Molecular Genetic and Physiological Studies on the Sex-determining *M/m* and *A/a* Genes in Cucumber (*Cucumis sativus* L.)

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Dedicated to

my grandfather, the late Beyene Buya

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Abstract

Sex determination in cucumber is the most extensively studied subject. Three major genes (*F/f, M/m* and *A/a*) have been described that control sex in cucumber. In addition to these genes, environmental and hormonal factors also influence the sex in cucumber plants. Because of its direct influence on sex expression ethylene is considered as a sex hormone of cucumber. In relation to the sex hormone ethylene, the assumed role of the sex-determining *F/f*, *M/m* and *A/a* genes is that the *F/f* gene controls the level of ethylene; the *M/m* gene influences ethylene perception and signaling and the *A/a* gene may control the level of ethylene biosynthesis, perception and signaling. Tremendous achievements have been made in characterizing the sex-determining *F/f* gene in relation to ethylene-related genes. In recent years, characterization using the ethylene synthesis-related genes enabled researchers to isolate the promoter region of the dominant *F* gene in cucumber. Only few studies have been conducted concerning the characterization of the *M/m* and *A/a* genes. Most of the previous investigations were on the *F* gene, since the amount of ethylene correlates with the *F* gene that provided a hint for intensive study on the *F* gene.

To understand the assumed role of the *M/m* gene in ethylene perception and signal transduction, the expression levels of ethylene receptor and ethylene signal transduction related genes were compared between nearly isogenic cucumber lines that differ on the *M/m* gene. Strong expression level of the ethylene receptor genes (*CsETR1, CsETR2* and *CsERS*) and weak expression levels of the ethylene signal transduction related-genes (*CsEIN3* and *CsEIL1*) are detected in developed stamen-primordia linked to the recessive *m* gene of hermaphrodite cucumber. The exact opposite result is detected in arrested stamen-primordia

linked to the dominant *M* gene in gynoecious cucumber. Further comparison of the expression level of the ethylene related genes after chemical application indicated that the dominant *M* gene in gynoecious controls the expression level of ethylene receptor and signaling genes in a different way than the recessive *m* gene in hermaphrodite cucumber. Physiological investigation also showed that the gynoecious cucumber with the dominant *M* gene responds differently than the hermaphrodite cucumber with the recessive *m* gene towards external chemical application. Thus, these results indicate that *M/m* gene control the effect of ethylene at a level of ethylene perception and signal transduction.

Identical expression level of ethylene receptor and signal transduction-related genes are detected in flowers and leaves of monoecious and androecious cucumber plants that differ by the *A/a* gene. Physiological studies by grafting, and ethylene measurement after chemical application also revealed that the response from the dominant *A* gene and the recessive *a* gene in relation to the sex hormone ethylene is identical. Therefore, the findings indicate that the *A/a* gene may not involve in ethylene perception and signal transduction.

In the attempt to isolate the sex-determining *M/m* gene, suppression subtractive hybridization was used on mRNA obtained from nearly isogenic gynoecious and hermaphrodite cucumber floral buds. 21 cDNA clones were isolated. The sequences of the clone ‘cl-38’ is homologous to nucleotide sugar epimerase, hence named as putative nucleotide sugar epimerase. This putative nucleotide sugar epimerase is shown to be poorly expressed in female floral buds than the male and hermaphrodite floral buds. The weak expression level of the putative nucleotide sugar epimerase in female floral buds is an indicative that the gene function is impaired in female flower and cause arrest of the stamen development. The isolated putative nucleotide sugar epimerase gene may be the product of the sex-determining *M/m* gene.

Preliminary analysis using Southern blot indicated that the copper transporter (*CsRAN1*) gene is linked to the sex-determining *A/a* gene. After further confirmation analyses, this approach may be used to isolate the sex-determining *A/a* gene in cucumber.

Key Words: Cucumber, Ethylene, Sex determination

Molekular genetische und physiologische Studien an geschlechts-bestimmenden *M/m* und *A/a*- Genen der Gurke (*Cucumis sativus* L.)

