *etr1* mutation seedlings bound 80% less ethylene than wild type seedlings, leading to the proposal that the *ETR1* gene encodes an ethylene receptor (Bleecher *et al.*, 1988). The *ETR1* gene was isolated by positional cloning and found to encode a protein with similarity to bacterial two-component histidine kinases which is a large family of environmental sensors initially characterized in bacteria (Chang *et al.*, 1993). These two-component regulators typically consist of at least two proteins: a sensor histidine kinase (the receptor) that autophosphorylates an internal histidine residue in response to environmental signals; and a response regulator that receives the phosphate group on a conserved aspartate residue from histidine kinase (Chang *et al.*, 1993). The amino-terminal region of ETR1 contains three hydrophobic stretches, each of which is predicted to span a membrane. The binding of ethylene was localized to the amino-terminal, hydrophobic domain of ETR1 (Schaller and Bleecker, 1995a). The carboxy terminus of ETR1 displays similarity to both the histidine kinase and response regulator domains of bacterial two-component sensing systems (Kieber J., 1997).

Table. 1.3. The ethylene response mutants

Mutant	Plant	Comments	Reference
etr1	Arabidopsis	ethylene insensitive ethylene receptor	Chang et al., 1993
Nr	Tomato	ethylene receptor	Yen et al., 1995
eTAE1	Tomato	ethylene receptor	Zhou et al., 1996
LeERT1, LeERT2 LeERT4, LeERT5	Tomato	ethylene receptor	Tieman et al., 2000
ein4	Arabidopsis	ethylene insensitive	McGrath & Ecker, 1988
ein2	Arabidopsis	ethylene insensitive	Roman et al., 1995
ein3	Arabidopsis	ethylene insensitive EIN3 is a nuclear protein	Chao et al., 1997
ein5, ein6, ein7	Arabidopsis	weak ethylene insensitive	van der Straeten, 1993
ers	Arabidopsis	ethylene response sensor	Hua et al., 1995
eto1, eto2, eto3	Arabidopsis	constitutive response	Kieber, 1997
ctr1	Arabidopsis	constitutive response	Kieber et al., 1993
ran1	Arabidopsis	responsive-antagonist	Hirayama et al., 1999
ain2	Arabidopsis	insensitive to ACC and ethylene	van der Straeten,1999
slo	Arabidopsis	delay leaf emergence	van der Straeten,1999

ETR1 is found as a membrane-associated, disulfide-linked dimer in extracts of Arabidopsis and when expressed in yeast (Schaller et al., 1995b). The four *etr1* mutations all occur in this amino-terminal hydrophobic domain within the three transmembrane segments, and one of these mutation, *etr1-1* , has been shown to disrupt ethylene binding both in Arabidopsis and when expressed in yeast, suggesting that the transmembrane segments are required for ethylene binding (Kieber , 1997). The ethylene binding region is the most conserved among the four *ETR1* homologs that have been analyzed.

The gene corresponding to the tomato *Never-ripe (Nr)* mutation has been found to encode a protein with high similarity to ETR1(Yen *et al.*, 1995). A second gene with homology to ETR1, eTAE1, has also been identified from tomato (Zhou *et al.*, 1996). So, based on ETR1 primary amino acid sequence and binding to ethylene, the ETR1 protein is predicted to contain a sensor component fused to a receiver domain of a response regulator.

Bleecker group constructed a chimeric gene consisting of the coding sequence for the first 128 amino acids of ETR1 protein fused to the glutathione S-transferase (GST) coding sequence and expressed in yeast cells. The fusion protein showed ethylene-binding activity. The fusion protein was solubilized and purified from yeast membranes in an active form (Rodriguez *et al.*, 1999). Addition of 300 μM $CuSO_4$ to the isolated yeast membrane expressing the fusion protein led to 10-20-fold increase in ethylene binding activity. They proposed that a copper ion associated with the

ethylene-binding domain in ETR1 is required for high-affinity ethylene-binding activity (Rodriguez *et al.*, 1999). As a result of these studies *responsive-to-antagonist1* (*ran1*) mutant was identified and *RAN1* was cloned. RAN1 protein shows similarity to copper-transporting P-type ATPases, including the human Menkes/Wilson proteins and yeast Ccc2p. Expression of *RAN1* complemented the defects of a ccc2 deletion mutant of yeast, demonstrating its function as a copper transporter to create functional ethylene receptors (Hirayama *et al.*, 1999).

Recent genetic studies of loss-of-function ethylene receptor mutants revealed that these proteins function as negative regulators of the signaling pathway and show significant functional overlap among the ethylene receptors (Hua and Meyerowitz, 1998). A family of ethylene receptors from tomato, designated *LeETR1, LeETR2, NR, LeETR4 and LeETR5*, with homology to the *Arabidopsis ETR1* ethylene receptor, have been cloned (Tieman *et al.*, 2000). Transgenic plants with reduced *LeETR4* gene expression display multiple symptoms of extreme ethylene sensitivity, including severe epinasty, enhanced flower senescence and accelerated fruit ripening. Therefore, LeETR4 is a negative regulator of ethylene responses. The transgenic lines with reduced *NR* mRNA levels exhibit normal ethylene sensitivity but elevated levels of *LeETR4* mRNA, indicating a functional compensation of *LeETR4* for reduced *NR* expression (Tieman *et al.*, 2000). By regulating receptor levels, the plant can modulate the ethylene response even when high levels of ethylene are present. The regulation of both ethylene synthesis and perception allows the plant to initiate an ethylene response in one tissue while suppressing the response in others.

The *CTR1* gene was isolated by T-DNA tagging, and was the first ethylene signaling gene to be cloned (Kieber *et al.*, 1993a). RNA blot analysis indicated that *CTR1* is constitutively expressed, and its expression is not altered by ethylene treatment. Sequence analysis indicated that the *CTR1* gene bore significant homology to the Raf family of mammalian serine/threonine protein kinases (Kieber *et al.*, 1993a). The carboxyl terminus of CTR1 has all the hallmark features of a serine/threonine protein kinase. In mammalian systems, *Raf* kinases have been demonstrated to be activated by the small GTP binding protein *Ras* , and to regulate the mitogen-activated protein kinase (MAPK) cascade. MAP kinases are activated by phosphorylation by MAP kinase kinase (MEK kinase), a class of proteins of which *Raf* kinases are members (McGrath and Ecker, 1998). Loss-of-function mutations in CTR1 result in plants that shows complete activation of all known ethylene-induced phenotypes in the absence of ethylene, indicating that CTR1 is a negative regulator of the pathway. And it is predicted to negatively regulate activity of the ethylene signal transduction pathway through the activation of a MAP kinase cascade (Roman *et al.*, 1995).

Epistasis analysis of *ers* mutants with *ctr1* indicates that CTR1 functions downstream of ERS. Double mutant analysis indicates that *EIN2* acts downstream of *CTR1*, because *ein2ctr1* plants display the Ein- phenotype (Roman *et al.*, 1995). Numerous other Ein- mutants of varying severity have been isolated, including *ein2, ein3, ein5, ein6 and ein7* (Roman *et al.*, 1995). These intermediate to weak mutants all mask the *ctr1* constitutive signaling phenotype, indicating that they act downstream of *CTR1* (Roman *et al.*, 1995).

The ETR1 and ERS1 ethylene receptors both interact with CTR1 (Clark *et al.*, 1998). A portion of CTR1 was co-expressed in yeast along with GST or GST-ETR1 fusions. GST proteins were then affinity purified by binding to glutathione-agarose beads, and the purified proteins examined by Western blot analysis using antibodies directed against GST and CTR1. The CTR1 co-purified with the GST-ETR1 fusion protein, but no CTR1 co-purified with control yeast expressing GST, indicating a specific interaction between CTR1 and ETR1 (Gamble *et al.*, 1999). These proteins interaction was also observed by the yeast two-hybrid protein interaction assay as well as *in vitro* protein association assays (Clark *et al.*, 1998). These two studies demonstrated that the presumed regulatory domain of CTR1 can interact directly with the histidine kinase domain of ETR1 and ERS.

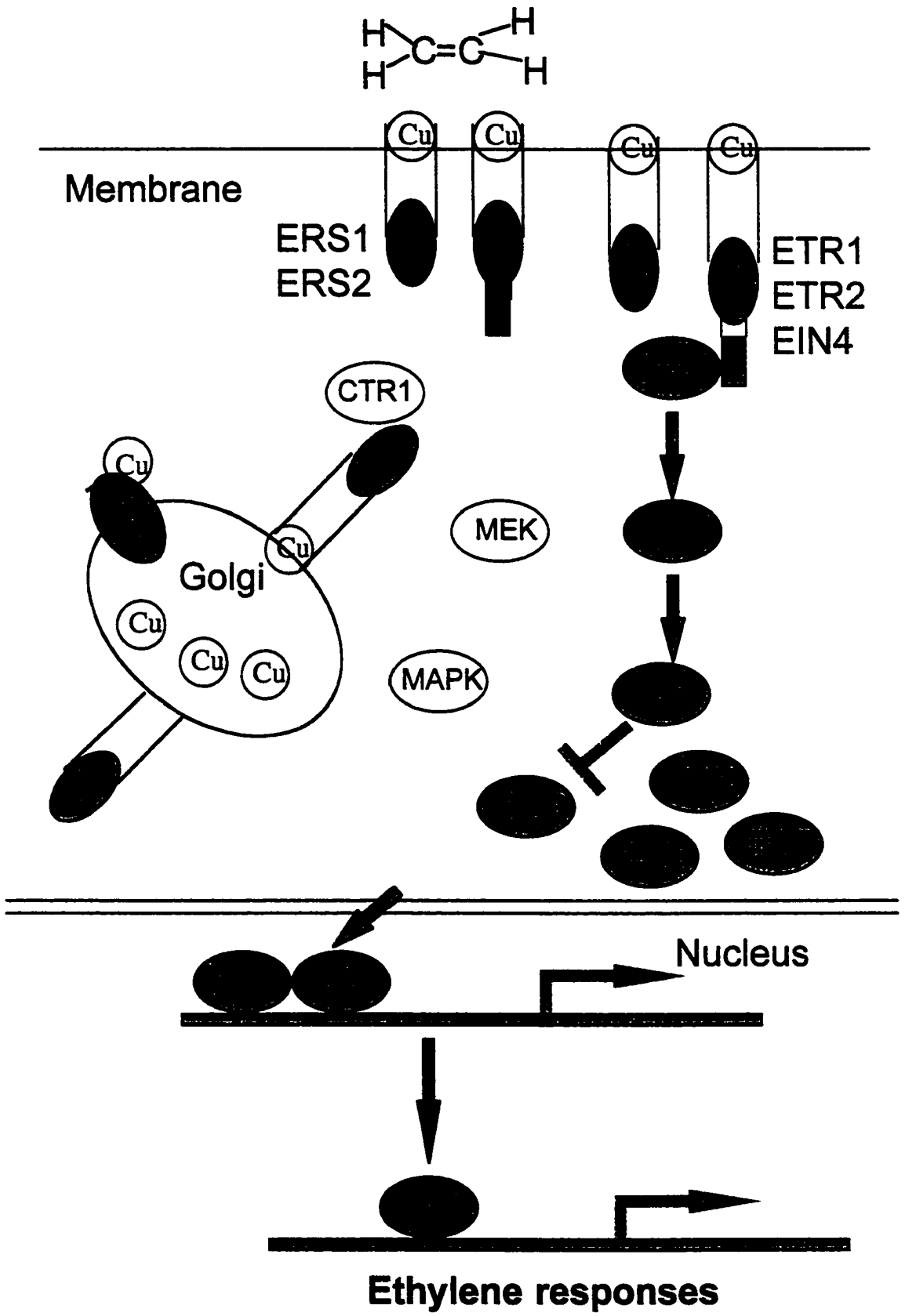
EIN3 (ethylene-insensitive3) gene is a novel postive regulator in the ethylene signaling pathway in Arabidopsis. Its protein displays several features commonly observed in nuclear proteins that serve as transcriptional regulators. *EIN3* was fused to *GUS* reporter gene and introduced into Arabidopsis leaf mesophyll protoplasts and soybean suspension cell protoplasts. The accumulation of GUS activity in the nuclei of cells transformed with fusion gene was confirmed in both Arabidopsis and soybean cells (Chao *et al.*, 1997) demonstrating that the native protein may function in the nucleus. Transgenic plants with *EIN3* gene displayed contitutive ethylene response phenotype throughout development and maturation in the absence of exogenous ethylene. This phenotype is due to increased amount of EIN3 protein, creating an increased sensitivity to endogenous ethylene. This result suggests that high level

expression of *EIN3* is sufficient for the activation of the ethylene pathway. The constitutive ethylene response observed in the *EIN3* expressing plants did not require a functional EIN2 protein as this phenotype was completely unaffected by the presence of homozygous ein2 mutation (Chao *et al.*, 1997). Thus, *EIN3* most likely acts after *EIN2*. Taken together, these results reveal that *EIN3* is a nuclear protein and both necessary and sufficient for activation of all known responses mediated by the ethylene pathway (Chao *et al.*, 1997). Additional classes of transcription factors have been implicated in ethylene-regulated gene expression, such as the ethylene response element binding proteins (EREBPs) (Ohme-Takagi *et al.*, 1995). Expression of some EREBPs is induced by the ethylene-releasing compound ethephan, suggesting that EIN3 is a direct regulator of the transcription factors that control ethylene-regulated genes (Ohme-Takagi *et al.*, 1995).

It is clear now that the ethylene signal is perceived and transduced through a largely linear pathway that begins at the membrane and proceeds to the nucleus [Fig.1.2]. Acting through a putative CTR1-MEK-MAP cascade of protein kinases, a family of transmembrane receptors (ETR1, ETR2, EIN4, ERS1, ERS2) functions as negative regulators of ethylene signaling events. In the nucleus, a family of positive regulatory proteins (EIN3) serves to activate transcriptional responses to the hormone upon repression of receptor function by the binding of ethylene.

Several novel ethylene-responsive (ER) genes were isolated from late immature-green tomato fruit (Zegzouti *et al.*, 1999). Among the isolated ER clones many correspond to regulatory genes involved either in signal transduction or in

Fig.1.2 *The ethylene signal transduction pathway in Arabidopsis.* This model shows that in the absence of the ethylene, the receptors interact and activate the CTR1 protein kinase, which in turn negatively regulates downstream signaling events by repression of the positive acting EIN2 protein. This regulation is through MAPK cascade. The ethylene inactivates the receptors and the CTR1 is no longer able to repress EIN2. The nuclear members of the signaling pathway, EIN3/EILs are positive regulators of ethylene responses, acting downstream of EIN2. RAN is the copper transportor protein, it brings copper to the ethylene receptors (Hirayama et al., 1999, Theologis et al., 1998, Solano and Ecker 1998).



transcriptional and post-transcriptional regulation. ER43 and ER50 share significant homology with a GTP-binding protein and a Raf kinase from the *CTR1* type, respectively. ER24 is homologous to the multibridging factor MBF1, a component of the TAF complex (TATA box binding protein associated factor). ER49 is putative mitochondrial translational elongation factor and potentially involved in the ethylene posttranscriptional regulation of gene expression (Zegzouti *et al.*, 1999).

III. Ethylene and flower senescence

Senescence includes the processes that follow physiological maturity and lead to the death of a whole plant, organ, tissue, or cell. The senescence of flowers is generally rapid and predictable, and therefore they provide an excellent organ for the study of the process. Ethylene is considered a major hormonal regulator of senescence of most plant organs (Abeles, 1992). A large group of flowers is classified as ethylene-sensitive, which includes carnation, orchids, petunia and rose (Woltering and Van Doorn 1988). In these flowers, ethylene production peaks toward senescence, application of exogenous ethylene enhances the process and inhibition of ethylene synthesis or action slows it down (Reid and Wu 1992). In ethylene-sensitive flowers, ethylene is regarded as a signal, mediating a sequence of events that eventually lead to the organ's death. The study of petal senescence provide not only methods to improve the postharvest longevity of cut flowers, but insights into the mechanisms underlying the control of plant senescence in general.

In climacteric flowers, such as carnation, petal senescence is accompanied by a series of biochemical changes including increased respiration, changes in hydrolytic enzyme activity, peroxidation of membrane lipids, and an increase in ethylene production (Borochov and Woodson, 1989). The increase in ethylene plays a critical role in the regulation of petal senescence. Treatment of flowers with inhibitors of ethylene synthesis or action prevents typical petal senescence, while exposure of flowers to exogenous ethylene induces petal senescence (Lawton *et al.*, 1989 and 1990). The increase in ethylene production and onset of petal senescence are associated with dramatic changes in gene expression (Borochov and Woodson, 1989). Two classes of senescence-related gene have been cloned or studied: they are ethylene synthesis required genes, such as ACC synthases gene, and ACC oxidase gene and ethylene response genes.

(1) ACC synthase and ACC oxidase genes:

The senescence of carnation flower petals is associated with a dramatic increase in the synthesis of ethylene. In carnation flower petals, ethylene-induced ethylene production was associated with the accumulation of mRNAs for ACC synthase and ACC oxidase (Drory *et al.*, 1993). CARACC3 is one of the ACC synthase genes cloned from carnation petals. CARACC3 mRNA accumulates during senescence of carnation flower petals concomitant with an increase in ethylene production and ACC synthase enzyme activity (Park *et al.*, 1992). Ethylene induced the accumulation of CARACC3 synthase mRNA in presenescent petals, but not in wound-induced ethylene production in leaves, further suggesting a correlation between flower

senescence and ethylene. These results indicate that CARACC3 represents an ACC synthase transcript involved in autocatalytic ethylene production in senescing flower petals (Park *et al.*, 1992). In presenescence flower petals ethylene production is limited by low activities of both ACC synthase and ACC oxidase. An increase in activities of these enzymes leads to the climacteric-like production of ethylene during petal senescence. It is clear ethylene plays a role in stimulation of both enzymes through an autocatalytic mechanism. Consistent with autocatalytic regulation, treatment of presenescence flowers with exogenous ethylene stimulates both ACC synthase and ACC oxidase activities, whereas interruption of ethylene action with the competitive inhibitor 2,5-norbornadiene inhibits these enzymes and reduces ethylene production in senescing petals (Woodson *et al.*, 1993).

Pollination of flowers often results in very rapid and controlled senescence of petals, and has been found to result in rapid increases in ethylene production by styles, ovaries, receptacles and petals (Nichols, 1977; Nichols *et al.*, 1983; Pech *et al.*, 1987). The interaction of the reproductive structures and the petals, along with the sequential nature of pollination-induced ethylene production, suggests that a transmissible factor is involved in ethylene production and petal senescence (Borochov and Woodson., 1989). Pollen of many flowers is extremely rich in ACC (Whitehead *et al.*, 1983) thus suggesting that early pollination-induced ethylene may be the result of diffusion of ACC out of the pollen to the stigma (Borochov and Woodson 1989). The roles of ACC and ethylene in interorgan signaling during senescence in orchid flowers was investigated by Woltering *et al.* in 1995. Following application of radiolabeled

ACC to the stigma, radiolabeled ethylene is produced by all flower parts. And following ethylene treatment of the central column, ethylene was released by the petals within approximately 0.5 hour. In these flowers, ethylene produced in the stigmatic region following pollination or emasculation serves as a mobile factor responsible for senescence symptoms observed in other flower parts (Woltering *et al.*, 1995). A cDNA sharing sequence identity with ACC synthase and three different cDNAs sharing sequence identity with ACC oxidase were isolated from self-pollination geranium pistils. Transcripts hybridizing with these probes increased slightly in response to self-pollination (Clark *et al.*, 1997).

(2) Ethylene response genes:

Expression of the genes for the ethylene biosynthetic enzymes ACC oxidase and ACC synthase, and the gene for a putative ethylene receptor *RhETR* in rose, were recently examined during flower senescence of a long-lasting cultivar and short flower life cultivar by using the probes from conserved regions of geranium ACC synthase and rose ETR genes (Muller *et al.*, 2000). The abundance of ACC oxidase transcript increased during the late stages of flower development in petals of the short flower life cultivar higher than in the long lasting cultivar. The ACC synthase transcript increased during flower senescence in the long lasting cultivar but remained constant at a low level in the short flower life cultivar. The expression of *RhETR* was distinctly higher in the cultivar with short flower life than in the long-lasting cultivar, and modulation of receptor levels was also observed during flower development (Muller *et al.*, 2000). Exposure to low ethylene concentrations resulted in an up-regulation of *RhETR* in

flowers of both cultivars. Differences in the expression of the putative ethylene receptor of both cultivars suggest that variation in flower longevity may be due to differences in receptor levels during flower development. A cDNA clone encoding a putative ethylene-response sensor *psERS* was isolated from pea flowers. The levels of *psERS* mRNA paralleled ethylene production in petals. Silver thiosulfate treatments were efficient at preventing *psERS* mRNA induction in petals (Orzaez *et al.*, 1999).

One of the flower senescence-related genes from carnation encodes a glutathione S-transferase (GST) (Itzhaki *et al.*, 1994). This gene (*GST1*) was shown to be transcriptionally activated by ethylene specifically during flower petal senescence. Deletion analysis of the 5' flanking sequences of *GST1* identified a single positive regulatory element necessary for ethylene-responsive expression--ethylene-responsive-element (ERE) (Itzhaki *et al.*, 1994).

IV Conclusion:

Ethylene response is regulated at multiple levels, from hormone synthesis and perception to signal transduction and transcriptional regulation. The first committed step in ethylene biosynthesis is conversation of S-adenosyl- methionine to ACC by ACC synthase and ACC is converted to ethylene by ACC oxidase. Wounding, pathogenic attack, flooding, fruit ripening, development, senescence and ethylene treatment itself induce ethylene production. As more genes in the ethylene response pathway are cloned and characterized, they illustrate the precision with which signaling can be regulated. Ethylene binding to copper activated receptors with homology to two-component regulators triggers a kinase cascade that is propagated

through the CTR1 Raf-like kinase and other components to the nucleus. It is likely that more components involved in kinase cascade will be uncovered in the future. Activation of the EIN3 family of nuclear proteins leads to induction of the relevant ethylene-responsive genes via other transcription factors, such as ethylene-responsive element binding proteins (EREBPs), eliciting or mediating a response to flower senescence, and flower senescence induces more ACC synthase, ACC oxidase and ethylene receptors transcripts. A more complete picture of apparently complex roles of ethylene in flower senescence inducible gene expression awaits the analysis of cDNA microarrays containing a larger number of genes.