Zusammenfassung

Geschlechtsdetermination ist einer der am häufigsten untersuchten Eigenschaften bei der Gurke. Drei Majorgene *F/f*, *M/m* und *A/a* sind beschrieben worden, die das Geschlecht dieser Pflanzenart kontrollieren. Zusätzlich zu diesen Genen beeinflussen noch hormonale Faktoren und Umweltfaktoren das Geschlecht der Gurke. Wegen seines direkten Einflusses auf die Geschlechtsexpression der Gurke wird Ethylen als Geschlechtshormon der Gurke betrachtet. Die bisherige Vorstellung bzw. Annahme bezüglich der Bedeutung der geschlechtsbestimmenden Gene *F/f*, *M/m* und *A/a* im Zusammenhang mit dem Ethylen kann wie folgt beschrieben werden: Das Gen *F/f* kontrolliert die Ethylenproduktion , das *M/m* Gen könnte die Ethylenperception und/oder -transduktion kontrollieren, und das *A/a* Gen könnte an der Ethylenbiosynthese und/oder -perception und/oder –transduktion beteiligt sein. Es sind bezüglich der Charakterisierung des *F/f* Gens im Zusammenhang mit den Genen, die mit dem Ethylen in Beziehung stehen, wichtige Aussagen gemacht worden. In den letzten Jahren hat z.B. die Nutzung der Charackterisierung der mit der Ethylenbiosynthese in Beziehung stehenden Genen den Forschern die Isolierung der Promotorregion des dominanten *F* Gens bei der Gurke ermöglicht. Bezüglich der Charakterisierung der *M/m* und *A/a* Gene existieren jedoch nur sehr wenige Untersuchungen. Dies liegt daran, dass die Korrelation der Ethylenmenge mit dem *F/f* Gen im Gegensatz zu den anderen Genen eine gute Ansatmöglichkeit für die Untersuchungen bietet.

Um die Vorstellung bezüglich der Rolle des *M/m* Gens in der Ethylenperception und -signal transduktion zu klären, wurde die Expression der Gene, die zu Ethylenperception und -signaltransduktion in Beziehung stehen, bei nahe isogenen Gurkenlinien untersucht, die sich

nur am *M/m* Gen unterschieden. An den entwickelten Stamen-Primordia der mit dem rezessiven *m* Gen in Verbindung stehenden hermaphroditischen Pflanzen konnte eine wesentlich stärkere Expression der Ethylenrezeptorgene *CsETR1*, *CsETR2* und *CsERS* und eine wesentlich schwächere Expression der Ethylensignaltransduktionsgene *CsEIN3* und *CsEIL1* festgestellt werden. Dagegen wurden an den in ihrer Entwicklung verhinderten Stamen-Primordia der mit dem dominanten *M* Gen in Verbindung stehenden gynözischen Pflanzen genau entgegengestzte Ergebnisse erzielt. Weiterer Vergleich der Expression der zur Ethylen in Beziehung stehenden Gene nach Verabreichung von Chemikalien zeigte, dass das dominante *M* Gen der gynözischen Pflanzen die Expression der Ethylenrezeptorgene und -signaltransduktionsgene auf einer anderen Art und Weise beeinflußt als das rezessive *m* Gen der hermaphroditischen Pflanzen. Auch bei physiologischen Studien zeigten die gynözischen Pflanzen mit dem dominanten *M* Gen gegenüber der Behandlung mit Chemikalien eine völlig andere Reaktion als die hermaphroditischen Pflanzen mit dem rezessiven *m* Gen. Diese Ergebnisse zeigen, dass das *M/m* Gen den Effekt des Ethylens auf der Ebene der Ethylenperception und Ethylensignaltransduktion kontrolliert.

Identische Expression der Gene, die mit der Ethylenperception und -signaltransduktion in Beziehung stehen, wurden bei den monözischen und andrözischen Pflanzen, die sich am *A/a* Gen unterscheiden, sowohl in den Blüten als auch in den Blättern festgestellt. Physiologische Studien nach Propfung und Ethylenmessungen nach Behandlung mit Chemikalien zeigten, dass die Reaktion des dominanten *A* Gens und des rezessiven *a* Gens bezüglich der Beziehung zu dem Geschlechtshormon Ethylen identisch ist. Diese Ergebnisse deuten darauf hin, dass das *A/a* Gen in Ethylenperception und Ethylensignaltransduktion höchstwahrscheinlich nicht involviert ist.

Bei den Versuchen, deren Ziel die Isolierung des Gens *M/m* war, wurde an mRNA aus Blütenknospen der nahe isogenen gynözischen und hermaphroditischen Linien die

„Supression Subtractive Hybridisation“ Methode angewandt. 21 cDNA Klone wurde isoliert. Es wurde gezeigt, dass der Klon ‚cl-38’, der nach Sequenzanalysen als „putative nucleotide sugar epimerase“ bezeichnet wurde, in weiblichen Blütenknospen wesentlich schwächer expremiert wird als in Knospen der männlichen und hermaphroditischen Blüten. Dies deutet darauf hin, dass dieses Gen an der Verhinderung des Stamenentwicklung der weiblichen Blüten involviert ist. Das isolierte „putative nucleotide sugar epimerase“ könnte das Produkt des *M/m* Gens sein.

Vorläufige Analysen durch die Nutzung der Southern Blot- Technik deuten darauf hin, dass das Kupfer Transporter Gen *CsRAN1* mit dem Gen *A/a* gekoppelt ist. Die Bestätigung dieses Ergebnisses könnte für die Isolierung des *A/a* Gens einen wichtigen Ansatzpunk bedeuten.

Schlüsselwörter: Gurke, Ethylen, Geschlechtsdetermination

Table of Contents

Abbreviations

ACC	=	1-aminocyclopropane-1-carboxylic acid
ACO	=	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	=	1-aminocyclopropane-1-carboxylic acid synthase
AGPs	=	Arabinogalactan-proteins
ATP	=	Adenosine triphosphate
BC_1	=	backcross 1
bp	=	base pairs
CCC2	=	Ca^{2+}-sensitive cross-complementer 2
cDNA	=	complementary DNA
cl	=	clone
cm	=	centimeter
Cs	=	*Cucumis sativus*
CTAB	=	Cetyltrimethyl ammonium bromide
CTR1	=	constitutive triple response 1
°C	=	degree Celsius
Cu(I)	=	copper ion
dATP	=	Deoxyadenosine-5'-triphosphate
dCTP	=	Deoxycytidine-5'-triphosphate
ddH_2O	=	double-deionized water
DEPC	=	Diethylpyrocarbonate
dGTP	=	Deoxyguanosine-5'-triphosphate
DIG	=	Digoxigenin
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
dNTP	=	Deoxy-ribonucleotide-5'-triphosphate
ds cDNA	=	double-stranded complementary DNA
DTT	=	Dithiothreitol
dTTP	=	Deoxythymidine-5'-triphosphate
EDTA	=	Ethylene-diamine-tetra-acetic acid
EIL1, 2	=	ethylene insensitive 3 like 1, 2
EIN2, 3, 4	=	ethylene insensitive 2, 3, 4

EREBP	=	ethylene-responsive element binding protein
ERF1	=	ethylene-responsive element binding factor 1
ERS1, 2	=	ethylene response sensor 1, 2
ETR1, 2	=	ethylene receptor 1, 2
F_1	=	first filial generation
F_2	=	second filial generation
g	=	gram
GTP	=	Guanosine triphosphate
h	=	hour
kb	=	kilo base
kPa	=	kilo Pascal
µg	=	microgram
µM	=	micromolar
M	=	molar
mbar	=	millibar
mg	=	milligram
min	=	minute
ml	=	milliliter
MNK	=	Menkes
MOPS	=	N-Morpholino-propanesulfonic acid
mRNA	=	messenger RNA
ng	=	nanogram
NILs	=	nearly isogenic lines
nM	=	nanomolar
nm	=	nanometer
Nr.	=	number
OP	=	Operon primer
PCR	=	Polymerase chain reaction
PK	=	Protein kinase
ppm	=	Parts per million
RAN1	=	responsive to antagonist 1
RAPD	=	Randomly amplified polymorphic DNA
RHD1	=	root hair deficient1
RNA	=	Ribonucleic acid

RNase	=	Ribonuclease
rpm	=	round per minute
rRNA	=	ribosomal RNA
RT-PCR	=	Reverse transcription PCR
SDS	=	Sodium dodecyl sulfate
SE	=	standard error
SSC	=	Saline sodium citrate
SSH	=	Suppression subtractive hybridization
SST	=	Serum separation tube
TAE	=	Tris-acetate EDTA
Tris	=	Tris (hydroxymethyl)-aminomethane
TS2	=	tasselseed 2
U	=	unit
UDP	=	Uridine 5’-diphosphate
UV	=	ultraviolet
V	=	volt
v	=	volume
w	=	weight
xg	=	gravity

1 General Introduction

Sex determination is a process that leads to the physical separation of male and female gamete-producing structures to different individuals of a species (Dellaporta and Calderon-Urrea, 1993; Ainsworth et al., 1998; Ainsworth, 1999). Cucumber, *Cucumis sativus*, has been serving as a model system for sex determination studies since the 1950s driven by breeding programs for hybrid seed production (Durand and Durand, 1984; Tanurdzic and Banks, 2004).