Therefore, the study of ethylene biosynthesis and transduction pathway in plant should provide insights into the mechanisms underlying the control of plant senescence and ripening in general, and provide the methods to improve the postharvest longevity of commercial cut flowers and climacteric fruits.

Rose is an ethylene-sensitive plant. The ethylene production peaks toward senescence (Woltering and Van Doorn, 1988), application of exogenous ethylene enhances the process and inhibition of ethylene synthesis or action slows it down (Reid and Wu, 1992). The increase in ethylene biosynthesis is the result of increased activities of both ACC synthase and ACC oxidase, which convert AdoMet to ACC and ACC to ethylene, respectively (Woodson *et al.*, 1992). Kardinal rose that is the focus of this research is widely used in the cut flower industry. In this plant it has been observed that the petals fall off shortly after the buds fully open. In order to investigate the role of ethylene in petal senescencing, we investigated the level of

expression of ACC synthase mRNA. For this purpose, a cDNA library was constructed from the aging rose petals and an ACC synthase cDNA was cloned. The present study appears to suggest that the expression of this ACC synthase gene is related to petals senescence in Kardinal rose.

Chapter 2 Rose petal cDNA library construction and ACC synthase gene cloning

I. Introduction

The phytohormone ethylene regulates a variety of physiological processes in plant growth and development. The role of ethylene in fruit ripening, seed germination, leaves and flowers senescence is now well documented (Abeles et al., 1992). Enhanced synthesis of ethylene is also observed when plants are subjected to stress such as wounding, drought and flooding. The biosynthetic pathway for the synthesis of ethylene has been worked out by Yang and coworkers (Adams and Yang, 1979). Three enzymes, namely S-adenosylmethionine synthase, ACC synthase and ACC oxidase are involved in conversion of methionine into ethylene. The conversion of S-adenosyl-L-methionine to ACC by ACC synthase appears to be the rate limiting step in the ethylene biosynthesis (Yang and Hoffman, 1984). This enzyme was first identified in homogenates of ripening tomato pericarp tissue by Boller (1979) and Yu (1979). Subsequently, Sato and Theologis identified the first cDNA clone of ACC synthase from zucchini (Sato and Theologis, 1989). Since then a variety of ACC synthase genes from different plant species have been cloned (Kende, 1993). The studies reveal that

ACC synthase is encoded by a multigene family (Huang et al., 1991; Subramaniam et al., 1996; Rottmann et al.,1991; Liang et al., 1992; Van der Straete et al., 1992, 1995; Botella et al., 1992; Zarembinski et al., 1993) which are differentially regulated in a tissue specific manner during plant growth and development; and in stress.

The role of ethylene in flower senescence is well established (Reid and Wu, 1992; Park et al., 1992; Bui and O'Neill, 1998; Tang et al., 1994; Clark et al., 1997). In carnation, flower senescence is accompanied by increased ethylene production concomitant with the increase in ACC synthase activity and an ACC synthase cDNA clone expressed in the petals has been identified (Park *et al.*, 1992). Rose is a major floriculture plant that commands a significant segment of the flower market. The longevity of cut flowers is an important consideration to the horticulture industry. Different varieties of roses appear to show differences in the rate of flower senescence. In certain rose varieties, such as Kardinal, the petals senescence rapidly on the opening of the bud and shortly thereafter fall off. The present study described the identification of a single ACC synthase cDNA clone from a cDNA library prepared from senescing rose petals.

II. Material and Methods

1. Plant material

Rosa x hybrida (commercial name Kardinal) senescing petals were used for nucleic acid extraction. The flower petals were harvested and immediately frozen in liquid nitrogen, then stored in -70°C until use.

2. Total RNA extraction

Rose total RNA was extracted from aging flower petals using the extraction method described by Manning (1991) with minor modification. The frozen petals (around 4 g) were powdered in liquid nitrogen and ground in a prechilled pestle and mortar. Then 15 ml of extraction buffer (0.2 M boric acid/Tris, 10 mM Na_2EDTA, pH 7.6) were added (2.5-10 ml/g of tissue). Just before use 300 µl (1/50 volume) of 25%(w/v) sodium dodecyl sulfate (SDS) solution and 300 µl (1/50 volume) of 2-mercaptoethanol were added to 15 ml of extraction buffer. The mixture was allowed to thaw and brought to room temperature, then was extracted with an equal volume of a phenol/chloroform (1:1) solution. The phenol used was pre-equilibrated with extraction buffer. Following centrifugation at 12,000 rpm for 15 minutes at room temperature, the upper aqueous phase was decanted and the interface pellet and lower phase were re-extracted with the same volume (15.6 ml) of extraction buffer. After recentrifugation the aqueous phase was combined with the aqueous phase from the first extraction and extracted with an equal volume of phenol/chloroform mixture as before. Forty-two mililiter (42 ml) of DEPC treated water was added to the phenol/chloroform extract (28 ml) to dilute it to

2.5 volume, and 5.5 ml of 1 M Na acetate/acetic acid buffer (pH 4.5) were added to adjust the final Na^+ concentration (including the contribution from Na_2EDTA) to 80 mM and then 30 ml (0.4 volume) of 2-butoxyethanol (2-BE) were added to precipitate polysaccharides. After 30 minutes on ice, the mixture was centrifuged at 12,000 rpm for 20 min. at 0°C and the supernatant was decanted without disturbing the gel-like pellet containing polysaccharides. The nucleic acids in 105 ml of supernatant were precipitated by addition of 45 ml of 2-BE to one volume. After 30 min. on ice, the nucleic acid precipitate was collected by centrifugation at 12,000 rpm for 10 min. at 4°C. The pellet was washed consecutively with 5 ml of extraction buffer: 2-BE mixture (1:1) to remove traces of polyphenols, 5 ml of 70% (v/v) ethanol containing 0.1 M K acetate/acetic acid (pH 6.0) , and 5 ml of absolute ethanol. The pellet was dried at room temperature. The total nucleic acids were dissolved in 600 µl DEPC treated water to give a concentration not less than 1 mg/ml and adjusted to 3 M LiCl by adding 200 µl of 12 M LiCl. It was kept at -20°C for at least 1 hour. RNA precipitate was collected by centrifugation at 12,000 rpm for 15 min. in a microcentrifuge, and the pellets were washed with 500 µl of 3 M LiCl, 500 µl of 70% ethanol and 500 µl of 100% ethanol respectively. Air dried RNA pellets were dissolved in 100 µl DEPC-treated water for mRNA isolation. The total RNA yield was around 300 µg and the 230/260 ratio was 2.5; the 260/280 ratio was 1.8 .

3. mRNA isolation

The rose mRNA was isolated from total RNA by using the protocol of DYNABEADS OLIGO $(dT)_{25}$ (DYNAL product). Dynabeads Oligo $(dT)_{25}$ is designed for rapid isolation of highly purified, intact polyadenylated RNA from eukaryotic total RNA, which relies on base pairing between the poly(A) residues at the 3' end of most messenger RNA and the oligo(dT) residues covalently coupled to the surface of the Dynabeads Oligo$(dT)_{25}$. Other RNA species lacking a poly(A) segment will not form hydrogen bonds with the Dynabeads Oligo$(dT)_{25}$ and are readily washed off.

One hundred microliter (100 µl) of 2X binding buffer (200 mM Tris-HCl pH 8.0, 1M LiCl, 20 mM EDTA, 2%LiDS, 10 mM DTT) and 100 µl of total RNA (300 µg) were added to Dynabeads. The mixture was incubated at 65°C for 2 min. to disrupt secondary structures. The Eppendorf tube with the mixture was rotated gently for 20 min. at room temperature to let polyadenylated RNA binding to oligo (dT). The Dynabeads bound with polyadenylated RNA were washed twice with 200 µl washing buffer (10 mM Tris-HCl pH 8.0, 0.15 M LiCl, 1 mM EDTA) using the Dynal MPC (hand held magnetic stand used to collect Dynabeads). The purified mRNA was eluted by 38 µl of DEPC-treated water by incubation at 65°C for 2 min. About 5 µg of mRNA was recovered from the Dynabeads based on the estimation of the concentration on the ethidium bromide agarose plate with different concentrations of DNA as standards (ZAP Express cDNA synthesis kit instruction manual, Stratagene).

4. cDNA library construction

The ZAP Express™ cDNA synthesis kit (Stratagene) was used to prepare cDNA and construct the rose cDNA library. In this kit the first-strand synthesis is primed with a linker-primer, the oligo(dT) primer linked with a *Xho* I site, and is transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) in the presence of 5-methyl dCTP. The use of 5-methyl dCTP during first-strand synthesis hemimethylated the cDNA, thus protecting it from digestion by *Xho* I. Therefore, on *Xho* I digestion of the cDNA, only the unmethylated site within the linker-primer will be cleaved. The hemimethylated DNA is no longer digested by the *mcr*A and *mcr*B restriction system when it is introduced into XL1-Blue MRF' which is *mcrA*⁻*mcrB*⁻ strain. The *Eco*R I site adapter is ligated to the other end of cDNA. The cDNA is then ready for insertion into the lambda vector [Fig. 2.1].

(1) First-strand synthesis

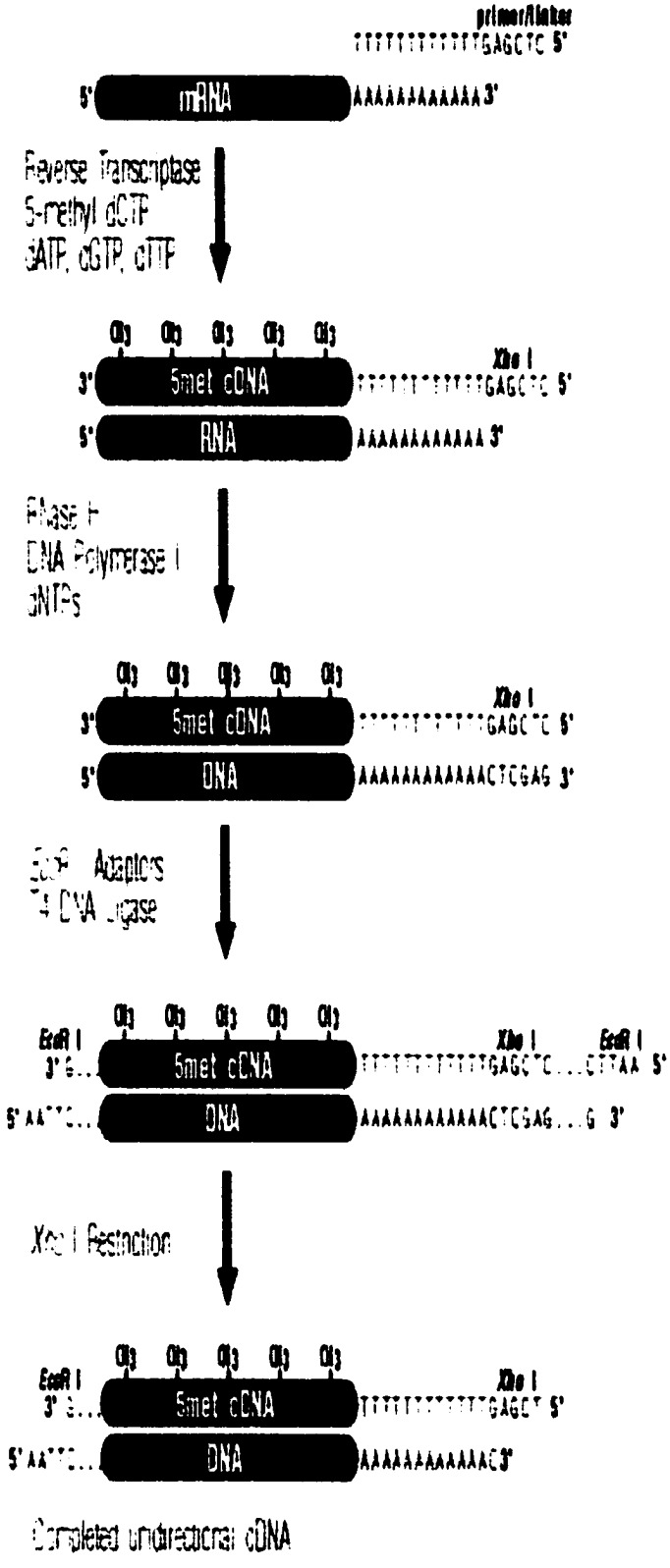
Rose mRNA (37.5 µl) was used as template to synthesize first-strand cDNA with MMLV-RT (75 U), in the presence of dATP, dGTP, dTTP, 5-methyl dCTP, buffer, and the primer:

Xho I

5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTT

The reagents were added in the following order: 5 µl of 10X first strand buffer (0.5 M Tris-HCl pH 8.3, 0.75 M KCl, 0.03 M $MgCl_2$); 3 µl of first strand methyl nucleotide

Fig. 2.1 *Flow diagram of synthesis of cDNA* (Adapted from Stratagene Instruction Manual).



mixture; 2 μl of linker-primer (oligo dT, 1.4 μg/μl); 1 μl of RNase Block Ribonuclease inhibitor (40 U/μl). The mixture was vortexed to ensure the RNase Block Ribonuclease Inhibitor mixed well, then 37.5 μl of rose mRNA was added. The mixture was kept at room temperature for 10 min. to allow the template and the primer to anneal. Finally, 1.5 μl of MMLV-RT (50 U/μl) was added to the mixture to a total reaction volume of 50 μl. The mixture was gently mixed and 5 μl of it was transferred into a separate tube containing 0.5 μl of [α-^{32}P]dATP (10 μCi/μl). This sample was used as the control sample to analyze the quality and quantity of the first strand synthesis. Both tubes were incubated in 37°C water bath for 1 hour. Two microli ter of radioactive control sample was applied onto a 1% agarose gel, subjected to electrophoresis and autoradiography. The results showed that the yield of the first strand of cDNA fragments was high and the size of the synthesized fragments ranged from 1900-2500 bases.

(2) Second-strand synthesis

The RNA bound to the first-strand cDNA was nicked by RNaseH to produce a multitude of fragments, which served as primers for DNA polymerase I. DNA polymerase I "nick-translated" these RNA fragments into second-strand cDNA. Twenty microliter (20 μl) of 10X second-strand buffer (0.188 M Tris-HCl pH 8.3, 0.906 M KCl, 0.046 M $MgCl_2$), 6 μl of second-strand nucleotide mixture, 88 μl of water; 2 μl of [α-^{32}P]dNTP, 2 μl (1.5 U/μl) of RNaseH and 11.1 μl (9.0 U/μl) of

DNA polymerase I were added to the first-strand mixture (45 μl) and the reaction mixture was incubated at 16°C for 2.5 hours, and then the tube was immediately placed on ice. [α-^{32}P]dNTP would serve as an indicator of the size of dsDNA fragments synthesized.

(3) Preparation of dsDNA

Preparation of blunt ended of cDNA termini: The uneven termini of dsDNA were nibbled back or filled in with 2 μl of *Pfu* DNA polymerase (2.5 U/μl) and 23 μl of dNTP mixture. The reaction tube was incubated at 72°C for 30 min. but not more than 30 min. The blunt ended DNA was purified by phenol/chloroform (1:1) and precipitated with ethanol. The pellet was dissolved in 9.0 μl of *Eco*RI adapters and incubated at 4°C for 30 min.. One μl of reaction mixture was subjected to electrophoresis on a 1% agarose gel. The result showed that the size of the second-strand DNA ranged from 500 bp to 2300 bp.

Ligation of the Eco*R I *adapters to cDNA: *Eco*R I adapters with the sequence shown below were ligated to the blunt ended cDNA.

5' -AATTCGGCACGAG-3'

3' GCCGTGCTC-5'

These adapters are composed of 9- and 13- mer oligonucleotides, which are complimentary to each other with an *Eco*R I cohesive end. The 9-mer oligonucleotide is kinased, which allows it to ligate to other blunt termini available in the form of cDNA

and other adapters. The 13- mer oligonucleotide is kept dephosphorylated to prevent it from ligating to other cohesive ends.

One µl of 10X ligase buffer (500 mM Tri-HCl pH 7.5, 70 mM $MgCl_2$, 10 mM DTT), 1 µl of 10 mM rATP and 1 µl of T_4 DNA ligase (4 U/µl) was added to a tube which contained 8 µl of blunted cDNA and the *Eco*R I adapters. The tube was incubated overnight at 4°C to ligate *Eco*R I adapter to the blunted ends dsDNA. After ligation, the tube was placed in a 70°C water bath for 30 minutes to inactivate the ligase.

***Phosphorylation of the Eco*R I *ends of cDNA*:** After the *Eco*R I adapter ligation was complete and the ligase had been heat inactivated, the 13-mer oligonucleotide was treated with kinase to allow its ligation to the dephosphorylated vector arms. One µl of 10X ligase buffer (500 mM Tri-HCl pH 7.5, 70 mM $MgCl_2$, 10 mM DTT), 2 µl of 10 mM rATP, 6 µl of distilled water and 1 µl of T_4 polynucleotide kinase (10 U/µl) were added to the ligation tube, and the mixture was incubated for 30 min. at 37°C. Then the T4 polynucleotide kinase was inactivated at 70°C for 30 min. The reaction mixture was briefly centrifuged and brought to room temperature for 5 min.

Xho* I *Digestion: *Xho* I buffer supplement (28 µl) and 3 µl of *Xho* I (40 U/µl) were added to the kinase treated dsDNA, the reaction mixture was incubated at 37°C for 1.5 hours. After being cooled to room temperature , 5 µl of 10X STE buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA) were added to it.

***Size Fractionation*:** Sephacryl S-500 was packed in a 1 ml plastic syringe, equilibrated with 1X STE buffer, the digested cDNA were pipetted into the Sephacryl S-500 column for size fractionation of cDNA. The column was centrifuged in a tabletop centrifuge (Beckman Model TJ-6) at 400× g. The column was washed twice by 300 µl of 1X STE buffer. Fifty seven (57)µl of *Xho* I digested cDNA were loaded on the column and centrifuged for 2 min. to collect fraction #1. Then 60 µl of 1X STE buffer were added and centrifuged for 2 min. to collect fraction #2. This step was repeated and a total of 10 fractions were collected. The 5 µl from each fraction were taken out to check the size of cDNA in each fraction by electrophoresis in a 1.0% agarose gel. The DNA size in fractions varied from 500 bp to 2300 bp. All fractions were purified with phenol-chloroform, chloroform and precipitated with 100% ethanol at -20°C overnight.

(4) Ligation of cDNA into the ZAP Express Vector Arms

The ZAP Express Vector allows the expression of both eukaryotic and prokaryotic cloned genes. The ß-galactosidase gene is flanked by multiple cloning sites and the inserts cloned into the ZAP Express vector can be excised out of the phage in the form of the kanamycin-resistant pBK-CMV phagemid vector. pBK-CMV phagemid vector is flanked by T_3 and T_7 promoters and has three standardized primer sites (T_3, T_7 and M13) for DNA sequencing.

Fractions #1 and #2 of cDNA (2.5 µl) (0.3 µg and 0.5 µg respectively) were ligated into 1 µl of ZAP Express Vector (1 µg/µl) in the present of 0.5 µl of 10X ligase buffer (500 mM Tri-HCl pH 7.5, 70 mM $MgCl_2$, 10 mM DTT), 0.5 µl of 10 mM rATP

and 0.5 μl of T_4 DNA ligase (4 U/μl). The reaction tubes were incubated at 4°C for 2 days.

(5) Packaging of DNA recombinant phage DNA

Gigapack III packing extracts have been developed in restriction minus *E. coli* ($HsdR^-McrA^-McrBC^-McrF^-Mrr^-$) to increase the efficiency and representation of libraries constructed from highly methylated DNA.

The packing extracts (25 μl) was warmed until the extract just began to thaw, 1 μl of ligated DNA was added to the tube with packing extracts, mixed with a pipette tip and incubated at 22°C for 2 hours (but no more than 2 hours). SM buffer (500 μl) and chloroform (20 μl) were added to the tube and mixed gently.

(6) Titration of λ phages generated by packaging:

The host cells XL1-Blue MRF' were cultured from a single colony in 10 ml of LB medium containing 10 mM $MgSO_4$ and 0.2% (w/v) maltose at 37°C till OD_{600} of 0.6-0.8 (no more than 1.0). The bacteria were harvested by centrifuging at 8,000 rpm for 10 min. and resuspended gently in sterile 10 mM $MgSO_4$ to make the cell suspension an OD_{600} of 0.5. The 1:2 and 1:10 dilution of packaging reaction were mixed with 200 μl of XL1-Blue MRF' cell suspension and the mixtures were incubated at 37°C for 15 minutes with gentle shaking to allow the phage to attach to the host cells. Then the phage-bacteria suspension was mixed with 2.5 ml of NZY top agar, 15 μl of 0.5 M IPTG and 50 μl of X-gal (250 mg/ml in N,N'-dimethylformamide, DMF), and plated

immediately onto the NZY media which contained 12.5 μg/ml tetracycline. The plates were incubated at 37°C overnight.

The expression of *lac* Z gene is required for the generation of a functional ß-galactosidase protein from the ZAP Express vector without a DNA insert. Insertion of a cDNA clone in the polylinker region within the *lac* Z gene of ZAP Express vector will destroy the activity of the *lac* Z gene product, resulting in white plaques. Noninsert-containing background plaques with a functional *lac* Z gene remain blue. By counting the white plaque numbers, the concentration of phage with insert was determined. On average 98% of plaques were white with a packing efficiency of 4×10^5 pfu/μg of cDNA insert.

5. Screening of cDNA library for ACC synthase cDNA

(1) Probe preparation:

The probe (kindly provided by Dr. Janguo Fan) used for screening ACC synthase DNA from the cDNA library was amplified from rose petal mRNA by RT-PCR using designed degenerated primers according to the ACC synthase genes conserved sequences (Zarembinski and Theologis, 1994). The degenerated primers are:

5'-GGIC/ TTICCIGGITC/ TC/ AGIG/ ATIGG-3'

5'-CAIAIICG/ TG/ AAAG/ CC/ AAICCIG/ AG/ CC/ TTC-3'

The amplified probe, 400 bp long, was collected and purified from 1.25% SeaPlaque^{GTG} low-melting temperature agarose gel. This DNA fragment was used as a probe and

labeled with [α-^{32}P]dATP by using the Amersham Megaprime^{TM} DNA labeling system.

The probe DNA (4 μl) was mixed with 17 μl of water and 5 μl of nonamer primer in a microcentrifuge tube. The mixture was incubated in boiling water for 5 minutes to denature the DNA template, then centrifuged at room temperature for 1 minute. The following reagents were added: 4 μl of each dCTP, dGTP, dTTP (300 μM each), 5 μl of 10X reaction buffer (250 mM Tris-HCl pH7.5, 50 mM 2-mercaptoethanol and 25 mM $MgCl_2$), 2 μl of Klenow polymerase (1 U/μl) and 5 μl of [α-^{32}P]dATP(10 μCi/μl). The tube was briefly spun and incubated in 37°C water bath for 1 hour. To stop the reaction, 5 μl of 0.2 M EDTA were added.

Labeling efficiency of the probe:

One microliter of labeled probe solution was taken out and mixed with 199 μl of water and 50 μl of carrier DNA solution to check the labeling efficiency. The tube was incubated at room temperature for 1 hour and 5 μl of this mixture were deposited onto a piece of 3 mm Whatman filter paper to determine the total input radioactivity. The remaining sample was mixed with 3 ml of 10% TCA and left on ice for 10 minutes. The mixture was vortexed and vacuum filtered through a Whatman glass fiber filter. The filter paper was washed 3 times with 10% TCA and air dried. The radioactivity on the filter paper was counted in a liquid scintillator. The probe labeling efficiency was calculated by the formula:

$$\% \text{ incorporation} = \frac{\text{mean count on washed filters}}{\text{mean counts on unwashed filters}} \times 100\%$$

The incorporation in the labeled rose ACC synthase cDNA probe was 65%. It was used for the screening of the cDNA library.

(2) Lifting of the plaque onto Hybond-N^+ Nylon transfer membrane:

The unamplified cDNA library was plated on large (150 mm) NZY plates with 50,000 pfu/plate. The certain amount of phage depending on the packing efficiency was mixed with 600 µl of host cell (XL1-Blue MRF') suspension at an OD_{600} of 0.5, incubated at 37°C for 15 min. with shaking, then transferred to the tube with 6.5 ml of NZY top agar/plate. The mixture was plated on the NZY media immediately. The plates were incubated in 37°C for 6-8 hours. When the plaques became visible (pin prick size), the plates were transferred to 4°C overnight.

The plaques on the NZY plates were transferred onto the Hybond-N^+ Nylon membranes (132 mm, Amersham) by spreading the membrane on the top of the medium for 2 minutes. The position of the membrane on the plate was marked by pricking through the membrane and agar with a 26 gauge needle dipped in India ink. Duplicate lifts of the plaques on membrane were made for each plate.

After plaque lifting, the phage DNA on the membrane were denatured by submerging the membranes in the denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 2 minutes; then in neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl, pH

8.0) for 5 minutes to neutralize the membrane. Finally, the membranes were rinsed with a 0.2 M Tris-HCl (pH 7.5) and 2X SSC buffer solution for 30 seconds. The phage DNA on the membranes were fixed by UV autocrosslinking (Stratagene) and then vacuum dried at 80°C for one hour.

(3) First screening of the plaques on the membrane:

Treatment of membrane with prehybridization solution: Forty-eight membranes with phage DNA were incubated in 100 ml of Rapid hyb-buffer (Amersham) at 65°C for 2.5 hours.

Hybridization of labeled probe: The [α-^{32}P]dATP labeled probe was denatured in boiling water for 10 minutes and mixed with 50 ml of preheated (70°C) Rapid hyb-buffer (Amersham). Then the denatured probe solution was poured onto the prehybridized membranes and the membranes were incubated at 60°C for 4 hours with gently shaking.

Washing of the membranes with the following solutions:

2X SSC with 0.1% SDS 1 liter for 15 min. at room temperature, with gentle shaking;

1X SSC with 0.1% SDS 1 liter for 30 min. at 55°C, with gentle shaking;

0.1X SSC with 0.1% SDS 1 liter for 30 min. at room temperature, with gentle shaking.

After washing, the membranes were dried at room temperature and exposed to X-Ray films (Fuji Medical X-Ray film) at -70°C overnight. The plaques that matched on the duplicate membranes were selected for further screening.

(4) Second screening:

Eight putative positive plaques in the first screening were removed by using Pasteur pipette tips and transferred into microcentrifuge tubes containing 0.5 ml of SM buffer and 20 µl of chloroform. The tubes were vortexed and stored in the refrigerator. This plaque solution was used as plaque stock solution for the second screening. The titer of phage in these samples was determined.

The second screening plating was done on large (150-mm) NZY agar plates with diluted sample expected to yield on the average 1000 plaques per plate. The plates were incubated at 37°C for 8-10 hours to allow plaques to fully develop (unlike in the first screening where the plaques were needle point size). The plating, lifting, blotting, prehybridization and hybridization were performed as in the first screening.

(5) Tertiary screening:

The single positive plaques from the second screening plates were taken out and transferred to a tube with 500 µl SM buffer and 20 µl of chloroform. This plaque solution was titered and used as stock solution for tertiary screening. The plaques for tertiary screening were plated on small (100-mm) NZY plates with about 100 plaques/plate. At this stage of screening, all plaques showed strong positive hybridization to radioactive probe [Fig. 2.3]. The three screenings were performed over a period of two weeks.

6. *In vivo* excision of the pBK-CMV phagemid vector from the ZAP Express Vector

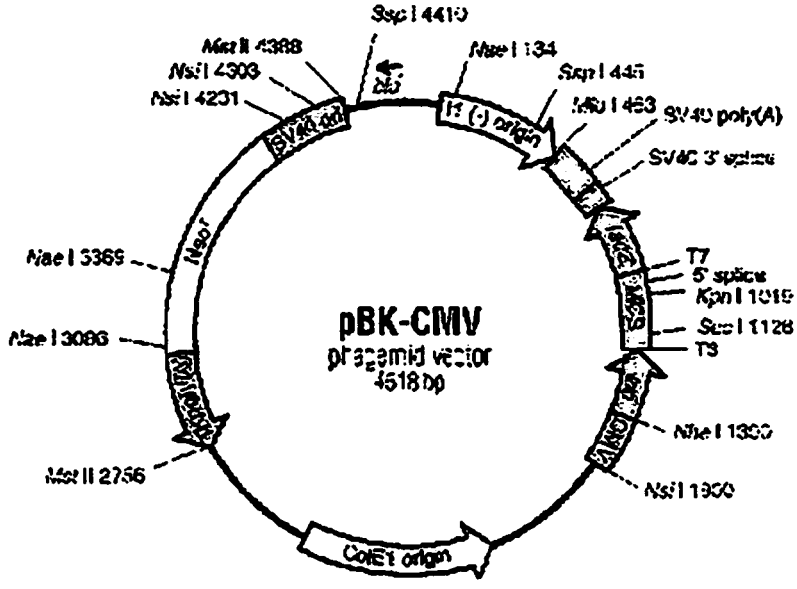
The ZAP Express vector has been designed to allow simple, efficient *in vivo* excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. The ExAssist helper phage with XLOLR strain is designed to allow efficient excision of the pBK-CMV phagemid vector from the ZAP Express vector, while eliminating problems associated with helper phage co-infection. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing *E. coli* strain such as XLOLR cells. This would allow only the excised phagemid to replicate in the host, removing the possibility of productive co-infection from the ExAssist helper phage. Since the ExAssist helper phage cannot be replicated in the XLOLR strain, single-stranded rescue cannot be performed in this strain using this helper phage. DNA from excised colonies can be used for analysis of insert DNA, including DNA sequencing, mapping and expression [Fig. 2.2].

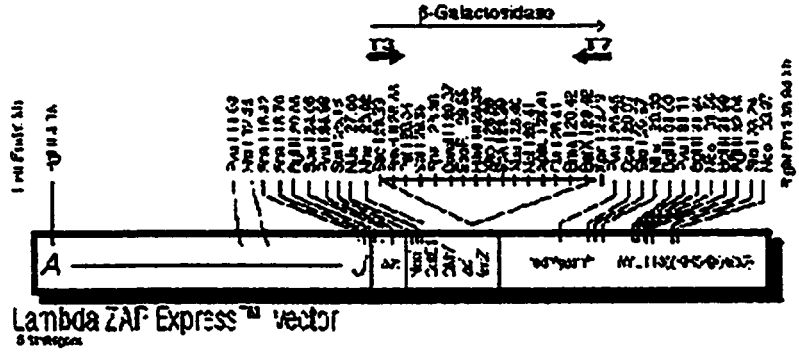
The protocol of in vivo excision:

The positive plaques from the tertiary screening plates were collected and put in a sterile microcentrifuge tube containing 0.5 ml of SM buffer and 20 µl of chloroform. The phage particles were released into the SM buffer by vortexing the tubes. The microcentrifuge tubes were kept in 4°C overnight.

Fifteen mililiter (15 ml) of bacteria XL1-blue MRF' and XLOLR cells were cultured in NZY broth separately at 30°C overnight. The XL1-Blue MRF' growth was supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$. XL1-Blue MRF' and

Fig. 2.2 *Lambda ZAP Express vector and pBK-CMV phagemid vector map.* The rose cDNAs were ligated into the Lambda ZAP Express vector and the ACC synthase positive clone DNAs were excised from Lambda ZAP Express vector and recirclized in pBK-CMV vector.





XLOLR cells were harvested by centrifugation at 1000x g and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 1.0. Two hundred microliters of XL1-Blue MRF' cells at an OD_{600} of 1.0, 250 µl of phage stock solution and 1µl of the ExAssist helper phage (>1 x 10^6 pfu/µl) were combined in a Falcon polypropylene tube and kept in a 37°C water bath for 15 minutes. Three ml of NZY broth were added to the Falcon tube and incubated at 37°C for 4 hours with shaking. After incubation, the Falcon tubes were heated at 65-70°C for 20 minutes and centrifuged at 4000 rpm for 15 minutes. The supernatants were decanted into a sterile Falcon 2059 tube. This stock solution contained the excised pBK-CMV phagemid vector packaged as filamentous phage particles. Two hundred microliters of fresh grown XLOLR cells suspension was added to each of two Falcon tubes with 100 µl and 10 µl of the phage stock solution separately. The tubes were incubated at 37°C for 15 minutes. The NZY broth (300 µl) was added into the tubes with phages and XLOLR cells. These tubes were shaken at 37°C for 45 minutes. Then the cell-phage mixture (200µl) was plated on LB media with 50 µg/ml of kanamycin and the plates were incubated overnight at 37°C.

The colonies appearing on the plate contained the pBK-CMV double-stranded phagemid vector with cloned DNA insert. These colonies were streaked on a new LB-kanamycin agar plate. For long-term storage, the bacteria were stored in 40% glycerol at -80°C.

The phagemid DNA (pBK-CMV) with cloned DNA inserts were purified by miniprep or by CsCl density gradient centrifugation method for sequencing, *in vitro*

transcription, translation and southern blot.

7. Digestion of cDNA with restriction enzymes and Southern hybridization:

The purified phagemid DNA (0.5 µl or 1µg) were digested with 0.5 µl of *Eco*R I (10 U/µl) and 0.5 µl of *Xho* I (10 U/µl), in the presence of 0.5 µl of 10X digestion buffer (60 mM Tris-HCl, 60 mM $MgCl_2$ and 1.5 M NaCl, pH 7.9) and 3 µl of distilled water, to release the inserts from the vector. The digested DNA clones were subjected to electrophoresis in a 1% agarose gel; the sizes of DNA insert varied from 1200 bp to 1800 bp. The cloned DNAs in the agarose gel were depurinized in 0.25 M HCl, denatured in 0.5 M NaOH/ 1.5 M NaCl and blotted onto a Hybond-N^+ Nylon membrane (Amersham) by capillary transfer in 10X SSC. The membrane was prehybridized in Rapid hyb-buffer and the hybridization was done at 60°C for 4 hours with the same [α-^{32}P]dATP labeled probe used in the screening of the cDNA library.

8. Protocol of DNA sequencing:

Amersham Thermo Sequenase radiolabeled terminator cycle sequencing kit was used for DNA sequencing (Fan et al., 1997). This system has two advantages: first, the label is incorporated into the DNA sequencing reaction products by the use of four [α-^{33}P]dideoxynucleotide terminators. The labeled ddNTPs are more efficient for labeling sequencing experiments than other labeled nucleotides because they label only the properly terminated DNA chains. Also, since prematurely terminated chains are not labeled, 'stop' artifacts and most background bands are eliminated. Second, Thermo Sequenase DNA polymerase has been engineered to efficiently incorporate

dideoxynucleotides, allowing the use of very low amounts of isotope ([α-^{33}P]ddNTP) for the termination reactions. Thermo Sequenase DNA polymerase is also thermostable and is convenient for sensitive cycle sequencing protocols.

The DNA templates were purified from the pBK-CMV clones by the miniprep method, and the concentration of DNA used for sequencing was 1 μg/μl.

The sequence reaction mixtures were prepared on ice as follows:

1. Termination mixes: the termination master mixture (7.5 μM dATP, dCTP,dGTP,dTTP) 2 μl were added to the tubes which were labeled 'G', 'A', 'T' and 'C'. And [α-^{33}P]ddGTP (0.5 μl) was added to the tube which was labeled 'G', 0.5 μl each of [α-^{33}P]ddATP, [α-^{33}P]ddTTP, [α-^{33}P]ddCTP were added to the 'A', 'T' and 'C' tubes respectively.

2. Preparation of reaction master mixture: the DNA template (2.0 μg), 2.0 μl of 10X reaction buffer (260 mM Tris-HCl pH 9.5, 65 mM $MgCl_2$) 0.5 μl of primer (5 μM); 2 μl of Thermo Sequenase (4 U/μl) and 13.5 μl of distilled water were mixed in the total volume of 20 μl. Four point five (4.5) μl of master mixture was transferred into the tubes labeled G,A,T,C with the terminator mixture. The reaction mixture was covered by mineral oil.

The cycle conditions were: 95°C for 3 min. one cycle; 95°C for 30 seconds, 55°C for 30 seconds, then 72°C for 1 min. for 40 cycles.

Four microliters (4 μl) of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) were added to each of the termination reactions. The samples were heated at 95°C for 5 min., cooled on ice and loaded on a 6% polyacrylamide gel. The samples were electrophoresed in 1X TBE buffer at 2000 V for 2.5, 4.5 and 6 hours and the temperature of the gel was kept at 55°C. After electrophoresis, the gel was dried under vacuum at 80°C for 1 hour and subjected to autoradiography.

The pBK-CMV contains T_3 primer and $M13\text{-}20$ primer which are universal primers for sequencing the DNA insert. So, the T3 and M13 primers were used as the first primers to sequence the cDNA insert from both the 5' and 3' ends. Also several internal primers were designed for DNA sequencing during the processing. All the positive clone DNAs were sequenced in this method.

9. MacDNAsis software:

MacDNAsis software (Hitachi) was used to align of cotigs of different clone sequences, and to search for homology of the rose ACC synthase DNA sequences with other ACC synthase genes. The software was also used to design the internal primers for sequencing and to analyze the restriction sites in the sequences. The BLAST program was used to search for and retrieve the other ACC synthase genes in GenBank.

10. Southern Blot

ACC synthase gene in the rose genome was investigated by Southern blotting. Genomic DNA was extracted from rose leaves. The protocol is described in Methods in

Plant Molecular Biology and Biotechnology (Taylor et al., 1993). Briefly, 100 mg frozen leaves were ground to a fine powder in liquid nitrogen; 500 µl of preheated (65°C) extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% β-mercaptoethanol) were added and incubated in 65°C water bath for 30 min. Then an equal volume of chloroform/isoamyl alcohol (24:1) was added and the sample was mixed gently for several minutes at room temperature. The sample was centrifuged at top speed (10,000 rpm) in a microfuge for 20 min. The aqueous phase was removed and re-extracted with chloroform/isoamyl alcohol (24:1). The supernatant was transferred to a clean tube and one volume of precipitation buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% β-mercaptoethanol) was added to precipitate DNA. The DNA precipitate were recovered by centrifugation at 12,000 rpm for 5 min. and resuspended in 200 µl of 1.0 M NH_4OAc. The NH_4OAc concentration was adjusted to 2.5 M by adding 120 µl of 5 M NH_4OAc. The sample was mixed well and the nucleic acid was precipitated with 2 volumes of isopropanol and centrifuged (10,000 rpm) for 15 min. The DNA pellet was resuspended in 200 µl of 1.0 M NH_4OAc again and treated with RNaseI at 37°C for 15 min. The pure DNA was precipitated by 2.5 M NH_4OAc and 2 volume of isopropanol. The pellet was washed with 70% ethanol.

The rose genomic DNA samples (30 µg) were digested with *Bam*H I, *Eco*R I and *Hind* III. The digested DNAs were electrophoresed in 0.8% agarose gel at 60V for 7 hours. The digested genomic DNA on the gel was depurinized in 0.25 M HCl for 20 min. and transferred to N+hybond Nylon membrane by capillary transfer in 0.4 M

NaOH overnight (Amersham product manual). The membrane was UV crosslinked and baked at 80°C for one hour.

The probe used for Southern hybridization was obtained from RKacc7 insert following digestion of pBK-CMV vector with *Sal* I and *Not* I. The insert fragment was extracted from 1.25% low-melting temperature agarose gel and purified by phenol, chloroform/isoamyl alcohol (24:1). The probe was labeled with [α-^{32}P]dATP. The protocol of labeling was modified from Megaprime DNA labeling system (Amersham) as follows:

Ten µl of purified ACC synthase cDNA insert (200 ng), 5 µl random nonamer primer solution and 35 µl of distilled water were mixed. The mixture was heated at 95°C for 10 min., then centrifuged in a microfuge at top speed for 1 min. The 10X reaction buffer (250 mM Tris-HCl pH7.5, 50 mM 2-mercaptoethanol and 25 mM $MgCl_2$) 5 µl, 4 µl of each of dCTP, dGTP, dTTP (300 µM) and 6 µl of [α-^{32}P]dATP (10 mCi/ml) were added to the tube with DNA template and primer. The total volume was adjusted to 98 µl with distilled water. Finally, 2 µl of Klenow fragment (2U/µl) was added to the mixture. The reaction was incubated in a 37°C water bath for 1 hour. The probe labeling incorporation was measured with 2 µl of reaction mixture. One µl of reaction mixture was used for total radioactivity and 1 µl was precipitated with 10% TCA for incorporation of label in the probe. Nearly 57% of [α-^{32}P]dATP label was incorporated in the probe.

The membrane was prehybridized in Rapid hyb-buffer at 55°C for 2 hours and hybridized with [α-^{32}P]dATP labeled RKacc7 probe at 55°C for 5 hours. The membrane was washed with 2X SSC containing 0.1% SDS at room temperature for 15 min., 1X SSC containing 0.1% SDS at hybridization temperature 55°C for 30 min., and 0.1X SSC with 0.1% SDS at room temperature for 30 min. The membrane was air dried and subjected to autoradiography. For the high stringency hybridization, the membrane with the probe was stripped in 0.4 M NaOH at 60°C for 1 hour. The stripped membrane was prehybridized in Rapid hyb-buffer at 55°C for 2 hours and hybridized at 60°C for 5 hours.

III. Result and Discussion

The strategy I have used for the isolation of ACC synthase gene from rose was to create a specific ACC synthase probe from rose mRNA and use this probe to screen the cDNA library. At first, two degenerate primers were designed based on the conserved regions of ACC synthase genes published previously (Zarembinski and Theologis, 1994). A cDNA fragment was amplified from rose petal mRNA by PCR priming with these degenerate primers. A 400 bp amplified cDNA fragment was isolated from the agarose gel, it was cloned into TA cloning vector (Invitrogene) and sequenced. The sequence of this amplified cDNA fragment showed high homology to the ACC synthase gene.

In this study, the mRNA extracted from senescing rose petals was used to prepare a cDNA library. The double strand cDNA fragments larger than 1500 bp were size selected and packaged in lambda ZAP expression vector. Approximately 1.28 x 10^6 clones from the rose unamplified cDNA library were screened using the specifically amplified probe from the rose petal mRNA fraction. After three cycles of screening, a total of eight putative positive clones were selected [Fig.2.3]. These positive clones were excised from phage and stored in phagemid pBK-CMV. The cloned rose cDNA inserts were released after digestion with *Xho* I and *Eco*R I at the multiple cloning sites and showed high homology to the probe by Southern hybridization [Fig.2.4]. The sizes of insert in these clones varied from 1200 bp-1800 bp.

All eight positive clones were sequenced from both 5' and 3' ends by using T_3 primer, M_{13} primer and designed internal primers. The sequence data showed that

except for differences in length seven out of eight clone sequences are identical [Fig.2.5]. Two of the clones are full-length cDNA and contained the start and stop codons; the others are partial cDNA without the start codon. The longest clone consisted of 1750 bp (RKacc7), and the shortest one consisted of 1270 bp (RKacc6). RKacc 7 contains an open reading frame of 480 amino acids sufficient to encode a protein of 58 KDa. The open reading frame is proceded by 269 bp of an untranslated leader and is followed by 38bp of untranslated sequences at the 3'-end [Fig.2.6]. Seven regions have been identified as highly conserved among all the ACC synthase sequences characterized thus far. One of the conserved regions includes the active-site lysine residue (GISKDLSLPGFRVR), which binds pyridoxal phosphate and AdoMet (Yip et al., 1990). These eleven amino acid residues are common to all ACC synthases and certain amino transferases. But in RKacc7 amino acid sequence, there is a serine at position 133 instead of tyrosine; an asparagine at 276 instead of aspartic acid and phenylalanine at 280 instead of tyrosine [Fig.2.7]. A comparison of other ACC synthase sequences in the GenBank Database with RKacc7 shows that the RKacc7 has 57% nucleic acid and 54% amino acid sequence homology to wheat ACC synthase gene (U35779) [Fig.2.8]. The phylogenetic analysis revealed that RKacc7 is very loosely clustered with the other dicots ACC synthase genes based on the nucleic acid sequences.