Cucumber plants may produce male (staminate), female (pistillate) and hermaphrodite (bisexual) flowers. The respective three bud types have at a young developmental stage both stamen and carpel primordia, that appear equally developed under the microscope and are morphologically bisexual (Shifriss and Galun, 1956; Atsmon and Galun, 1960; Perl-Treves, 1999). Unisexual male flowers develop from the bisexual buds as a result of inhibition of the carpel primordia as stamens develop, while female flowers form when stamen primordia are arrested and the carpel differentiate. A rudimentary carpel is still present in mature male flowers and vestigial stamens are still present in mature female flowers.

Cucumber plants are originally monoecious bearing separate staminate and pistillate flowers on the same plant. Cucumber plants can also be either of the following sex types: gynoecious- producing pistillate flowers only; andromonoecious- having staminate and bisexual flowers on the same plant; hermaphroditic- caring only bisexual flowers; androecious- bearing staminate flowers only (Malepszy and Niemirowicz-Szczytt, 1991; Tatlioglu, 1993; Perl-Treves, 1999)

Genetic and non-genetic factors that control sex expression in cucumber

The inheritance of sex expression in cucumber has been elucidated by Galun (1961) and Kubicki (1969a-d). Regardless of complex trait that leads to the appearance of different

flower types, three major genes (*F/f*, *M/m* and *A/a*) account for most sex phenotypes in cucumber (Tatlioglu, 1991; 1993; Perl-Treves, 1999) (Table 1.1). *F/f* gene- is a partially dominant gene that controls femaleness. The *F* allele shifts the monoecious sex pattern 'downstream', causing the female phase to start much earlier. *A/a* gene- increases maleness; *F/f* is epistatic to *A/a*: in *ff* genotypes, the recessive *a* allele intensifies the male tendency. *M/m* gene controls bisexual flower expression. The recessive *m* allele allows the formation of bisexual flowers and the dominant *M* allele controls the selective arrest of stamens. In buds determined to develop a carpel, the dominant *M* gene may act, directly or indirectly, as a stamen-suppressor (Galun, 1961; Kubicki, 1969d; Pierce and Wehner, 1990; Perl-Treves, 1999).

Table 1.1 Major genes involved in sex determination in cucumber and their interaction (taken from Tatlioglu, 1993)

	F	*f*	
	A or *a*	*A*	*a*
M	Gynoecious	Monoecious	Androecious
m	Hermaphrodite	Andromonoecious	Androecious

In addition to the sex-determining genes, several factors such as environmental conditions, chemical treatments and plant hormones influence sex expression in cucumber. For example, short days and low night temperatures enhanced femaleness (Atsmon and Galun, 1962; Atsmon, 1968; Matsuo, 1968; Lower, 1975; Cantliffe, 1981; Takahashi et al., 1983); application of gibberellic acid on cucumber plants favor male flower formation (Wittwer and Bucovac, 1962; Clark and Kenney, 1969; Pike and Peterson, 1969; Fuchs et al., 1977; Atsmon and Tabbak, 1979); compounds that inhibit gibberellic acid synthesis (anti-gibberellins) promote femaleness (Hansen et al., 1976; Atsmon and Tabbak, 1979); auxins, such as naphthalene acetic acid promote female flower formation (Galun, 1959a; b; Saito and Ito, 1964; Shannon and de le Guardia, 1969; Friedlander et al., 1977a; Takahashi and Jaffe,

1984; Trebitsh et al., 1987); ethephon (2-chloroethylphosphonic acid), an ethylene releasing compound increase the number of female flowers (Iwahori et al., 1969; Robinson et al. 1969; Rudich et al. 1969; George, 1971; Retig and Rudich, 1972); aminoethoxyvinyl glycine (AVG) inhibit ethylene synthesis and promote maleness (Owens et al., 1980; Rudich, 1990); silver ion act as inhibitors of ethylene action and application of $AgNO_3$ induced staminate flowers (Beyer, 1976a; b; Beyer, 1979; Nijs and Visser, 1980; Goren et al., 1984).