A multigene family for ACC synthase sequences has been reported for zucchini (Huang et al., 1991), tomato (Rottmann et al., 1991), Arabidopsis (Liang et al., 1992; Van der Straeten et al., 1992), mungbean (Botella et al., 1992), rice (Zarembinski et al., 1993) and potato (Van der Straeten et al., 1995). Different factors, such as auxin

(Botella et al., 1992), wounding (Hyodo et al., 1983), pollination (Bui et al., 1998; Clark et al., 1997), can induce expression of different ACC synthase genes. In the present study, mRNA was extracted from rose flower petals, without any treatment, and the cDNA library was established using this mRNA fraction. The probe used in screening the library was amplified from rose senescence petals mRNA, therefore, the sequence of the probe may be specific to the ACC synthase gene which is related to senescence. The ACC synthase gene isolated from this library may be related to senescence of rose petals and would suggest that this RKacc7 gene may be involved in flower senescence. The other ACC synthase genes, which are related to the other developmental stages or stimulated by other factors, may not be represented in this cDNA library or have low homology to the probe, so that they would not be selected in the screening process. Whether this is the only gene related to flower senescence in rose remains an open question until other ACC synthase genes from rose have been identified.

The hybridization temperature used in library screening was 60°C, which represents high stringency In tomato, five ACC synthase genes have been isolated from a genomic library with one probe under low stringency hybridization conditions (Rottmann et al., 1991), suggesting that it may be possible to select more than one gene from the rose cDNA library under low stringency hybridization condition.

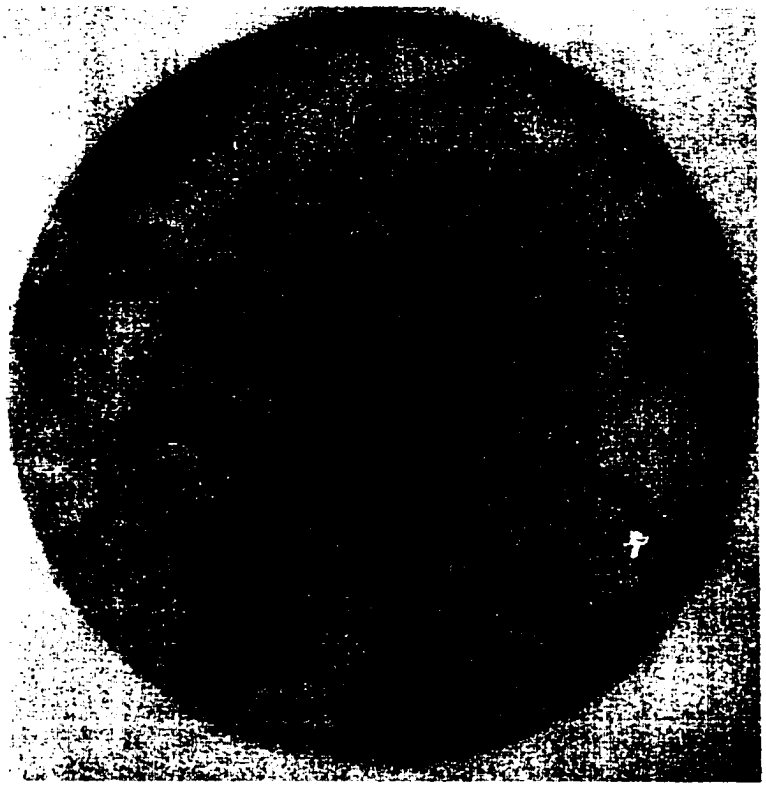
The length and primary sequence at the C-terminal region of varius ACC synthases sequenced thus far are hypervariable (Li and Matto, 1994). And Theologis (1992) also pointed out that the carboxyl termini of different ACC syntases are most divergent (Park et al., 1992, Fluhr and Matto, 1996). From Fig 2.5, the probe used for library

screening has high homology with the rose ACC synthase sequences in the caboxyl region, which is varible in ACC synthases. Therefore, the sequences selected by this probe may be the specific and is present only in Rkacc7 synthase.

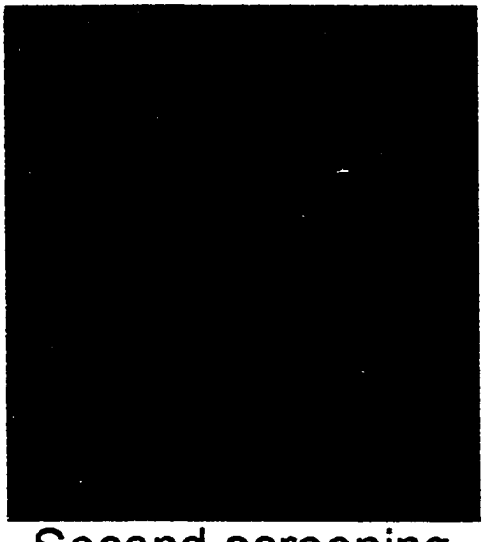
Another clone, RKacc27 was identified. DNA sequencing results showed it contains an insert of 2500 bp with the entire coding region of RKacc7 except for the 397 bp from the 5'-end. In addition the 5'-end of RKacc27 is ligated to the poly(A) tail of another gene of unknown origin. These results suggested this chimaric construct may have been generated during cDNA construction and ligation steps.

Rose genomic DNA was digested by *Eco*R I, *Bam*H I and *Hind* III and resolved by electrophoresis. The blotted membrane was hybridized with RKacc7 at high and lower stringency conditions. From the Southern blot results [Fig.2.10], it is clear that there are multiple bands generated by digestion with each enzyme. The RKacc7 restriction map shows one *Eco*R I site, one *Hind* III site and two *Bam*H I sites [Fig.2.10]. A comparison of the RKacc7 restriction map with the restriction pattern generated by digestion of genomic DNA by Southern blotting suggests a much more complex picture. These results raise strongly the possibility that a multiple gene copy may be involved. This may be due to the hybrid nature of rose. With lower stringency hybridization conditions, additional bands are observed in each lane, suggesting again that a multiple gene family may be responsible for the results.

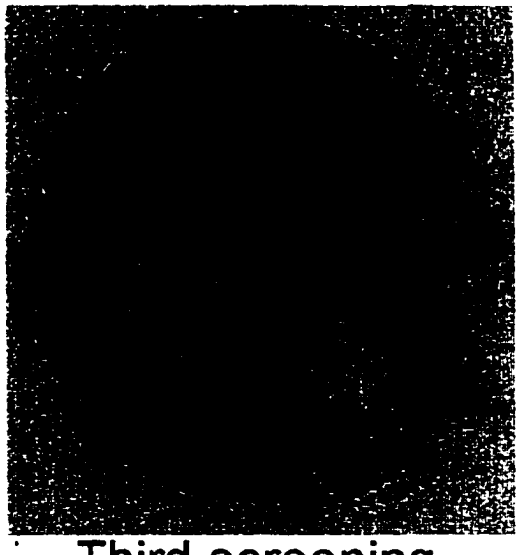
Fig. 2.3 *Screening of rose cDNA library with ACC synthase probe*. The details are described in Material and Method section.



First screening



Second screening



Third screening

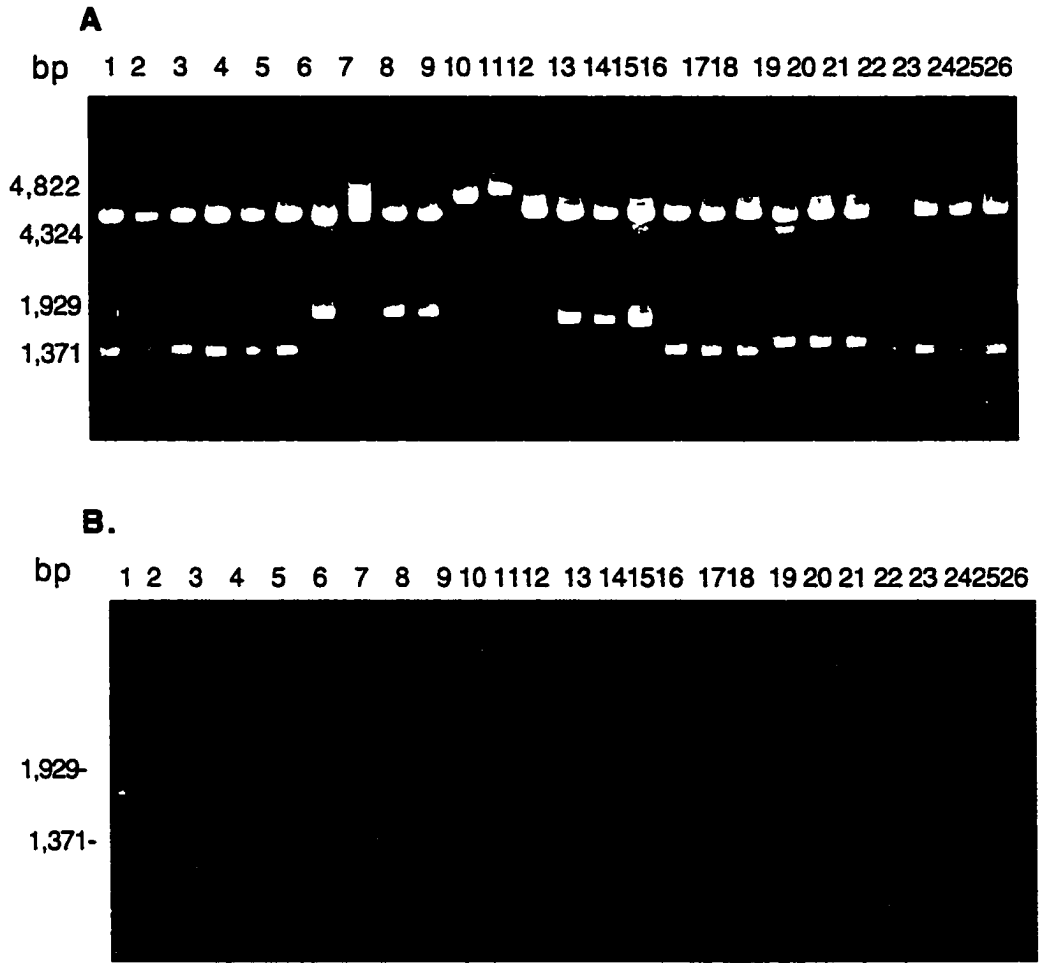


Fig. 2.4 *Analysis of positive rose ACC synthase cDNA clones by Southern hybridization*. The rose ACC synthase cDNA inserts were released from pBK-CMV by digestion with *Eco*RI and *Xho*I (**A**). The inserts were hybridized by the same specific probe (Fig.2.5) used in cDNA library screening (**B**).The molecular size of the insert varied from 1200 bp to1900 bp. Lane 1-3: different colonies of clone 1; lane 4-6: clone 6; lane 7-10: clone 7; lane 11-13: clone 18; lane 14-16: clone 22; lane 17-19: clone 24; lane 20-22: clone 25; and lane 23-26: clone 27. Details are described in Material and Method section.

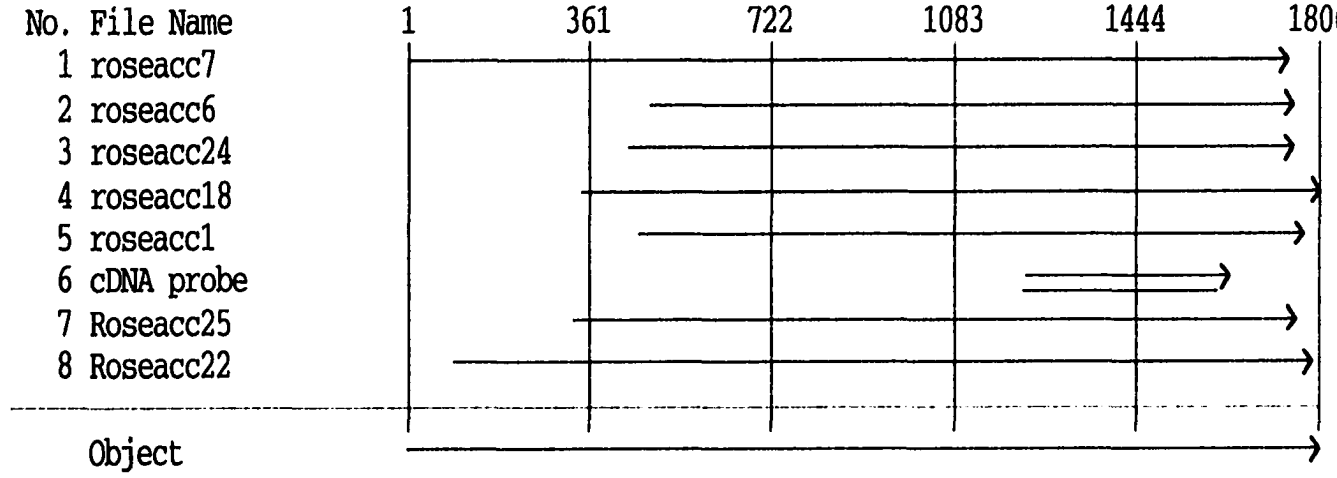


Fig. 2.5 *The aligment of cDNA sequences of rose ACC synthase clones.* Except for differences in length, the sequences of these clones are otherwise identical.