The plant hormone ethylene perception and signaling

The biochemistry of ethylene biosynthesis has been a subject of intensive study in plant hormone physiology (Adams et al., 1979; Yang, 1980; Yang and Hoffman, 1984; Kende, 1993; Davies, 1995). Major breakthroughs in the ethylene synthesis pathway were the establishment of *S*-adenosyl methionine and 1-aminocyclopropane-1-carboxylic acid (ACC) as the precursors of ethylene (Yang and Hoffman, 1984; Davies, 1995; Fluhr and Mattoo, 1996). On the basis of this knowledge, genes that catalyze these reactions, such as ethylene biosynthesis enzymes ACC synthase (ACS) and ACC oxidase (ACO) have been characterized and cloned (Sato and Theologis, 1989; Hamilton et al., 1991; Spanu et al., 1991; Fluhr and Mattoo, 1996). ACS catalyzes the conversion of S-adenosyl methionine to ACC, the immediate precursor of ethylene. ACO catalyzes the last ethylene biosynthesis step from ACC to ethylene (Fluhr and Mattoo, 1996; Wang et al., 2002). Such genes were readily isolated from many plants including cucumber based on sequence homology (Trebitsh et al., 1987; 1997; Perl-Treves et al., 1998; Kamachi et al., 2000; Saraf-Levy et al., 2000).

After its synthesis, ethylene is perceived and its signal transduced through transduction machinery to trigger specific biological responses. Ethylene is perceived by a family of membrane-localized receptors (Kieber et al., 1993; Kieber, 1997; Bleecker et al., 1998; McGrath and Ecker, 1998; Bleecker, 1999; Wang et al., 2002). Five ethylene receptors exist in *Arabidopsis*: *ETR1, ETR2, ERS1, ERS2*, and *EIN4* (Chang et al., 1993; Hua et al., 1995;

Hua and Meyerowitz, 1998; Hua et al. 1998; Sakai et al., 1998). Isolation of the loss-of function alleles of *ETR1, ETR2, EIN4*, and *ERS2* by screening for intragenic suppressors of the dominant receptor mutants provides genetic evidence of how the ethylene receptors actually work (Hall et al., 1998; Hua and Meyerowitz, 1998; Hall et al., 2000). The constitutive triple response observed in a quadruple-receptor mutant indicates that the receptors negatively regulate this ethylene response. The synthesis of the results from both genetic and biochemical studies leads one to conclude that ethylene receptors are inactivated by ethylene binding. Characterization of ethylene binding to *ETR1* has revealed that it occurs in a hydrophobic pocket located at the N terminus of the receptors and requires a transition metal, copper, as a cofactor (Schaller and Bleecker, 1995; Gamble et al., 1998; Rodriguez et al., 1999).

Further evidence for a role of copper in ethylene signaling comes from the characterization of the Arabidopsis responsive-to-antagonist (*RAN1*) gene (Hirayama et al., 1999; Hirayama and Alonso, 2000). Co-suppression of the *RAN1* gene led to a constitutive ethylene response phenotype. This is consistent with a loss-of-receptor function (Hirayama et al., 1999; Woeste and Kieber, 2000). This phenotype can be partially rescued by exogenous copper application. Cloning and subsequent functional analysis of *RAN1* revealed that it encodes a copper transporter that shares similarity with copper-transporting P-type ATPases such as the yeast Ca^{2+}-sensitive cross-complementer2 (*CCC2*) and human Menkes/Wilson disease proteins (Hirayama et al., 1999). Taken together, these findings indicate that *RAN1* is involved in delivery of copper to the ethylene receptor and that this copper-delivery pathway is required to create functional ethylene receptors in plants.

In the courses of ethylene signaling (Fig. 1.1), ethylene receptors directly interact with constitutive triple response (*CTR1*) which is a negative regulator of downstream signaling events (Kieber et al., 1993; Clark et al., 1998; Fluhr, 1998). The *CTR1* then interacts

downstream with *EIN2*- an essential positive regulator in the ethylene signaling pathway. In the nucleus, *EIN2* interacts with *EIN3* and its family, *EIN3*-like (*EIL1* and *EIL2*). *EIN3* and EILs bind primary ethylene response elements in the promoters of ethylene response factor (*ERF1*) or as ethylene-response-element binding proteins (*EREBPs*) (Solano et al., 1998; Wang et al., 2002). Finally the ethylene signaling process wind up with ethylene induced gene expression.