Fig. 2.6 *The cDNA sequence of RKacc7.* There are 273 bp of untranslated sequence at the 5'-end and 37 bp sequence at the 3'-end shown in lower case. Data also shows amino acid sequence of polypeptide and asterisk donates termination codon.

```
5'gccttggctttcctcccttcgctttcttcttcttcttcatcatcgtactctcc      56
gacgacccgaaaccccaccgcgacccggcccggatgtctccaatatgacccggacccg    114
agacgaagaccggcgacccagcagcagcagcagcggcggcggaggaggcgccgatgag    172
agttatagtccctctacaaggcgtggttcaaggcagaggaggactcgttctcggctcc    230
gtcataccatgcgcgctcttctatttctccagctttatcATGAAACGTCACCGTTCCAAC   290
                                          M  K  R  H  R  S  N
TCCAACCCGCCGACTCCGCCGCCTTCTCCGGACTCGGACTCGGACCACCACCCCGCCGGG   350
S  N  P  P  T  P  P  P  S  P  D  S  D  S  D  H  H  P  A  G
CAGTTGGTGGAAGTTCCGGTTCTGCCCCGGTCGATGTCGAGGTCCCATCTCTCTCCGAGG   410
 Q  L  V  E  V  P  V  L  P  R  S  M  S  R  S  H  L  S  P  R
AACCCGGGTCCGGTACATGTCTCGGGTCGGGCCAATTCGGTTTTGAAAGGCGGTGAGCCG   470
 N  P  G  P  V  H  V  S  G  R  A  N  S  V  L  K  G  G  E  P
CCGTATTATGTCGGCTTGAGGAAGGTGGCGGAGGATCCGTACGACGAGTTGGGTAACCCG   530
 P  Y  Y  V  G  L  R  K  V  A  E  D   P  Y  D  E  L  G  N  P
GATGGGGTTATTCAGCTGGGTTTGGATGAAAACAAGTTAGCTTTGGACTTGGTTCGAGAT   590
 D  G  V  I  Q  L  G  L  D  E  N  K  L  A  L  D  L  V  R  D
TGGCTACTGGAGAATGCAAAGGATGCAATACTGGGTGGTGAGGAGCTTGGGATTAGTGGG   650
 W  L  L  E  N  A  K  D  A  I  L  G  G  E  E  L  G  I  S  G
ATTGCTTGTTACCAGCCTTCTGATGGTTTAATGGAGCTCAAACTGGCTGTGGCAGGATTC   710
 I  A  C  Y  Q  P  S  D  G  L  M  E  L  K  L  A  V  A  G  F
ATGTCTAAGGCCATCGGAAATTCAGTTACGTACAACCCCTCACAAATTGTATTGACAGCT   770
 M  S  K  A  I  G  N  S  V  T  Y  N  P  S  Q  I  V  L  T  A
GGTGCAACCCCTGCAATTGAGATTCTAAGCTTCTGCCTAGCAGACAGTGGAAACGCATTT   830
 G  A  T  P  A  I  E  I  L  S  F  C  L  A  D  S  G  N  A  F
CTCGTTCCGGCACCATATTACCCTGGTTTGGACAGAGATGTGAAGTGGCGAACTGGAGTG   890
 L  V  P  A  P  Y  Y  P  G  L  D  R  D  V  K  W  R  T  G  V
GAGATAATACCTGTTCCATGCCGCAGTGCTGACAAATTCAATTTAAGTATAACTGCACTT   950
 E  I  I  P  V  P  C  R  S  A  D  K  F  N  L  S  I  T  A  L
GATCGAGCATTCAACCAGGCAAAGAAACGTGGTGTAAAAGTTCGTGGGATTATAATTTCA  1010
 D  R  A  F  N  Q  A  K  K  R  G  V  K  V  R  G  I  I  I  S
AATCCTTCAAATCCTGGTGGCAGTTTACTTACTCGTGAATCACTTTACAACCTTCTGGAC  1070
 N  P  S  N  P  G  G  S  L  L  T  R  E  S  L  Y  N  L  L  D
TTTGCCCGAGAGAAGAACATTCATATAATCTCAAATGAATTGTTTGCTGGATCCACGTAT  1130
 F  A  R  E  K  N  I  H  I  I  S  N  E  L  F  A  G  S  T  Y
GGAAGTGAAGAGTTTGTTAGCATGGCAGAAATCGTTGATTTGGAAGATCTCGACCAGAAC  1190
 G  S  E  E  F  V  S  M  A  E  I  V  D  L  E  D  L  D  Q  N
AGAGTGCATATAGTATATGGCATATCGAAAGATCTCTCACTTCCAGGTTTCAGGGTGGGT  1250
 R  V  H  I  V  Y  G  I  S  K  D  L  S  L  P  G  F  R  V  G
GCCATCTACTCCTTTAACAAGAATGTCTTGACTGCTGCTAAAAAGTTGACAAGGTTCTCT  1310
 A  I  Y  S  F  N  K  N  V  L  T  A  A  K  K  L  T  R  F  S
TCTATCTCCGCCCCATCCCAACGGTTGCTTATCTCTATGCTTTCAGACACCAAATTTATG  1370
 S  I  S  A  P  S  Q  R  L  L  I  S  M  L  S  D  T  K  F  M
CATAAGTTCATCGAGATTAACAGAGAAAGGCTCCGTGGAATGTATCTTAGATTTGTGACA  1430
 H  K  F  I  E  I  N  R  E  R  L  R   G  M  Y  L  R  F  V  T
GGATTGAAGCAATTGGGCATTGAGTGCACAAAGAGCAATGGGGGTTTCTACTGTTGGGCA  1490
 G  L  K  Q  L  G  I  E  C  T  K  S  N  G  G  F  Y  C  W  A
GACTTGAGTGGGTTAATTCGCTCTTACAGTGAGAAAGGGGAGCTTGAGCTCTGGGATAGG  1550
 D  L  S  G  L  I  R  S  Y  S  E  K  G  E  L  E  L  W  D  R
TTGTTGAATGTAGGTAAGCTCAATGTTACTCCTGGATCTTCTTGTCATTGTATTGAACCG  1610
 L  L  N  V  G  K  L  N  V  T  P  G  S  S  C  H  C  I  E  P
GGATGGTTCCGGTTTTGTTTTACGACGTTGACTGAAAAAGATATCCCTGTTGTTATAGAA  1670
 G  W  F  R  F  C  F  T  T  L  T  E  K  D  I  P  V  V  I  E
CGAATTCGGAATATTGCCGAAACATGTAAATCACACAGTTGAaatgttcgttcattctac  1730
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tcaaaaaaaaaaaaaaaaa                                          1742
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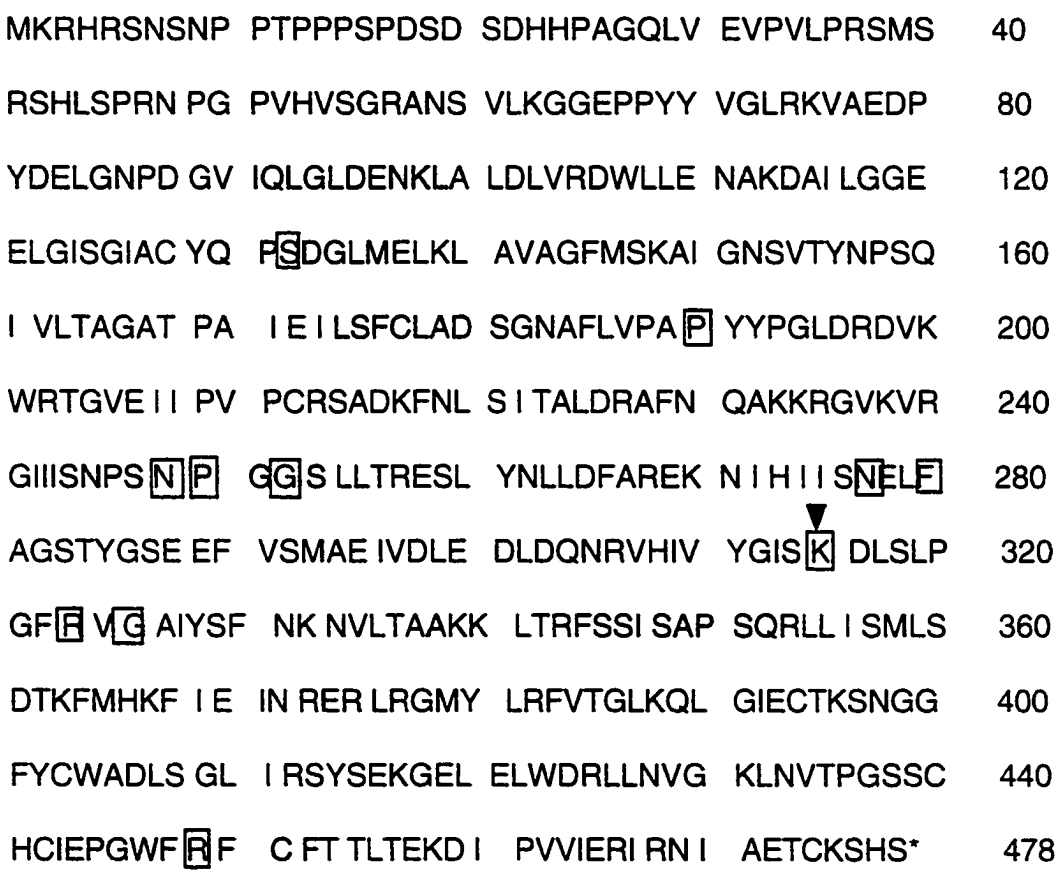


Fig. 2.7 *The deduced amino acid sequence of RKacc7.* Seven conserved regions in all ACC synthases are shaded. The residues in the boxes are the conserved residues in all ACC synthases and amino acid transferases. The arrow indicates the active site lysine which binds to pyridoxal phosphate and AdoMet.

Fig. 2.8 *The phylogenetic tree of ACC synthase constructed by using MacDNAsis software.* The Rkacc7 has 54.5% homology with wheat deduced ACC synthase amino acid sequence, but is different from the ACC synthases in other plants.

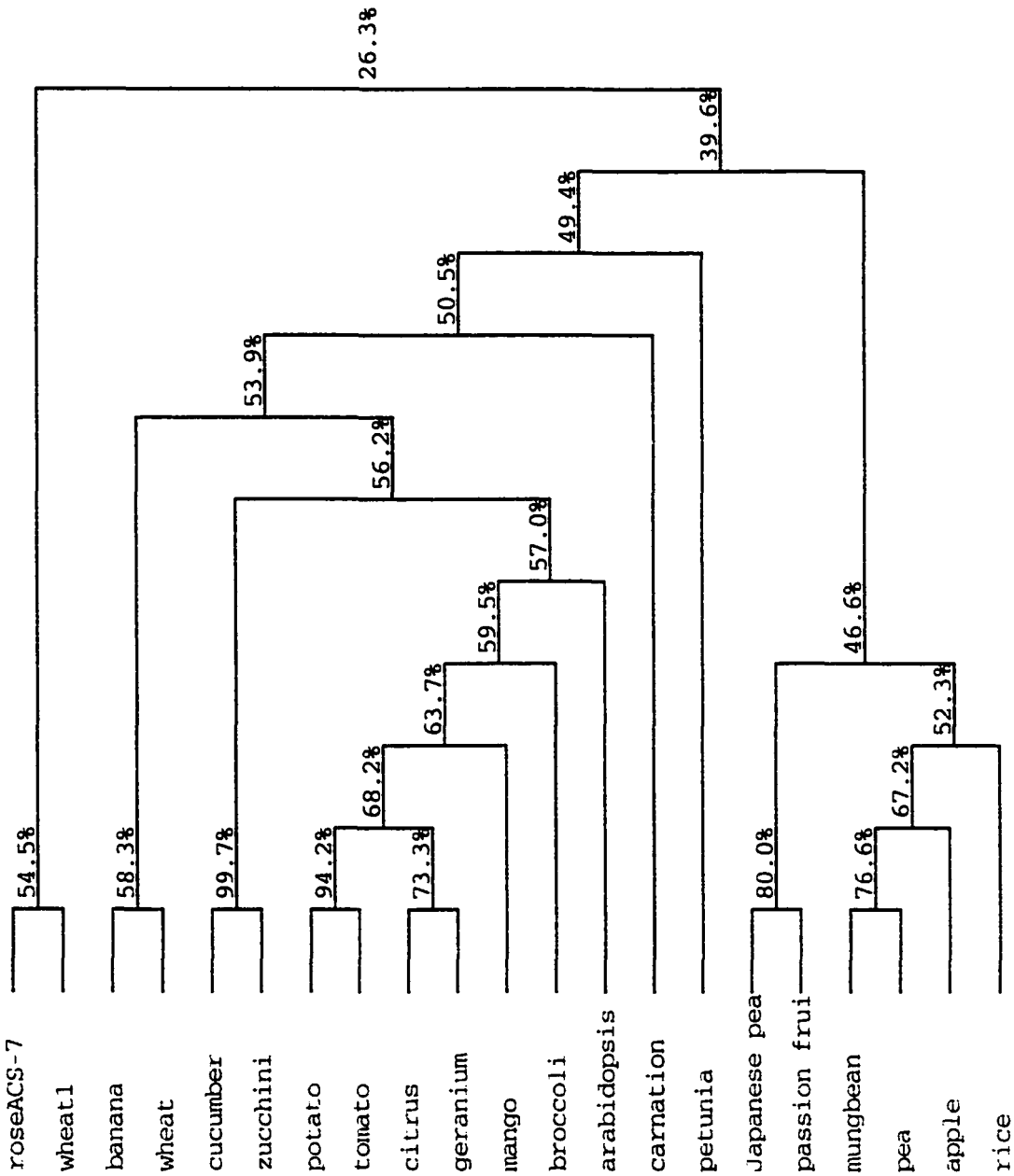


Fig. 2.9 *The most conserved amino acid sequences regions of ACC synthases.* The stars mark the homology amino acid residues in RKacc7 and other ACC synthase amino acid sequence.

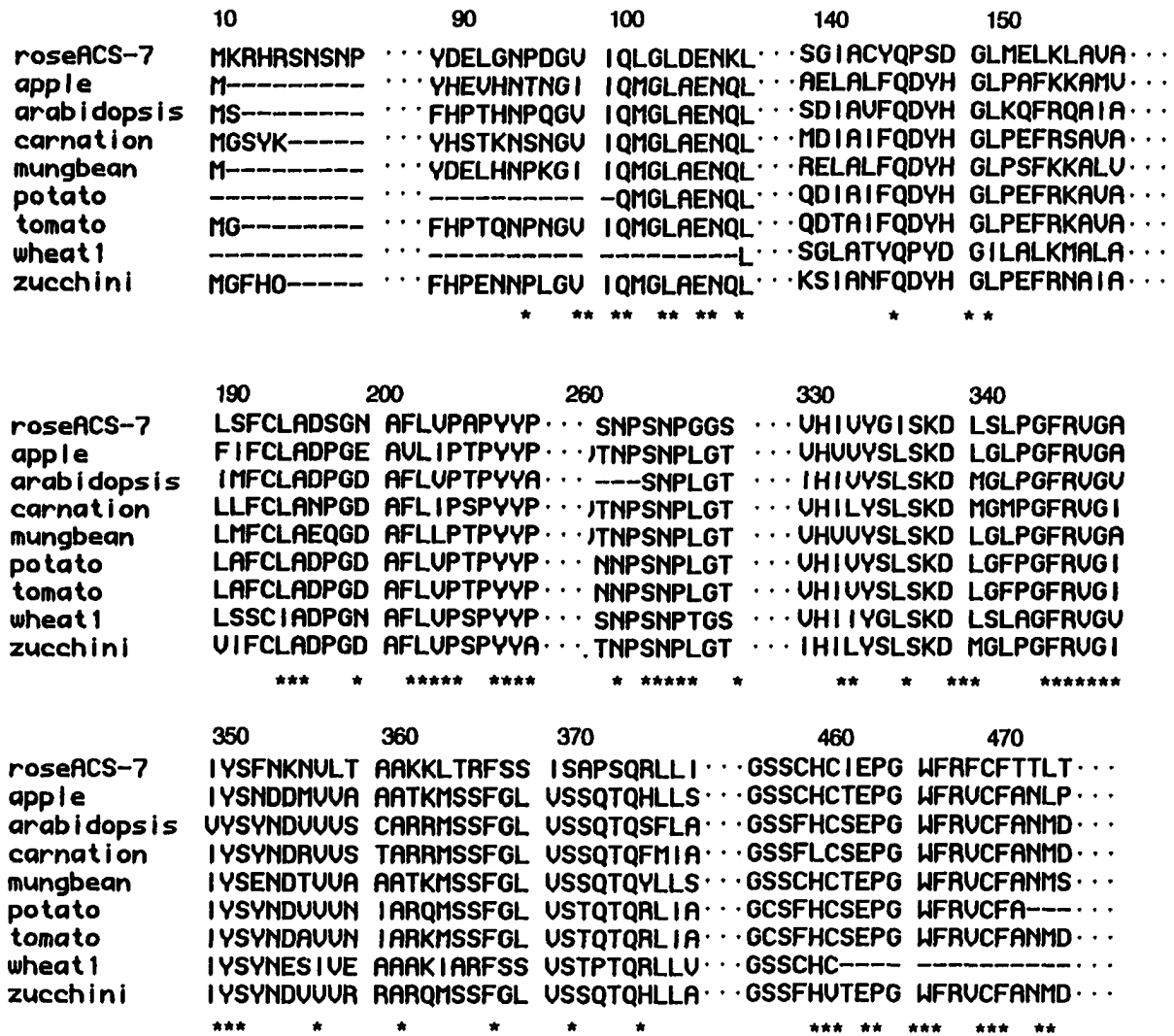
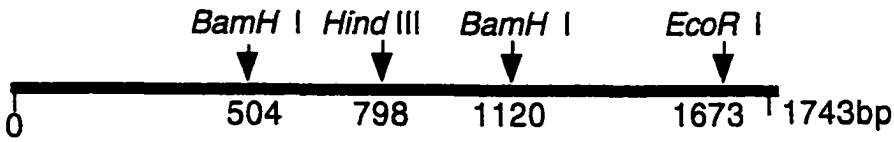


Fig. 2.10 *GenomicSsouthern blot of RKacc7*

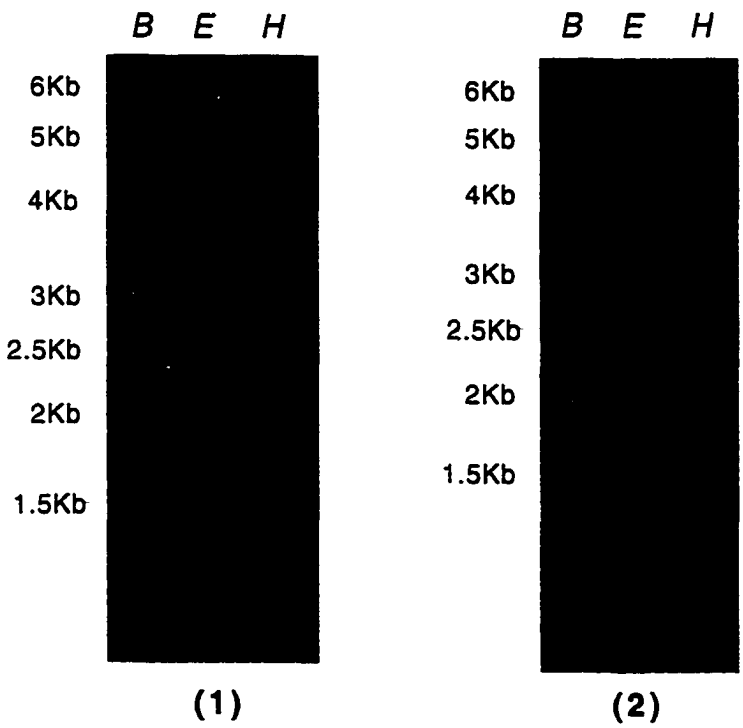
Panel A: The restriction enzyme *BamH*I, *Eco*RI and *Hind* III sites in roseACS-7 cDNA sequence.

Panel B: Genomic Southern blot of RKacc7-7. The 20μg of rose genomic DNA from leaves were digested with *BamH*I (B), *Eco*RI(E)and *Hind*III (H), the digested DNA were fractioned in 0.8% agarose gel and transferred onto positive charged Hybond nylon membrane. The genomic DNA were hybridized with [^{32}P]-labeled roseACS-7 cDNA at 50°C (1) and 60°C (2). The membranes were washed in high stringency condition and subjected to autoradiography.

A.



B.



Chapter 3 Rose ACC Synthase cDNA Expression

I. Introduction

The gaseous plant hormone ethylene plays an important regulatory role in growth and development. In plant tissues ethylene production typically is low, but increases at developmental stages such as ripening and senescence and in response to mechanical and environmental stresses (Yang and Hoffman,1984). From ethylene biosynthesis to ethylene signal transduction in plants, a lot of work has been done within the last twenty years. The cloning of ACC synthase genes from a number of different species has demonstrated that the enzyme is encoded by a multigene family, the members of which are differentially regulated in a tissue-specific manner by a variety of signals, including auxin treatment, wounding, anaerobiosis, ripening, senescence, and Li^+ (Kende, 1993). Although there is evidence for the regulation of ACC synthase at the posttranscriptional level (Chappell et al., 1984; Felix et al., 1991, 1994; Spanu et al., 1994), expression studies indicate that the induction of ACC synthase activity is most often the result of the increased accumulation of ACC synthase mRNAs (Kende, 1993; Zarembinski and Theologis, 1994). In carnation flowers and orchid flowers, senescence is accompanied by an increase in ethylene production and of ACC synthase activity

(ten Have and Woltering 1997; Bui and O'Neill, 1998). The expression of ACC synthase transcripts in carnation flower was up-regulated by auxin, ethylene, LiCl, pollination, and senescence in a floral-organ-specific manner (Jones and Woodson, 1999).

As the first step toward understanding of the regulation of the ACC synthase genes in rose flower senescence, a complementary DNA sequence to ACC synthase mRNA has been isolated from the kardinal rose petals. To determine whether this cDNA sequence has properties of ACC synthase, the cDNA was transcribed *in vitro* and the transcript was translated in a rabbit reticulocyte lysate system. Also, the rose ACC synthase gene was expressed as a fusion protein from a pET plasmid in *E.coli*. The immunoselection of the *in vitro* synthesized protein and fusion protein synthesized in *E.coli* by antiACC synthase antibodies are consistent with the fact that the rose ACC synthase polypeptide is coded by RKacc cDNA

II. Material and Method

1. ACC synthase gene expression in rose:

RT-PCR was used to detect the expression of ACC synthase gene in rose plant. For this purpose, total RNA was prepared from ovary, anther, sepal, leaves and from unopened buds, partially opened buds, petals at 1,2,4,5,6,7 days after the flower fully opened. Rose total RNA was extracted from these selected tissues. The extraction method was described by Manning (1991). The frozen tissue (around 4 g) was powdered in liquid nitrogen and ground-up in a prechilled pestle and mortar with extraction buffer (0.2 M boric acid/Tris, 10 mM Na_2EDTA, pH 7.6) (15 ml) to which 1/50 volume of 25%(w/v; 300 µl) sodium dodecyl sulfate (SDS) solution and 1/50 volume of 2-mercaptoethanol (300 µl) were added immediately before use. The mixture was extracted with an equal volume of a phenol/chloroform solution. The liquid phenol used was pre-equilibrated with extraction buffer. The upper aqueous phase was decanted following centrifugation at 12,000 rpm for 15 minutes and the pellet at interface and lower phase were re-extracted with the same volume of extraction medium. After recentrifugation (12,000 rpm for 20 min at 22°C)the aqueous phase from the second extraction was combined with the aqueous phase from the first extraction and again extracted with an equal volume of phenol/chloroform mixture as before. One volume of the aqueous phase from the phenol/chloroform extract was diluted to 2.5 volume with DEPC treated water (42 ml) and the Na^+ concentration (including the contribution from Na_2EDTA) was adjusted to 80 mM with 1 M Na

acetate/acetic acid buffer, pH 4.5 (5.5 ml). To precipitate polysaccharides, 0.4 volume (30 ml) of 2-butoxyethanol (2-BE) were added. After 30 min. on ice, the mixture was centrifuged at 12,000 rpm for 20 min. at 0°C and the supernatant collected without disturbing the gel-like pellet containing polysaccharides. The nucleic acids were precipitated by addition of 2-BE (45 ml) to the supernatant equal to 1.0 volume. After 30 min. on ice the nucleic acids precipitate was collected in the form of a pellet by centrifugation at 12,000 rpm for 10 min. at 4°C. The pellet was washed consecutively with 5 ml of extraction buffer/2-BE (1:1) mixture, 70% (v/v) ethanol containing 0.1 M KoAC (pH 6.0), and absolute ethanol. The total nucleic acid pellet was dried and dissolved in 600 µl of water to give a concentration not less than 1 mg/ml and then adjusted to 3 M LiCl by adding 1/3 volume (200 µl) of 12 M LiCl. After being kept at -20°C for more than 1 hour, RNA precipitate was centrifuged at 12,000 rpm for 15 min 4°C. The pellets were washed with 3 M LiCl, 70% ethanol and 100% ethanol respectively at 4°C. The total RNA in the pellet was dried and dissolved in 100 µl DEPC-treated water for mRNA isolation. The total RNAs (10 µg) were converted to first strand cDNA by using Ready-to-Go T-primed First-strand cDNA kit (Amersham Pharmacia Biotech Inc), and 2 µl of cDNAs were used as the templates to perform PCR. The PCR was primed by the primers:

Forward primer (377-394 bp) 5'-GGTCGATGTCGAGGTCC

Reverse primer (1519-1500 bp) 5'-CTGTAAGAGCGAATTAACCC

which were designed based on the RKacc7 cDNA sequence [Fig. 2.6]. To determine the expression of constitutively expressed gene, the ubiquitin cDNA fragment was amplified from rose 1st strand cDNA preparations. The primers used were:

Forward primer: 5'-GTGGTGCTAAGAAGAGGAA

Reverse primer: 5'-CTCCTTCTTTCTGGTAAACG

The design of the primers are based on *Arabidopsis thaliana* ubiquitin extension protein (UBQ5) gene sequence (Callis et al., 1990). The RT-PCR conditions for ubiquitin and RKacc7 were the following:

94°C for 3 minutes for 1 cycle; then 94°C for 1 minute; 59°C for 1 minute; 72°C for 2 minutes for 5 cycles; followed by 95°C for 30 seconds; 58.5°C for 30 seconds; 72°C for 1 minute for 35 cycles and finally an extension cycle at 72°C for 10 minutes. The best conditions for ubiquitin amplification were: 94 °C for 3 min, 94°C for 30 sec. 55°C for 30 sec. 72 °C for 1 min with 30 cycles and then 72°C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

2. ACC synthase antibody preparation

Based on amino acid sequence homologies data from ACC Synthase, three peptide regions exhibiting maximum homology were identified. These peptides varying from 33 to 38 amino acids in length were synthesized at the Colorado State University Macromolecular facility. The three peptides are:

(1)NVSPGSSFLCSEPGWFRVCFANMDNATLDVALNRI;

(2)YFDGWKAYDRDPYHSTKNSNGVIGMGLAENCLC;

(3)YSLSKDMGMPGFRFRVGIIYSYNDRVVSTARRMSSFGLVS.

A 1:1 emulsion of 200 μg/ml of each peptide in complete Freund's adjuvant was prepared and 0.1 ml volumes of each were injected subcutaneously (s/c) in different rabbits at 17 to 18 sites on the back of the rabbits. Before injection a preimmune serum sample was obtained from each rabbit. On day nineteen after first immunization, each rabbit received two intramuscular (i/m) injections of 0.35 ml of a 1:1 emulsion of each peptide in incomplete Freud's adjuvant at 100 μg/ml. On day 35 after the first immunization, the day 19 injections were repeated. On day 92, each rabbit was given a booster i/m with the same peptide emulsion as on day 19. Seven days later, the rabbits were bled and serum prepared.

Western blot analysis of antiserum with three peptides showed the presence of antibodies against each of their peptides with strong signal suggesting that the immunization was successful. Negative results were observed with preimmune serum. The antiserum of each rabbit was pooled, and kept in -20°C. Consistent with conserved amino acid sequences of these peptides, these antisera also cross reacted with tomato ACC synthase (Ranu, unpublished).

3. *In vitro* transcription

pBK-CMV vector contains T_3 and T_7 polymerase binding sites which can be used to transcribe a cloned insert (Ranu et al., 1996), and then the RNA transcripts can be used for *in vitro* translation (Ranu et al., 1979). T_3 and T_7 RNA polymerase also

incorporate 5'-7MeGpppG-5', cap analog to the 5'-end of the transcript (Ranu et al., 1996).

(1) DNA linearization:

The pBK-CMV vector DNAs with rose ACC synthase cDNA inserts were purified by cesium chloride density gradient centrifugation (Ranu et al., 1996). *E. coli* cells containing plasmid pBK-CMV with PKacc-cDNA insert were inoculated in 500 ml of LB broth and allowed to grow overnight at 37°C. The cells were harvested by centrifuge at 5000 rpm for 10 min., and the cell pellet was suspended in 16 ml of Tris-sucrose buffer (50 mM Tris-HCl pH 7.6, 60 mM EDTA, 25% sucrose). One ml of lysozyme (10 mg/ml in TE buffer) and 2 ml of 0.5 M of EDTA (pH 8.0) were added. The cell suspension was kept on ice for 5 min. to allow lysis of the cells, then 19 ml of Tris-TritonX-100 buffer (50 mM Tris-HCl pH 7.5, 60 mM EDTA, 10% TritonX-100) were mixed. The sample was centrifuged at 40,000 rpm for 2 hour at 4°C in a Ti 50.1 rotor. NaCl powder was added to the supernatant in a final concentration of 1.5 M. DNA was precipitated with 1/2 volume of 30% polyethylene glycol (PEG). The mixture was kept at 4°C overnight and centrifuged at 6000 rpm for 20 min. The supernatant was discarded and the pellets were dissolved in 5 ml (1/10 volume) of Tris-EDTA buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 10 mM NaCl). CsCl at 1.05 g/ml and 50 µl of ethidium bromide (5 mg/ml) were added to the sample. To remove undissolved material the sample was centrifuged at 10,000 rpm for 10 min. The supernatant was subjected to centrifugation at 60,000 rpm for 18 hours at 15 °C in the

Beckman ultracentrifuge NVT 65 rotor. The clean plasmid band was recovered with a syringe. The ethidium bromide was removed by extraction with water-saturated butanol and the plasmid DNA was precipitated with two volume of ethanol.

The purified DNAs were digested with restriction enzyme to prepare run off RNA transcripts. There is no *Xho* I restriction site in the rose ACC synthase cDNA insert, but there is one in the vector pBK-CMV close to the 3' end of insert DNA. The purified DNA was linearized by digestion with *Xho* I and extracted with phenol/chloroform, chloroform, and then precipitated by the addition of 1/10 volume of 3 M NaoAC (pH 5.2) and 2 volumes of 100% ethanol. The linearized DNA was used as template to transcribe run off transcript.

(2) Protocol for the preparation of *in vitro* transcript:

The transcription reaction mixture contained: 5 μl of 5X transcription buffer (200 mM Tris-HCl pH.7.9, 50 mM NaCl, 30 mM $MgCl_2$ and 10 mM spermidine), 1.0 μl of 0.75 M DTT, 1.0 μl of RNasin (40 U/μl), 1.0 μl of each 10 mM rATP, 10 mM rCTP, 10 mM rUTP, 1.5 μl of 0.5 mM rGTP, 1.0 μl of 10mM Cap anolog (3 mM M^7G, Biolab), 1 μg of DNA template, and 0.5 μl of T3 polymerase (80U/μl). The final reaction volume was adjusted to 50 μl with distilled water (Ranu et al., 1996). The mixture was incubated in 37°C for 1.5 hour, then 10 μl of transcript was taken out and denatured in 67°C for 10 min. with 10 μl of gel loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF). The denatured transcript was subjected to electrophoresis in a 1.2% agarose gel to