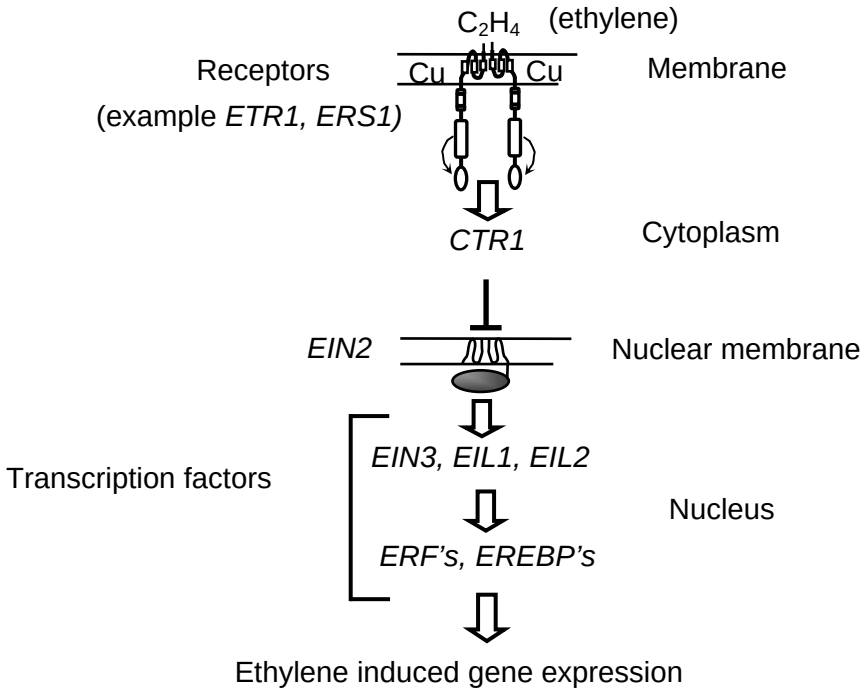

Figure 1.1 Ethylene signal transduction in *Arabidopsis* (modified from Chang and Shockey, 1999; Mibus, 2003)

Evidence on the ethylene theory of sex determination

Gas chromatographic measurements of ethylene produced by various sex-types of cucumber provided important evidence for the involvement of endogenous ethylene in cucumber sex expression. Comparison of the ethylene levels between gynoecious, monoecious and androecious cucumber plants clearly indicated that these plants differ in the level of endogenous ethylene (Rudich et al., 1976; Trebitsh et al., 1987). Higher amount of ethylene

was detected in gynoecious followed by monoecious and the least amount was detected in androecious plants (Rudich et al., 1976). Such measurement clearly indicated strong correlation between high ethylene level and femaleness tendency in cucumber plants. In a later study, Yin and Quinn (1995) indicated that ethylene is the main regulator of sex-determination in cucumber.

Recent results from several laboratories have provided molecular evidence in favor of the ethylene theory of sex determination in cucumber. Two 1-aminocyclopropane-1-carboxylic acid synthase genes, *CsACS1* and *CsACS2*, key enzymes in the ethylene biosynthesis, have been identified in cucumber, and one of them (*CsACS1*) mapped to the *F* allele (Trebitsh et al., 1997) and more recently, Mibus and Tatlioglu (2004) isolated the sequence of the promoter region of the *CsACS1G* postulated by Trebitsh et al. (1997) and confirmed as a dominant *F* allele. The monoecious cucumber genome has only one copy of *CsACS*, whereas the gynoecious genome has two copies. The expression of both *CsACS* genes correlates with sexual phenotype, with gynoecious plants accumulating more transcript than monoecious or andromonoecious plants (Kamachi et al., 1997; Yamasaki et al., 2001). Although these studies are consistent with the female-promoting effects of ethylene, they do not address the question of how ethylene inhibits stamen abortion in gynoecious and not in andromonoecious plants.