determine the size and integrity of the transcript. The result showed that the size of the transcripts were consistent with the size of cDNA inserts [Fig. 3.4]. The rest of the samples were kept in -70°C for *in vitro* translation.

4. *In vitro* translation of the rose ACC synthase transcript:

In vitro translations were performed using a cell-free rabbit reticulocyte lysate translation system made dependent for mRNA by treatment with micrococcal ribonuclease (Pelham et al., 1976). Rabbit reticulocyte lysate is optimized further for mRNA translation by the addition of the following:

- an energy generating system consisting of pretested phosphocreatine and phosphocreatine kinase
- a mixture of tRNAs to expand the range of mRNAs which can be translated
- hemin, to prevent inhibition of initiation of polypeptide synthesis
- potassium acetate and magnesium acetate.

(1) *In vitro* translation protocol:

Details of *in vitro* protein synthesis assay have been described except that [^{35}S] methionine was substituted for [^{14}C] leucine (Ranu et al., 1976). The master mixture contained 62.5 µl of rabbit reticulocyte lysate; 5 µl of RNasin (40 U/µl); 2.5 µl of amino acid mixture minus methionine, 7.5 µl of ^{35}S-Methionine (11.04 µCi/µl). An aliquot of 25 µl of master reaction mixture was transferred into a clean tube and 2 µl of RKacc7, RKacc18 or RKacc24 transcript pre-denatured at 67°C for 10 min. was added to each tube. Luciferase mRNA was used as positive control and 2 µl of distilled

water was added to the control reaction mixture. The reaction mixtures were incubated at 30°C for 1 hour. Hot trichloroacetic acid (TCA) precipitable radioactivity incorporated was measured by liquid scintillation. The translation products were also analyzed by electrophoresis in SDS-PAGE gel and by autoradiography.

(2) TCA assay

One microliter of translation reaction mixture was transferred to a 3 mm Whatman filter, and air dried for 1 min. The filters were transferred to a 10% TCA solution (ice cold), and kept on ice for 5 min. TCA solution was replaced with 25 ml of 5% TCA along with 4 ml of 30% H_2O_2 and the filters were kept in boiling TCA for 5 min.. They were washed twice with distilled water and acetone. The filters were air dried, placed in a vial individually and counted in a scintillation counter.

(3) SDS-PAGE:

An aliquot (10µl) of translation reaction mixture was denatured with 2X protein denaturation buffer (100 mM Tris-HCl pH 6.8; 200 mM dithiothreitol; 4%SDS; 20% glycerol and 0.2% bromophenol blue) at 95°C for 10 min., then the samples were subjected to electrophoresis in 11% SDS-PAGE gel . The SDS-PAGE gel was vacuum dried and autoradiographed.

(4) Immunoselection of *in vitro* synthesized polypeptides:

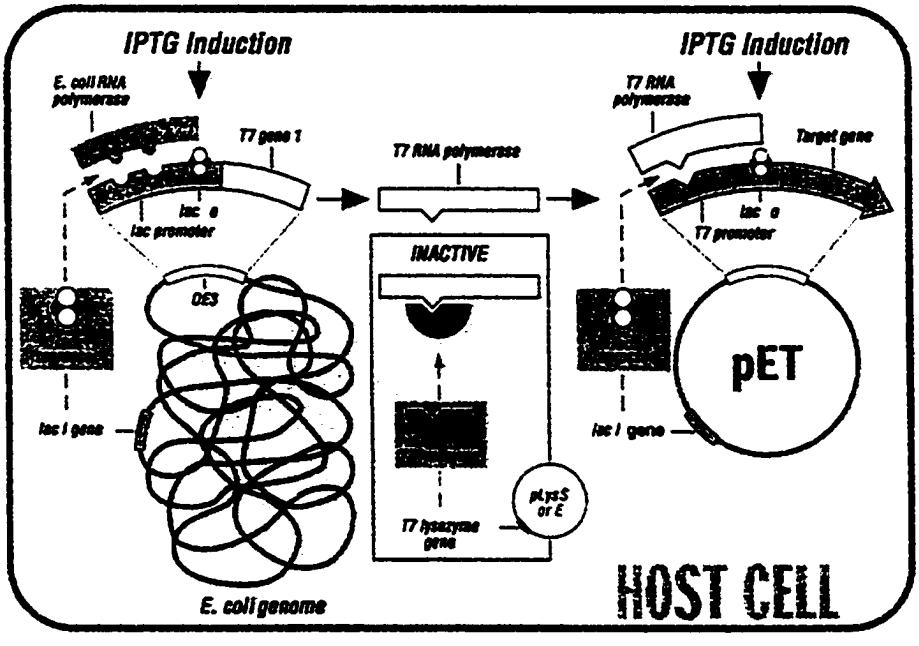
In another assay, the *in vitro* translated products were specifically immunoprecipitated. To 20 µl of translation reaction, 20 µl of antibody (1:10) preparation against ACC synthase were mixed along with 10 µl of PBS buffer (2.7

mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4 and 137 mM NaCl) and the reaction mixtures were incubated at 4°C for 1 hour. After incubation, 30 µl of Pansorbin® (*S. aureus* cell protein A) cell was added to absorb the antigen-antibody complexes. The reactions were incubated on ice for 30 min., the precipitate was recovered by centrifugation at 10,000 rpm in 1 min. The sediment was washed three times by NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH7.5 and 0.05% NP-40), finally dissolved in 2X protein denaturation buffer and heated in boiling water for 10 minutes. After centrifugation at 10,000 rpm for 5 minutes, the clear supernatant was analyzed by SDS-polyacrylamide gel electrophoresis.

5. Expression of rose ACC synthase gene in *E. coli*

The pET Expression System is one of the most powerful systems developed for expression of recombinant proteins in *E. coli* (Studier and Moffat et al., 1986). pET-30 vectors contain a highly efficient ribosome binding site and T7*lac* promoter. The T7*lac* promoter contains a *lac* operator sequence immediately downstream from the promoter region. Binding of the *lac* repressor at this site effectively reduces transcription by T7 RNA polymerase, thus providing a second *lacI*-based mechanism to suppress basal expression in λDE3 lysogens [Fig. 3.1]. In addition, pET-30 vector encoded affinity His-Tag in N-terminus or C-terminus of fusion protein which is available for purification of recombinant protein by metal chelation chromatography, and S-Tag for assaying expression levels by western blot (Novagen). The target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription

Fig. 3.1 *The pET protein expression system.* The expression hosts contain a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control and the expression is induced by the addition of IPTG. The target genes are cloned in pET plasmid under control of strong bacteriophage T7 transcription and the expression is induced by providing a source of T7 RNA polymerase in the host cell (Novagen pET system manual).



and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. Those hosts are lysogens of bacteriophage DE3, a lambda derivate that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lac*UV5 promoter, and the gene for T7 RNA polymerase. This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). T7 RNA polymerase is selective and active so that almost all of the cell's resources are converted to target gene expression; the desired product can in some systems comprise more than 50% of the total cell protein within a few hours after induction. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once plasmids are transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under *lac*UV5 control, the expression is induced by the addition of IPTG.

1. Construction of recombinant DNA, pET vector for expression of ACC synthase in *E.coli*

(1) Vector preparation

Sal I and *Not* I restriction enzyme sites were selected as the cloning sites in pET-30(a)-(c) vector. The digestions were performed sequentially (*Sal* I was from USB; *Not*

I was from Boehringer), because *Sal* I site and *Not* I site are close to each other in the vector.

The PET-30 vector DNA (5 μg) was digested with 2 μl (48 U/μl) of *Sal* I in 5 μl of 10X reaction buffer in a final volume of 50 μl. The reaction mixture was incubated in a 37°C water bath for 5 hours.The linearized vectors were purified as following: the 50 μl of water were added to the digestion mixture, and the mixture were extracted with 100 μl of phenol, 100 μl of phenol:chloroform/isoamylalcohol mixture (1:1), 100 μl of chloroform/isoamylalcohol (24:1). Finally, the digested vectors were precipitated by 1/10 volume of 3 M NaoAC (pH 5.2), 2 volume of 100% ethanol, and kept in –20°C overnight.

Then the vector pellets were dissolved in 40 μl of water and digested with *Not* I. The *Sal* I digested pET-30 vector (40 μl), 5 μl of 10X reaction buffer and 5 μl (10 U/μl) of *Not* I were mixed. The reaction mixture was incubated in a 37°C water bath for 5 hours. After digestion, the vectors were dephosphorylated with shrimp alkaline phosphatase (SAP from USB) to reduce the vector self-ligation background. To the 50 μl of pET-30 digestion reaction mixture, 6 μl of 10X SAP reaction buffer (200 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$)1.5 μl of SAP (0.1 U/μl) and 2.5 μl of distilled water were added. The reaction mixture was incubated at 37°C for 1 hour, then SAP was inactivated by incubation at 65°C for 15 minutes. The vectors were separated in 1.25% SeaPlaque low melting agarose gel from the undigested vectors and the digested

vectors were recovered from the agarose gel by Promega Wizard PCR Preps DNA Purification System following the low-melting temperature agarose DNA purification protocol (Promega Inc). The pieces of low-melting temperature agarose containing digested vector DNA were incubated in 70°C to melt the agarose, and then 1 ml of DNA purification resin was mixed with the melted agarose and kept in room temperature for 2 min. The resin bound with DNA was passed through a mini column and the column was washed with 2 ml of 80% isopropanol. The purified vector fragments were eluted by 40 μl of TE buffer from the column after centrifugation at top speed (10,000 rpm) for 30 seconds.

(2) Preparation of insert DNA

Two kinds of rose ACC synthase cDNA were used for expression in *E.coli*. One of the cDNA contains an untranslation region, as in RKacc22, and the other one lacks an untranslation region and the first 38 amino acids, such as RKacc18 [Fig. 3.2]. The RKacc18 insert was released from pBK-CMV by digestion with *Sal* I and *Not* I simultaneously. The RKacc18 DNA (5 μg), 8 μl of 10X reaction buffer, 2 μl (48 U/μl) of *Sal* I , 6 μl (10U/μl) of *Not* I and 60 μl of distilled water were mixed together and incubated at 37°C in a water bath for 6 hours. Afterwards, 6X loading dye was added to the reaction mixture and the RKacc18 insert was separated by electrophoresis on 1.25% SeaPlaque low-melting temperature agarose gel. The digested RKacc18 was recovered by Promega Wizard PCR Preps DNA purification System (Promega Inc.).

For preparation of the RKacc22 insert, two special primers were designed based on the RKacc7 sequence to amplify the RKacc22 insert from pBK-CMV vector without the untranslated region, but retaining the *Sal* I and *Not* I restriction enzyme sites. The two primers are:

Sal I
5' GCAGAG**GTCGAC**TCGTTCTCGGCTCC-3' (205-222 bp)

Not I
5' GGGCCC**GCGGCCGC**TCTSGSSG-3' (1731-1719 bp)

The PCR reaction mixture contained 68 ng of RKacc22 DNA template, 5 µl of 10X PCR buffer (35 mM Mg^{+}), 5 µl of dNTP (2 mM), 2 µl of primer1 (5 µM) and 2 µl of primer 2 (5 µM), and 2 µl of KlenTaq (2 U/µl) in the total volume of 50 µl. The PCR program was: 95°C for 5 min.; 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 min. for 35 cycles and finally 72°C for 10 min. The PCR products were applied to a 1.25% SeaPlaque low melting agarose gel and purified by Promega Wizard PCR Preps DNA purification System as described before. The DNA fragments were eluted by 40 µl of distilled water; 40 µl (about 3 µg) of purified PCR products were digested with 2.5 µl (12U/µl) of *Sal* I and 2.5 µl (10 U/µl) of *Not* I in 5 µl of 10X high salt buffer. The digested PCR RKacc22 fragments were purified by phenol, phenol:chloroform/isoamyl alcohol mixture (1:1) and chloroform : isoamyl alcohol (24:1), the DNA fragments were precipitated with ethanol and washed with 70% ethanol. The DNA pellet was dissolved in 20 µl of distilled water.

(3) Cloning of inserts into pET-30 vectors

The prepared vectors pET-30(a)-(c) and insert RKacc18 or RKacc22 were ligated with T4 DNA ligase (New England BioLabs) as follows:

An insert to vector ratio of 3:1 was used for ligation; 2 μl of RKacc22 or RKacc18 (150 ng), 2 μl of pET-30 vector (150 ng), 1.5 μl of 10X ligase buffer and 1 μl (2 Wesis Unit) of T4 ligase were mixed and the total volume was adjusted to 15 μl with distilled water. The reaction mixture was incubated at 12°C overnight. 1X T4 ligase buffer contains: 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 μg/ml BSA, pH 7.8. The ligation mixture then was used to transform JM109 to determine whether the insert was ligated into the vector and was in the correct reading frame.

2. The pET-30RKacc18 and pET-30RKacc22 modification

Because the His-Tag and S-Tag sequences in the pET30 vector may influence folding of the target protein, the deletion of His-Tag and S-Tag was carried out from the pET-30RKacc18 and pET-30RKacc22 by digestion with *Nde* I and *Sal* I [Fig. 3.2]. For this purpose 5 μg of pET-30RKacc plasmid DNA was digested with 1.5 μl (20U/ μl) of *Nde* I and 2.5 μl (12 U/μl) of *Sal* I in the presence of 4 μl of 10X high salt digestion buffer in a final reaction volume of 40 μl. The reaction mixture was incubated at 37°C for 6 hours. The sticky ends were filled-in in the reaction mixture containing 5 μl of 10X Klenow buffer, 2 μl of dNTP (1 mM) and 1 μl of

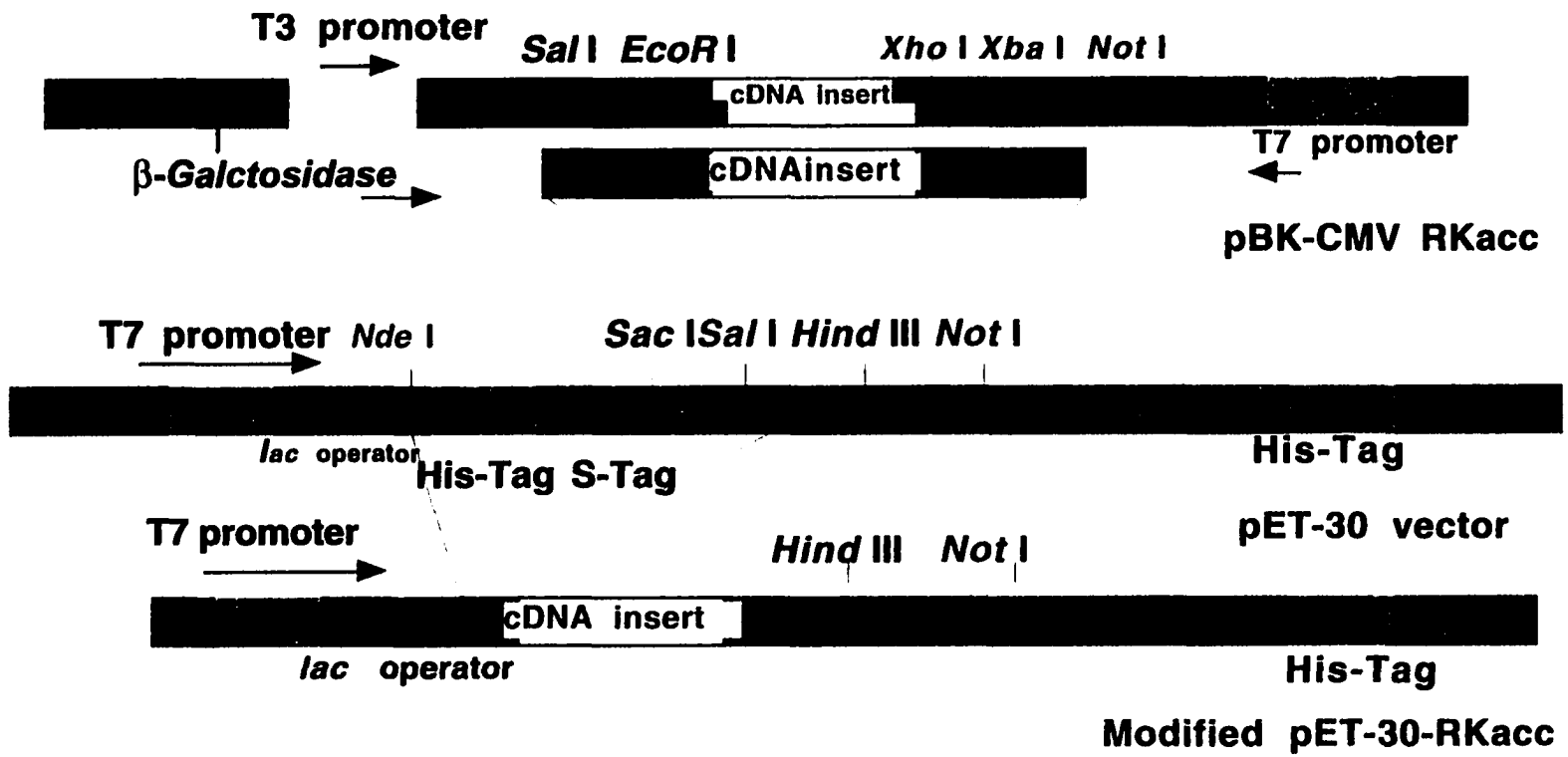


Fig. 3.2 *Recombinant DNA construction*

Klenow fragment (5 U/μl) in a total volume of 50 μl. The reaction was incubated at 37°C for 30 min., then the Klenow fragment was inactivated by incubation at 75°C for 10 min.. The modified plasmid DNA fragments were purified by phenol, phenol:chloroform mixture (1:1), chloroform/isoamyl alcohol (24:1), and the DNA fragments were precipitated by 2 volumes of ethanol. The DNA pellets were dissolved in 70 μl of distilled water.

To ligate the modified plasmid DNA again , the modified plasmid DNA 17 μl (300 ng), 1 μl of 10X T4 ligase buffer, 1 μl of 500 mM Tris-HCl pH 7.5, 1 μl of T4 ligase (400 U New England Biolab unit/μl) were mixed. This ligation mixture was modified to enhance blunt end ligation.The reaction mixtures were incubated at 8°C for overnight, and 10 μl of ligation mixture was used to transform expression host *E. coli* BL21(DE3). The transformants were analyzed to determine the sizes of modified plasmid DNA after restriction enzyme digestion with *Not* I or *Xho* I .

3. Transformation of recombinant DNA into the host cells

The pET system uses the bacteriophage T7 promoter to direct the expression of target genes. Since *E. coli* RNA polymerase does not recognize the T7 promoter, there is little if any transcription of the target gene in the absence of T7 RNA polymerase and the cloning step is thus effectively uncoupled from the expression step. The initial cloning was done in JM109 to avoid the possibility of difficulties in growth and plasmid instability that can be caused even by low levels of basal expression. JM109 is a convenient host for initial cloning of target DNA into pET vectors and for

maintaining plasmids because it is *recA*$^-$ and gives high transformation efficiencies and yield of plasmid DNA. For protein production, the recombinant plasmid was transferred to *E. coli.* BL21(DE3) containing a chromosomal copy of the gene for T7 RNA polymerase. This host is lysogen of λDE3 which has the immunity region of phage 21 and carries the *lacI* gene, the *lacUV5* promoter and the gene for T7 RNA polymerase. Once a λDE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter. Addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

The same protocol was used for transformation of recombinant vector into the cloning cell JM109 and expression host cells BL21(DE3). LB media (15 ml) was inoculated with an overnight culture of JM109 or BL21(DE3) cells, and continuously incubated in 37°C till OD_{600} reached 0.3-0.4. The cells were kept on ice for 10 minutes then harvested at 7,000 rpm for 5 minutes. The cell pellets were resuspended in 1/3 volume of TFB(10 mM morpholinoethane sulfonic acid pH 6.3; 100 mM RbCl; 45 mM $MnCl_2.4H_2O$; 10 mM $CaCl_2.2H_2O$; 3 mM hexamine cobalt (III) chloride), placed on ice for another 10 minutes and centrifuged at 7,000 rpm for 5 minutes. The pellets were resuspended in TFB again. For each 200 µl of cells suspension 7 µl of dimethyl sulfoxide (DMSO) was added. The tubes were kept on ice for 5 minutes; afterward 7 µl of 2.25 M DTT was added and the tubes were kept on ice for 10 minutes. Treatment with DMSO (7 µl) was repeated and the tubes were incubated on ice for 5

minutes. Competent cell suspension (200 μl) wase dispensed into microtubes. Eight μl of ligation reaction mixture were incubated with JM109 or BL21(DE3) competent cells on ice for 30 minutes and heat pulsed at 42°C for 90 seconds. The tubes were immediately kept on ice for 2 minutes. SOC broth (200-400μl) was added to each tube and the tubes were incubated at 37°C for one hour with shaking. The cell suspension in SOC media (150 μl) was plated on LB agar containing 30 μg/ml of Kanamycin. The plates were incubated at 37°C overnight.

The recombinant plasmid DNAs were isolated from the JM109 colonies by miniprep. PCR and the restriction enzymes digestion were performed to check that the insert was the right size. The correct reading frame of insert DNA was also confirmed by DNA sequencing. Plasmids with the right reading frame insert were then transferred into *E.coli* BL21(DE3) for expression.

4. The induction of ACC synthase gene expression:

After the target plasmid was established in the λDE3 lysogen, expression of the target DNA was induced by the addition of IPTG to a growing culture.

LB medium (100 ml) containing 30 μg/ml kanamycin was inoculated with an overnight culture of *E.coli* BL21(DE3) harboring the pET30-ACC recombinant plasmid and incubated at 37°C until an OD_{600} of 0.6; IPTG to a final concentration of 0.6 mM was added to induce T7 RNA polymerase gene expression in BL21(DE3) cell. The cells were continuously cultured at 25°C for 4 hours and then kept on ice for 10

minutes before centrifugation at 7000 rpm for 5 minutes. The cell pellets were washed with buffer (50 mM HEPES, 50 mM EDTA) and kept at –70°C till use.

The frozen cells were thawed and resuspended in the protein extraction buffer (50 mM HEPES, 150 mM NaCl, 4 mM DTT, 10 μM PLP and 1 % TritonX-100), and lysed with 200 μg/ml of lysozyme on ice for 30 minutes. Then the final concentration of 10 mM $MgCl_2$ and 40 μg/ml of DNase I were added to shear the chromosomal DNA by incubation on ice for one hour. The samples were centrifuged at 40,000 rpm for 30 minutes to remove the cell debris. The supernatants were mixed with 10% streptomycin to a final concentration of 0.5% and incubated at 20°C for 30 min. The mixture was centrifuged at 15,000x g for 15 minutes. The supernatant was mixed with protamine (1%) to a final concentration of 0.05% and incubated at 20°C for 15 minutes. The mixture was centrifuged again. To the supernatant saturated $(NH4)_2SO_4$ was added to a final concentration of 30% saturation. It was centrifuged at 15,000x g for 20 min.; the protein fraction was precipitated with $(NH4)_2SO_4$ to 80% saturation and collected by centrifugation. The pellet was dissolved in PBS buffer. An equal volume of 2X protein denaturation dye solution (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol and 4% SDS, 20% glycerol and 0.2% bromophenol blue) was added. The pellets from crude cell extraction were dissolved in protein denaturation solution (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol and 2%SDS). The supernatant samples and the dissolved protein pellets were subjected to electrophoreses on an SDS-polyacrylamide gel, followed by staining with Coomassie Brilliant blue.

5. Purification of rose ACC synthase protein expressed in *E.coli*

The frozen cells were thawed and treated with lysozyme and DNase I. The crude cell extract after centrifugation at 40,000 rpm for 30 min. was purified by affinity chromatography in His-Tag system. The target ACC synthase carries consecutive histidine residues at the N-terminal end. The His-Tag sequence binds to divalent cations Ni^{2+} immobilized on the His-Bind metal chelation resin. After unbound proteins are washed away, the target protein is recovered by elution with imidazol.

The His-Bind resin was suspended completely and allowed to settle in a small glass chromatography column (1.5 X 5 cm). When the level of storage buffer (20% ethanol) dropped to the top of the gel, the column was washed with the following sequence of buffers to charge and equilibrate the column:

a) 15 ml (3 volumes) of sterile deionized water

b) 25 ml (5 volumes) of 1X charge buffer (50 mM $NiSO_4$)

c) 15 ml (3 volumes) of 1X binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9)

The crude cell extract was mixed with His-Bind resin with gentle shaking at 4°C for 1 hour, and the resin bound with protein was allowed to settle in the column. When the cell extraction solution dropped to the top of the column, the column was washed with 50 ml (10 volume) of 1X binding buffer. The target ACC synthase was eluted by 60 mM imidazole and collected by fraction collector. Approximate 1.5 ml of each fraction was collected in total 30 fractions. The protein concentration in each fraction was measured by absorption at $O.D_{280}$. The fractions with high protein

concentration were dialyzed against PBS overnight. The dialyzed proteins were mixed with protein denaturation solution and subjected to electrophoresis in a 12% SDS-PAGE gel. The proteins from the SDS-PAGE were transferred onto Immobilon-P Membrane (Millipore) by electrophoresis in the transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol), and the ACC synthase was confirmed by western blotting as described below.

6. Detection of ACC synthase by Western Blotting

(1) S-Tag western blot

The S-Tag western blot kit (Novagen) was used to check target protein. The S-Tag system is a protein taggingand detection system based on the interaction of the 15 amino acids S-Tag peptide with ribonuclease S-protein. This is a specific high affinity binding. The 15 amino acids S-Tag sequence on the pET-30 vector is located on the N-terminal end. The highly purified S-protein conjugated with alkaline phosphatase (AP) is available. The membranes with purified protein were blocked briefly with 1% gelatin in TBST (10 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.1% Tween 20) at room temperature for 15 min., and incubated with 1:5000 dilution of S-protein alkaline phosphatase conjugate in TBST for 30 min. at room temperature. The membranes were washed 3 times with TBST at room temperature in 30 min.. Target protein bands were visualized with supplied NBT/BCIP substrates (60 µl NBT + 60 µl BCIP per 15 ml of 1X Alkaline Phosphatase buffer).

(2) Western blot against ACC synthase antibody:

The ACC synthase was also confirmed by western blot using the specific antiACC synthase antibody. The Immoblin membrane with expression protein was blocked in 1% gelatin+TBST at room temperature for 30 min., and then incubated with antiACC synthase antibody solution (1:50 of each antibody in TBST+1% gelatin) at room temperature for 2 hours. The membrane was washed 3 times with TBST and then incubated with Anti-Rabbit IgG Fc fragment (Goat) conjugated with alkaline phosphatase (1:5000 dilution in 50 mM Tris-HCl pH8.0, 1 mM MgCl2, 1% BSA) at room temperature for 1 hour. The membranes were again washed 3 times with TBST. The color was developed with NTB and X-phosphate as above.

7. ACC synthase activity assay

The rose ACC synthase activity can be assayed by detection of ACC by Thin Layer Chromaotography or by the conversion of ACC into ethylene by Gas Chromatography.

(1) Thin Layer Chromatography (TLC)

ACC synthase converts S-AdoMet (SAM) to ACC. And SAM, ACC can be separated by TLC. The reaction was mixed with 50 mM HEPES, 400 μM SAM, 2.5 mM PLP and target protein crude cell extract or purified target proteins. The reaction was incubated in 30°C water bath for one hour. The concentrated reaction mixture was applied to Cellulose Pre-Coated TLC plate (EM Reagents), and pure SAM and ACC were applied as positive control. The samples were separated in the solvent containing

butanol: acetic acid: H_2O: 70:10:20. The colors were developed by 0.25% ninhydrin in H_2O.

(2) Gas Chromatography (GC):

Ethylene can be produced from ACC by NaOCl when the reaction was carried out in the cold and in the presence of Hg^{2+} (Lizada and Yang, 1979).

The reaction mixture (500 µl) containing 400 µM SAM, 2.5 mM PLP, 50 mM HEPES and target protein cell extract or purified target protein was incubated at 30°C for 1 hour, then 200 µl of 5 mM $HgCl_2$, and 200 µl of H_20 were added to the reaction mixture followed by 100 µl of NaoCl/NaOH (2:1). The mixture was vortexed and kept on ice for 3 minutes. The reaction tubes were sealed with serum rubber stopper. One ml of gas was removed from the reaction tube with a one ml syringe (Hamilton) and injected into the gas chromatography column to measure ethylene. The positive control consisted of ACC which was transformed into ethylene by additional of NaOCl and $HgCl_2$.

The Gas chromatography (GC) was performed on Hewlett Packard-Model 5890, Hayesep-T (polymer) column and the oven temperature was set at 80°C.

III. Results and Discussion

(1) Expression of RKacc7 in rose:

The temporal and spatial expression patterns of RKacc7 gene was investigated in kardinal rose. Leaves, sepals, ovary and anther were selected as the tissues to study the RKacc7 gene spatial expression pattern. The petals of un-opened buds, partially opened buds and flower on 1,2,4,5,6,7 day after flowering were used to investigate the pattern of temporal expression.

RT-PCR is an easy and highly sensitive method to study gene expression in the tissues by amplification of mRNA. The forward primer (5'-CTGTAAGAG CGAATTAAC CC-3') and reverse primer (5'-GGTCGATGTCGAGGTCC-3') were designed based on the RKacc7 sequences [Fig.2.6]. The sequence amplifed by these primers spans all the conserved regions in RKacc7. Therefore, this amplification can serve as a measurement of presence of the RKacc7 mRNA, hence expression of the gene. The relative peak areas of the amplified signals were measured by the image scanner.

The results show that there is no detectable RKacc7 transcripts in the rose leaves [Fig. 3.3A] and suggest that RKacc7 gene is expressed specifically in rose floral organs. RKacc7 is expressed in ovary and sepals, but not in anthers [Fig. 3.3A]. The ovary and sepals are at the base of petals, and ACC is the signal molecule for ethylene biosynthesis which is related to flower senescence (Nichols et al., 1983; Reid et al., 1984). It is likely that the expression of ACC synthase gene in ovary and sepals results in production of ACC which in turn stimulates ethylene synthesis in petals

and flower senescence. The ACC synthase gene expression in ovary and its stimulation by pollination has been investigated in orchid flower (Bui and O'Neill., 1998) carnation (Henskens et al., 1994; ten Have and Woltering., 1997) and geranium (Clark et al., 1997). A specific ACC synthase gene is expressed in ovary and is regulated by pollination. Whether RKacc7 expression is regulated by pollination remains to be investigated.

The expression of RKacc7 during different stages of flowering was also investigated [Fig. 3.3B]. In unopened or partially opened bud petals, there were no detectable RKacc7 transcripts, but these could be detected as the rose flower began to open and continued to increase and peaked after 5 days when senescence becomes detectable [Fig. 3.3B]. A simultaneous assay of ubiquitin transcript, a constitutively expressed gene, by RT-PCR showed no detectable change during different stages of flowering process (results not shown).

The expression of ethylene biosynthetic genes and subsequent senescence in petals is dependent on the processes in floral organs. This has been studied in carnation. Several observations suggest that following pollination mobile senescence factors are involved in the coordination of the senescence process (ten Have and Woltering., 1997). Senescence in carnation flowers may, in addition to ethylene treatment, be induced by pollination. Ethylene production following pollination in carnation has been shown to start in the styles and sequential increases in ethylene production and ACC concentrations in different flower parts indicated that ACC may be the translocated senescence factor (Nichols et al., 1983). In addition, treatment of the

carnation stigma with radiolabelled ACC resulted in the production of radiolabelled ethylene by the petals, another indication that ACC may be translocated into petals (Reid et al., 1984). The site(s) of ethylene biosynthesis in the senescing carnation flower has been identified in carnation and the role of the different ethylene biosynthesis genes in ethylene production and senescence has also been investigated (ten Have and Woltering, 1997). Their results indicate that, during natural senescence, ethylene biosynthesis starts in the ovary as a result of the expression of ACC synthase and ACC oxidase genes. Gaseous ethylene, diffusing to the petals, presumably triggers the expression of a petal-specific ACC synthase and an ACC oxidase gene leading to increased petal ethylene production and senescence (ten Have and Woltering, 1997). In our studies, sepals and ovary show expression of ACC synthase mRNA. Thus a correlation may exist between expression of ACC synthase gene and ACC as the trigger of flower senescence.

(2) Expression of RKacc7 gene *in vitro* and in *E.coli:*

To determine the sizes of proteins encoded by the rose ACC synthase genes, *in vitro* transcription [Fig. 3.4] and translation of RKacc7 were performed. These *in vitro* translation results show that the rose ACC synthase cDNA clones can be translated into proteins ranging in molecular weight of 48 KDa to 55 KDa. They also match the predicted sizes of proteins encoded by these clones [Fig. 3.5].

In order to confirm that rose ACC synthase cDNAs cloned from the rose cDNA library encode the functional ACC synthases, constructs from RKacc22 and RKacc18 were prepared into pET-30 vector and expressed in *E.coli* BL21(DE3) cells. The

expression was induced by the addition of 0.6 mM IPTG. The ACC synthase was purified from crude cell extraction by affinity chromatography. The purified target proteins were eluted by 60 mM imidazole and fractions were collected. The crude cell extract and purified target protein were subjected to electrophoresis to determine target protein expression. The dominant bands in the crude cell extract and purified protein exhibit the same mobilities [Fig. 3.7]. Western blotting was use to confirm that the dominant band(s) in 12% SDS-PAGE gel is the target protein by S-Tag detection system [Fig. 3.8]. The expression of RKacc6, RKacc22 and RKacc18 constructs were compared. The expression of RKacc18 was more than RKacc22 and RKacc6 expression [Fig.3.6]. The western blot analysis also showed that antiACC synthase antibody recognized the RKacc18 polypeptide [Fig.3.8]. These results further confirm that RKacc18 encodes an ACC synthase.

The crude cell extracts of BL21(DE3)::pET-30 RKacc18 and 22 were used to analyze ACC synthase activity. The reaction mixture was prepared as described in Material and Method. Each reaction was repeated three times. TLC was used to detect ACC production from the reaction mixture, whereas gas chromatography was used to detect ethylene production from ACC in the presence of $HgCl_2$ and NaoCl/NaOH. Neither synthesis of ACC, nor ethylene production were detected by TLC and gas chromatography. The lack of ACC synthase activity from these constructs may be due to the presence of His-Tag and S-Tag. Therefore, the deletion of His-Tag and S-Tag was carried out in pET-30RKacc18 and 22 constructs [Fig. 3.9]. But the proteins

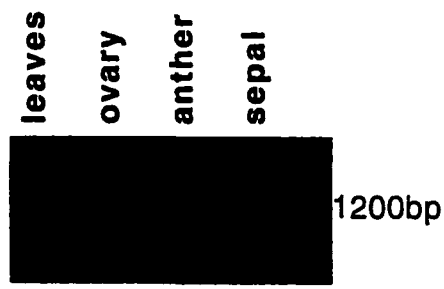
expressed from these constructs in *E.coli* BL21(DE3) also failed show detectable ACC synthase activity.

There are two reasons that may affect rose ACC synthase activity:

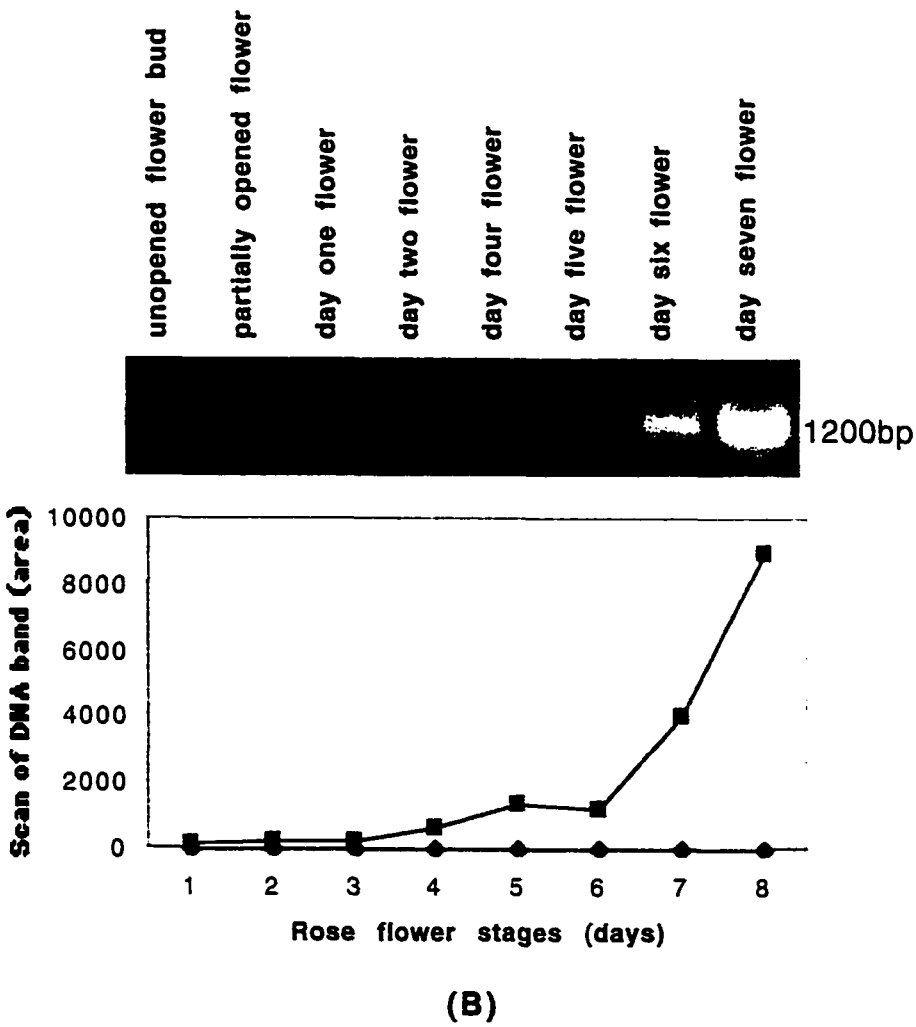
1) The expressed target protein in *E.coli* did not fold correctly. This may be due to the absence of appropriate molecular chaperones and a rate of translation incompatible with the solubility of the protein (Huxtable et al., 1998). The refolding of zucchini ACC synthase, a dimeric, PLP-requiring plant enzyme, with a molecular weight of 110 kDa and a number of possible disulfide bonds, falls into the category of more complex refolding protocols. Optimum folding conditions for one system may not work in another. Therefore, a protocol for refolding must be established on a case by case basis. The optimum conditions for renaturation of a wound-inducible zucchini ACC synthase cDNA were worked out by Huxtable et al.,(1998). The zucchini ACC synthase refolding protocol is a combined process of dialysis and dilution in 100 mM Mops, pH8, 30 mM Chaps, and 5 mM GSH (reduced glutathione) at a protein concentration of 45 μg/ ml (Huxtable et al., 1998). Development of a regeneration protocol for rose ACC synthase would be desirable.

2) Rose ACC synthase has a very short half life and as a result readily loses its activity during and after extraction and purification.

Fig. 3.3 *Expression of Rkacc7 during various stages of flower development.* Total RNA from early rose bud (unopened) to mature flower was used to prepare cDNA and then used as template for PCR (RT-PCR) to determine the level of RKacc7mRNA as described in the methods section.