Three ethylene receptor-related genes, *CsETR1*, *CsETR2* and *CsERS*, have been isolated in cucumber and found that *CsETR2* and *CsERS* are strongly expressed in gynoecious line, suggesting that sensitivity to hormone may also be additional factor underlying sex-expression (Yamasaki et al., 2000). Following ethephon treatment, Yamasaki et al. (2001) detected increased accumulation of *CsETR2* and *CsERS* in gynoecious and monoecious but not in andromonoecious cucumber. Furthermore, they provided evidence suggesting that the product of the *M* allele mediates the inhibition of stamen development by ethylene (i.e., *M*

affects sensitivity to ethylene). These findings indicate that ethylene concentration, which is likely to be dependent on the *F* allele, and the differential sensitivity of males and females to ethylene, which is likely to be dependent on the *M* allele, are both important in regulating sexual phenotype in cucumber. Definitive cloning of the *F, M* and *A* genes will allow these hypotheses to be tested directly. Partial sequences of ethylene signal transduction-related genes, *CTR*, *EIN3*, *EIL1*, *EIL2*, *ERF1* and *EREBP* have been cloned in cucumber (Mibus, 2003) and their relationship with the sex-determining *M/m* and *A/a* genes has been under investigation.

Model for cucumber sex expression

By treating monoecious and andromonoecious cucumber plants with various combinations of gibberellic acid and ethephon and their inhibitors, Yin and Quinn (1995) demonstrated that ethylene is the main regulator of sex determination, with gibberellic acid functioning upstream of ethylene, possibly as a negative regulator of endogenous ethylene production. These findings led them to propose a model for how sex determination might occur (Yin and Quinn, 1995), with ethylene serving both as a promoter of the female sex and an inhibitor of the male sex. The basic tenets of the model are that the *F* gene should encode a molecule that would determine the range and gradient of ethylene production along the shoot, thereby acting to promote femaleness, whereas the *M* gene should encode a molecule that perceives the ethylene signal and inhibits stamen development above threshold ethylene levels. This model is consistent with how unisexual flowers might arise very early and very late during shoot development.

Objectives of the study

The aim of this study was to understand the molecular events that control sex determination and expression in cucumber, in particular the relationship between sex-determining loci and the sex hormone, ethylene. Hence the following objectives were formulated:

- To isolate the sex-determining *M/m* and *A/a* genes in cucumber
- To analyze linkage relationships between sex-determining genes *M/m* and *A/a*, and ethylene perception and signal transduction genes.
- To elucidate the mode of action of sex-determining *M/m* and *A/a* genes at the physiological level compared to the better understood *F/f* gene.

The possible achievements in relation to the stipulated objectives are presented and discussed in the following four major sections.

2 Molecular genetic characterizations of the sex-determining *M/m* and *A/a* genes by using ethylene receptor and signal transduction-related genes in cucumber

2.1 Introduction

The cucumber sex expression is mainly determined by three major genes, *F/f*, *M/m* and *A/a* (Galun, 1961; Shifriss, 1961; Kubicki, 1969a-d; Pierce and Wehner, 1990; Malepszy and Niemirowicz-Szczytt, 1991). Thus the genotypes (*F-M---)* are gynoecious, (*ffM-A-*) are monoecious, (*F-mm--*) are hermaphrodite, (*ffmmA-*) are andromonoecious and (*ff--aa*) are androecious (reviewed in Tatlioglu, 1993; Perl-Treves, 1999).

Although sex in cucumber plants is genetically controlled mainly by the three genes mentioned above, it can also be modified by plant hormones and environmental conditions. Ethephon (2-chloroethylphosphonic acid), auxins, short days and low temperature promote femaleness, whereas silver nitrate ($AgNO_3$), aminoethoxyvinyl-glycine (AVG), gibberellins and other substances counteracting the ethylene effect promote maleness (reviewed in Malepszy and Niemirowicz, 1991; Perl-Treves, 1999). Amongst these sex modifying plant hormones, ethylene acts more directly on sex expression and is thought to be the most important sex hormone in cucumber (Yin and Quinn, 1995).

Series of studies have been carried out to understand the relation between ethylene and the different sex types in cucumber. High ethylene production is found in gynoecious plants than in monoecious (Rudich et al., 1972; Rudich et al., 1976; Trebitsh et al., 1987). Increased level of 1-aminocyclopropane-1-carboxylic acid (ACC) and increased activity of ethylene forming enzyme (EFE) are detected in gynoecious compared to monoecious cucumber plants (Trebitsh et al., 1987). An additional copy of 1-aminocyclopropane-1-carboxylate (ACC) synthase (*ACS1*) designated as *CsACS1G* is found in gynoecious plants linked to the *F*-locus (Trebitsh et al., 1997). In another investigation, Kamachi et al. (2000) also detected the transcript of

CsACS1 linked to the *F*-locus in gynoecious but not in monoecious plants. Recently Mibus and Tatlioglu (2004) isolated the promoter region of the *CsACS1G* and confirmed that *CsACS1G* is the *F*-allele indeed.