Panel A: the RKacc7 gene expressed in rose and Panel B: Accumulation of Rkacc7 mRNA during flower petal senescing. Lane 1: un-opened flower bud, lane 2: partially opened flower, lane 3: day one flower, lane 4: day two flower, lane 5: day four flower, lane 6: day five flower, lane 7: day six flower and lane 8: day seven flower. The graph shows the relative peak area of the amplified signals measured by using iscon program.

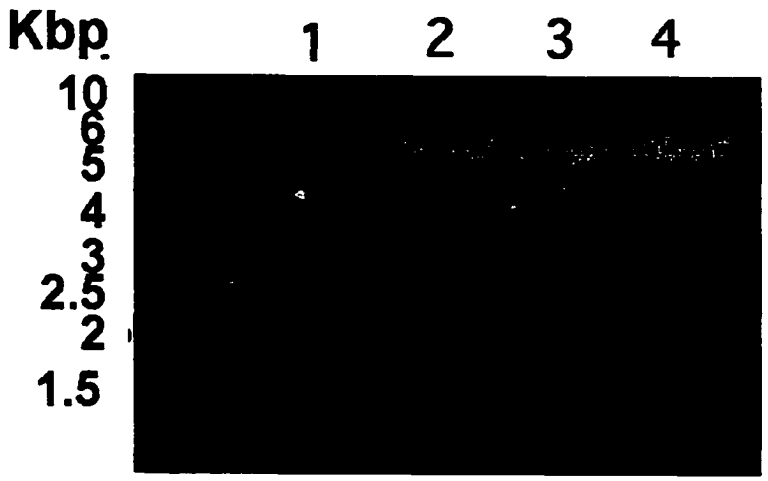


(A)

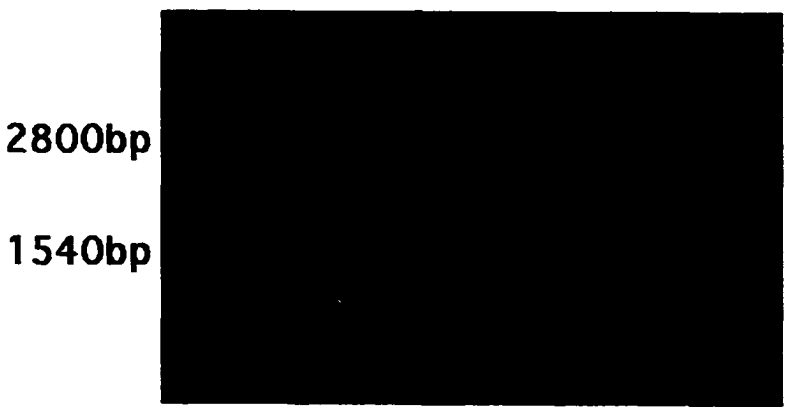


(B)

Fig. 3.4 *In vitro transcription of various PKacc clones.* pBK-CMV phagemids containing various PKacc cDNA were linearized by digestion with *Xho* I and then used for transcription with T3 RNA polymerase .
Panel A shows the size of cDNA insert released after digestion of pBK-CMV plasmid with *Sal* I and *Xho* I. Lane1: RKacc1; lane2: RKacc7; lane3: RKacc18 and lane4: RKacc22.
Panel B shows the sizes of RNA synthesized from the RKacc-cDNAs after electrophoresis in 1.2% agarose gel



A



B

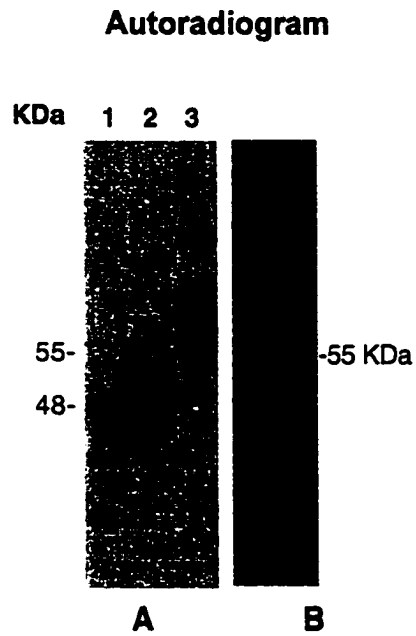


Fig. 3.5 *Translation of in vitro synthesized transcripts of RKacc cDNA in rabbit reticulocyte lysates:* Details are described in the Methods section.
(A) Lane1: RKacc24; lane2: RKacc18 and lane3:RKacc7.
(B)The RKacc7 protein translated and immuno precipitated with anti ACC synthase antibody.

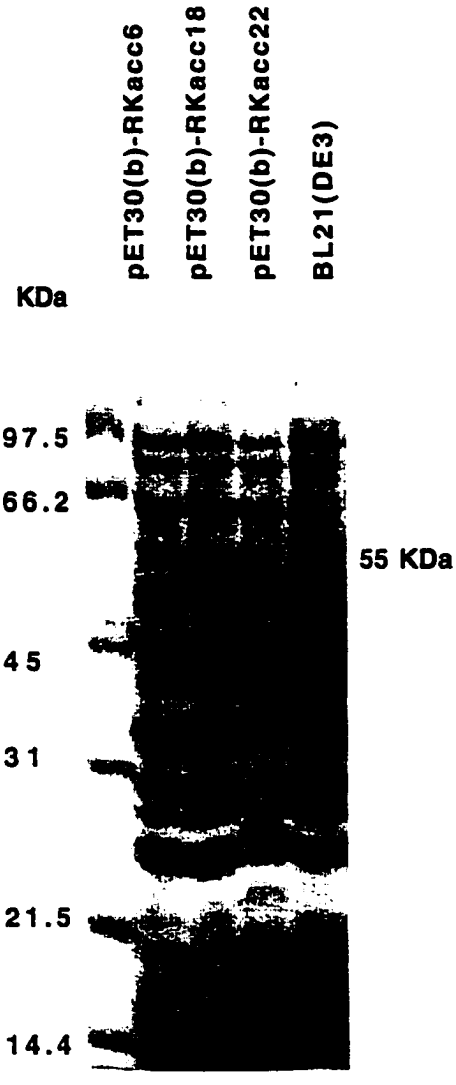


Fig. 3.6 *Expression rose ACC synthase in E. coli after induction with IPTG from pET-30 system.* The proteins in crude cell extracts were resolved by electrophoresis in 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Lane1: pET30(b)-RKacc6; lane2: pET30(b)RKacc18; lane3: pET30(a)-RKacc22 and lane4: BL21(DE3) cell extract. The induced full length ACC synthase polypeptide is indicated on the right (55KDa).

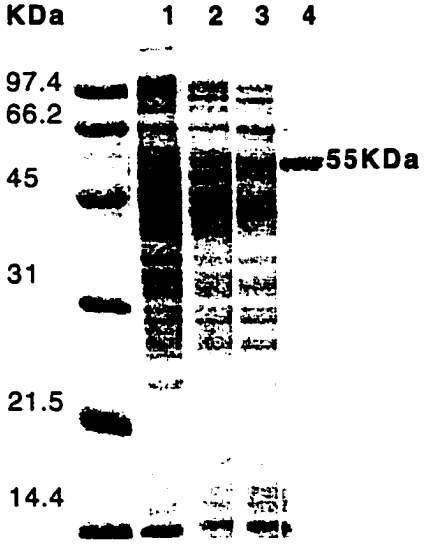


Fig. 3.7 *Rose ACC synthase cDNA expression in pET-30 system.* Lane1: BL21(DE3) cell extract; lane2: uninduced pET30(b)RKacc18 cell extract; lane3: 0.6 mM IPTG induced pET30(b)-RKacc18 cell extract and lane4: protein purified from pET30(b)-RKacc18 extracts by affinity chromatography. 10μg of protein sample was loaded in 1-3 lanes and 3 μg in lane 4.

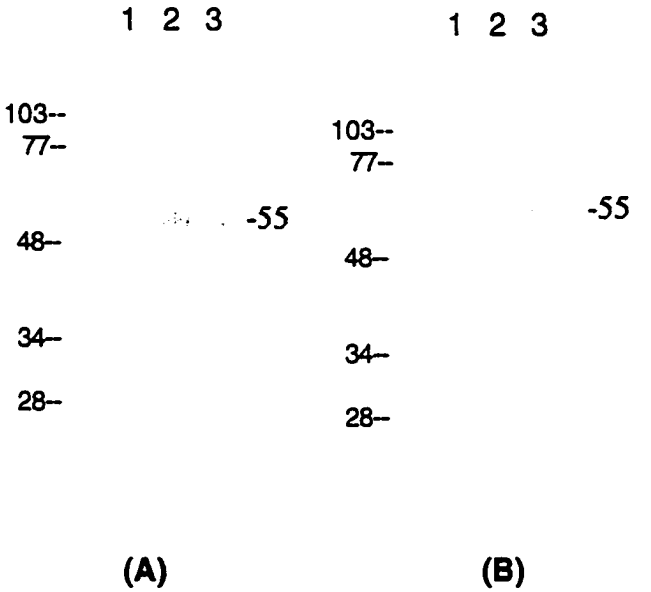
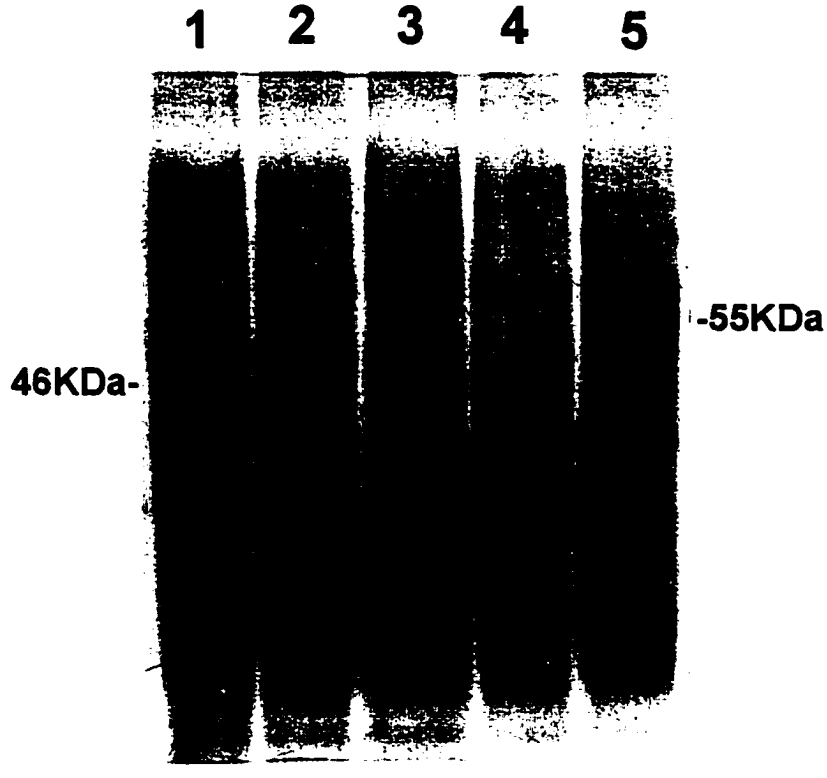


Fig. 3.8 *Analysis of expression of ACC synthase in E. coli by Western Blot.*

Panel A is Western blot analysis by S-protein and Panel B shows western blot analysis using anti-ACC synthase antibodies. In both panels, lane1: BL21(DE3) cell extract; lane2: pET30(b)-RKacc18 cell extract; lane 3: purified pET30(b)-RKacc18 protein purified by affinity chromatography. The protein amount loaded is 5 μg.

Fig. 3.9 *Expression of ACC synthase from modified pET30(b)-RKacc18 in which His-Tag and S-Tag sequences have been removed by Nde I and Sal I.* Lane1: BL21(DE3) cell extract; lane2: uninduced BL21(DE3) cell extract harboring modified pET30(b)-RKacc18; lane3: Extract from BL21(DE3) harboring modified pET30(b)-RKacc18 induced with 0.6mM IPTG; lane4:uninduced BL21(DE3) cell extract harboring pET30(b)-RKacc18; and lane5: Extracts from BL21(DE3) harboring modified pET30(b)-RKacc18 induced with 0.6mM IPTG.



Chapter 4 Conclusion

Ethylene is very important in flower senescence. The role of ethylene in flower senescence was not established until the development of analytical methods sufficiently sensitive to measure the minute quantities of ethylene produced by the minimal tissues of a flower. Using gas chromatography, researchers have shown that, depending on the species, ethylene may be involved in several phases of flowering, from induction through growth of floral tissues to petal senescence (Reid and Wu, 1992). Woltering and van Doorn (1988) studied the role of ethylene in petal senescence of flowers of 93 species representing 23 families. In Rosaceae, petal abscission was the initial symptom of senescence and the flowers were mostly sensitive to exogenous ethylene. In ethylene-contaminated air, rose cultivars with high sensitivity to exogenous ethylene exhibited short flower life. In ethylene-free environments, rose flower longevity was in most cultivars related to the onset of ethylene production in flowers (Muller et al., 2000).

The plant hormone ethylene is synthesized from the amino acid ACC by the enzyme ACC oxidase (Fluhr and Mattoo, 1996; Kende, 1993). In most plant tissues, the rate of ethylene production is determined by the activity of ACC synthase, which converts S-adenosylmethionine to ACC (Kende, 1993). Genes encoding ACC synthase

and ACC oxidase have been identified in a number of plant species, and in both cases multigene families that exhibit differential patterns of expression have been identified (Fluhr and Matto, 1996; Lasserre et al., 1996; Liang et al., 1992; 1996; Rottmann et al., 1991; Tang et al., 1993, 1994). ACC synthase genes have been shown to be regulated by auxin, ethylene, wounding, ozone, anaerobiosis, Li+, senescence, cycloheximide, and/or ripening (Kende, 1993; Fluhr and Mattoo, 1996). Expression studies have revealed that the induction of ACC synthase activity is most often the result of increased accumulation of ACC synthase mRNAs (Kende, 1993; Zarembinski and Theologis, 1994).

To investigate the role of ethylene involved in kardinal rose petal senescence, an ACC synthase complementary DNA (RKacc7) was isolated from senescing rose petal cDNA library with a rose specific probe. This probe consists of 400bp and was amplified from rose RNA fraction by PCR. The PCR was primed by two degenerated primers which were designed based on the conserved regions of ACC synthase genes published previously (Zarembinski and Theologis, 1994). A total of eight positive clones were selected, only one of these clones Rkacc7 presented the full length of cDNA which contains the start and the stop codons. Rkacc7 consisted of 1750 bp with an open reading frame of 480 amino acids sufficient to encode a protein of 58 Kda. The open reading frame is proceeded by 269 bp of an untranslated leader and a 37 bp of untranslated sequences at the 3'-end. The RKacc7 polypeptide contains the conserved dodecapeptide SKDLSLPGFRVG, that is part of the active site of ACC synthases (Fluhr and Mattoo, 1996; Yip et al., 1990). RKacc7 also contains the 11 invariant amino

acid residues conserved in ACC synthases and amino transferases (Fluhr and Mattoo, 1996). The comparison of RKacc7 to other known ACC synthases found in the protein database shows that it has more homology to the ACC synthase from wheat than to the ACC synthase from other closely related dicot species. These results suggest that the polymorphism of ACC synthase arose before the time of divergence of monocotyledonous and dicotyledonous plants and are probably presented in all higher plants. Similar early origin of gene family in plant evolution has also been described in phytochrome gene which is the most ancient regulatory photoreceptor family in plants (Liang et al., 1992).

Southern blot studies with the rose genomic DNA, ACC synthase gene appears to have multiple copies in the genome. This may be due to the fact that kardinal rose is a hybrid. ACC synthase is encoded by a divergent multigene family in numerous plant species. For example, seven ACC synthase genes have been identified in tomato. The expression of these genes is developmentally and spatially regulated by mechanical wounding, fungal elicitor, flooding stress, auxin treatment, ozone exposure and fruit ripening (Van der Straeten et al., 1990; Olson et al., 1991, 1995; Rottmann et al., 1991; Yip et al., 1992; Spanu et al., 1993; Oetiker et al., 1997; Tuomainen et al., 1997; Shiu et al., 1998). There are at least seven and five divergent members of ACC synthase genes in Arabidopsis and rice, respectively (Liang et al., 1992; Zarembinski and Theologis, 1993; Vahala et al., 1998; Arteca and Arteca, 1999; Woeste et al., 1999). Five members of the ACC synthase gene family have been isolated from potato plants, with the expression of each member of the family being increased differentially during the

progression of leaf and petiole senescence, in response to auxin in hypocotyls, in wounded leaves and tubers, and by ozone and Cu^{2+} treatment in leaves (Destefano-Beltran et al., 1995; Schlagnhaufer et al., 1997). In the present investigation only a single ACC synthase cDNA has been isolated from the kardinal rose cDNA library. However, results of Southern blots from genomic DNA under high stringency show multiple DNA bands beyond those expected from restriction sites within the cDNA. These multiple bands could be generated due to introns which are lacking in cDNA. Alternatively, they may be the results of other ACC synthase genes which show a high degree of homology with RKacc synthase 7. This possibility is strongly suggested by the molecular weight of DNA bands which are much larger than the 2 kb to 3 kb expected as a result of the presence of introns. Southern blots performed at lower stringency further revealed additional bands which may be the result of other ACC synthase genes with lower degrees of sequence homology with RKacc 7. It is possible that if lowered hybridization condition used in library screening, additional rose ACC synthase gene members may be selected from the rose petal cDNA library. The Southern blot data suggests to conclude that here too, rose ACC synthase is represented by a multigene family.

The rose ACC polypeptide contains all the conserved and characteristic domains found in the ACC synthase isoenzymes from various plant species, but no ACC or ethylene could be detected when rose ACC synthase cDNA was expressed in *Escherichia coli*. Since RKacc7 was transcriptionaly active in rose, the possibility exists that the RKacc7 polypeptide may be posttranslationally modified and is indeed active

in the intact plant. In some situations the activity of ACC synthase has been shown to be regulated at the posttranslational level (Felix et al., 1991). On the other hand, rose ACC synthase may be a dimeric protein, like ACC synthases in mung bean (Tsai et al., 1988) and zucchini (Sato et la., 1991). The optimum condition for refolding of a dimeric ACC synthase with a high molecular weight and a number of possible disulfide bonds falls into the category of more complex refolding protocol (Huxtable et al., 1998) which may be the case with rose ACC synthase.

Expression studies have revealed that the induction of ACC synthase activity is most often the result of increased accumulation of ACC synthase mRNAs (Kende, 1993; Zarembinski and Theologis, 1994). The RKacc7 is transcribed in rose petals and the amount of mRNA increased as the petals aged, suggesting that its expression correlates with petal senescence. This gene is also expressed in rose flower organs, such as: ovary and sepals, but not in rose leaves. These results indicate a specificity of expression in rose flower tissues. To further validate expression of RKacc7 in flower tissue, it will be necessary to isolate the RKacc7 promoter. A promoter-driven reporter gene expression will show whether RKacc7 expression is restricted to flower tissue.

In unopened buds and partially opened buds, there are no detectable RKacc7 mRNA. When the rose petals were fully opened, the RKacc7 transcripts started to accumulate suggesting that Rkacc7 is related to rose petals aging. How the Rkacc7 transcript is regulated in rose is unsolved problem. It has been found recently that ACC synthase activity can be triggered by pollination in carnation (Jones et al., 1999),

petunia (Lindstrom et al., 1999), geranium (Clark et al., 1997) and orchid (Bui and O'Neil, 1998).

It is now widely appreciated that both ethylene biosynthesis and ethylene perception contribute to the regulation of ethylene responses in plant (Wilkinson et al., 1995). In minature potted roses, the expression of the receptor gene RhETR was distinctly higher in the cultivar with short flower life than in the long-lasting cultivar, and modulation of receptor levels was also observed during flower development (Muller et al., 2000). Differences in the expression of the putative ethylene receptor of cultivars with short or long flower life suggest that variation in flower longevity may be due to differences in receptor levels during flower development.

To inhibit the ethylene response during the senescence in carnation flowers, the *Arabidopsis etr1-1* allele has been introduced into transgenic carnation plants. The transgenic carnation flower senescence was delayed by at least 6 days relative to the control flowers (Bovy et al., 1999). And in these flowers expression of ACC oxidase gene *ACO1* was down-regulated. This indicated that the autocatalytic induction of ethylene biosynthesis, required to initiate and regulate the flower senescence process, is absent in *etr1-1* transgenic plants due to dominant ethylene insensitivity (Bovy et al., 1999). Previously, the genes encoding key enzymes in the ethylene biosynthesis pathway have been successfully used to inhibit ethylene biosynthesis in different plant species. This has led to inhibition of fruit ripening in tomato (Good et al., 1994; Hamilton et al., 1990; Klee et al., 1991; Oeller et al., 1991) and melon (Ayub et al., 1996) or a delayed flower senescence in carnation (Savin et al., 1995). Transfer of the

ethylene biosynthesis pathway genes or ethylene receptor mutant genes in antisense orientation into plants is likely to find applications in climacteric or non-climacteric plants, flowers and fruits to prevent damage caused by ethylene.

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Appendix I

Plasmid DNA miniprep

Solution I: Glucose 5 mM

Tris-HCl 25 mM (pH 8.0)

EDTA 10 mM

Solution II: NaOH 0.2 N

SDS 1%

Solution III: KoAC 5M pH 4.8

1. suspend cell pellet in solution I (100 µl);

2. add solution II 200 µl, mix it and incubate at room temperature for 10 min;

3. add 150 µl solution III, mix and centrifuge at top speed for 5 min;

4. recover the supernatant and add equal volume of isopropanol, mix, incubate on ice for 10 min;

5. centrifuge at top speed for 10-15 min at 4C;

6. use 70% ethanol to wash the DNA pellet, twice; and the pellet air dried;

7. dissolve DNA in TE buffer.

Large scale isolation of plasmid

1. pellet bacteria cells at 5000 rpm for 10 min;

2. the cell pellet is suspended evenly in Tris-sucrose buffer (50 mM Tris-HCl pH 7.5) and then add lyzozyme solution (5 mg/ml) and 0.5 M EDTA,

incubate on ice for 5 minutes;

3. add Triton-X buffer (50 mM Tris-HCl pH 7.5, 60 mM EDTA, 10% Triton-X100), mix well, centrifuge at 40,000 rpm for 2 hours at 4∞C;

4. take out the supernatant and measure the volume;

5. add NaCl to the final concentration of 1.5 M and mix well; add 1/2 volume of 30% polyethylene glycol (MW 6000) to precipitate DNA;

6. centrifuge at 6000 rpm for 10 min to pellet DNA;

7. resuspend the DNA pellet in 1/10 volume of Tris-EDTA buffer (0.1M Tris-HCl pH7.5, 10mM EDTA);

8. add CsCl 1.05 g/ml and 80 µl of EtBr (10 mg/ml);

9. centrifuge at 10,000 rpm for 30 min to get rid of undissolved material;

10. ultracentrifuge at 60-65,000 rpm for 16-18 hours at 15∞C;

11. take out the lower band (plasmid), extract EtBr with butanol (water saturated);

12. delute the DNA solution with water or TE buffer (1:3);

13. priciptate DNA with 2 volume of 100% ethanol;

14.centrifuge to collect DNA and wash the pellet with 70% ethanol, dry it.

Appendix II

Media and Reagent Preparation

LB Broth (per liter)

NaCl	10 g	tryptone	10 g
yeast extract	5 g		

Adjust to pH 7.4, and add deionized H_2O to a final volume of 1 liter, autoclave

For LB agar, 13g of agar is added to 1 liter LB broth and autoclave.

NZY Agar (per liter)

NaCl	5 g	NZ amine	10 g
$MgSO_4 \cdot 7H_2O$	2 g	Agar	13 g
yeast extract	5 g		

Add deionized H_2O to a final volume of 1 liter and adjust the pH to 7.4, autoclave.

SOC Medium

2.0% Tryptone	2.5 mM KCl
0.5% Yeast Extract	10.0 mM $MgCl_2 \cdot 6H_2O$
10.0 mM NaCl	20.0 mM glucose

Prepare 1 M $MgCl_2$ solution and autoclave separately; 2 M glucose filter-sterilize

NZY Top Agar (per liter)

1 liter of NZY broth

Add 0.7% (w/v) agarose, autoclave.

SM Buffer (per liter)

NaCl	5.8 g
$MgSO_4 \cdot 7H_2O$	2.0 g
1 M Tris-HCl (pH 7.5)	50 ml
2% (w/v) gelatin	5.0 ml

Add deionized H_2O to a final volume of

1 liter

10 X STE Buffer

1 M	NaCl
200 mM	Tris-HCl (pH 7.5)
100 mM	EDTA

20 X SSC Buffer (per liter)

NaCl	17.3 g
sodium citrate	88.2 g
deionized H_2O	800 ml

Adjust to pH 7.0 and add deionized H_2O to a final volume of 1 liter