In addition to the ethylene synthesis related genes, ethylene receptor related genes were also studied in relation to the *F*-allele. Yamasaki et al. (2000) isolated *CsETR1, CsETR2* and *CsERS* in cucumber and found increased accumulation of *CsETR2* and *CsERS* in gynoecious cucumber than in monoecious cucumber plants. In their further investigation after ethephon application, Yamasaki et al. (2001), found increased accumulation of *CsETR2*, *CsERS* and *CsACS2* in monoecious and gynoecious but not in andromonoecious cucumber plants. This was the first evidence for the possible relationship between the ethylene-related genes and the *M/m* genes.

Although such multiple studies have been conducted, nothing has been reported except the prelude work by Mibus (2003), concerning the possible relationship between the ethylene signaling-related genes (*CTR1, EIN2, EIN3, EIL1, EIL2, ERF1* and *EREBP*) and the sex types in cucumber plants. Mibus (2003) for the first time isolated the partial sequences of some of the ethylene signaling genes in cucumber and laid a basic foundation to conduct this research. Particularly the correlation studies between the *M/m* gene and ethylene signal transduction-related genes has paramount importance since the *M/m* gene most probably involve in ethylene perception or signaling, unlike the *F/f* gene.

In this part of the investigation, the expression levels of ethylene receptor and ethylene signal transduction-related genes were studied by using total RNA obtained from the arrested stamen-primordia of gynoecious (*FFMMaa*) and developed stamen-primordia of the nearly isogenic hermaphrodite (*FFmmaa*) cucumber plants. The transcript levels of the above mentioned ethylene related-genes were also studied by using total RNA obtained from the shoot apices of nearly isogenic gynoecious and hermaphrodite cucumber plants treated with

chemicals. The possible role of the dominant *M* gene in gynoecious and the recessive *m* gene in hermaphrodite plants on the expression levels of the ethylene-related genes is discussed. Identical expression analysis was conducted by using total RNA obtained from flowers, leaves and roots of monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants and the possible relationship between the transcript levels of ethylene-related genes and the *A/a* gene is also presented.

2.2 Materials and Methods

2.2.1 Plant materials

Nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber lines from three different genetic backgrounds, WrD, ED and ECD, were used in these investigations. These plant materials were established after Gönen (1975) and further selected by Franken (1979) in the Institute of Applied Genetics, University of Hannover. Since 1979 a backcross program has been used in the same institute to maintain the materials and further enrich isogenicity between gynoecious and hermaphrodite cucumber lines of each genetic background (Tatlioglu, 1981; 1983). The backcross was conducted as indicated in Figure 2.1. In the parental generation a gynoecious cucumber line was crossed with a hermaphrodite one (a). The gynoecious line was maintained by using $AgNO_3$ to induce male flowers. The heterozygous plants derived from the first parental cross were backcrossed with a gynoecious line (b). The offspring was phenotypically female, but 50% of the plants were in a heterozygous condition and the remaining 50% were homozygous dominant for *M* gene. Therefore, half of the inbreeds segregate into gynoecious and hermaphrodite plants (c). The inbreeds segregated into hermaphrodite plants were backcrossed again with the gynoecious line (d). The procedure was continued for at least nine backcrosses in all the three different genetic backgrounds (WrD, ED and ECD) to obtain nearly isogenic lines (NILs) that share about 99.8% of their genome.

In addition to the nearly isogenic gynoecious and hermaphrodite cucumber plants given above, monoecious (*ffMMA-*) and androecious (*ffMMaa*) cucumbers from the three genetic backgrounds, WrD, ECD and ED were also used in the subsequent investigations. Additional monoecious cucumber line, Shimshon (*ffMMAA*) and androecious line, Erez (*ffMMaa*) (kindly provided by Dr. Rafael Perl-Treves, Bar-Ilan University, Faculty of life sciences, Israel), were

also used in the investigations. F_1 and F_2 populations of the cross between gynoecious and hermaphrodite as well as monoecious and androecious cucumber plants were also used in some studies. In every section of this dissertation the specific cucumber plant type used was stated and also referred back to the materials given here.

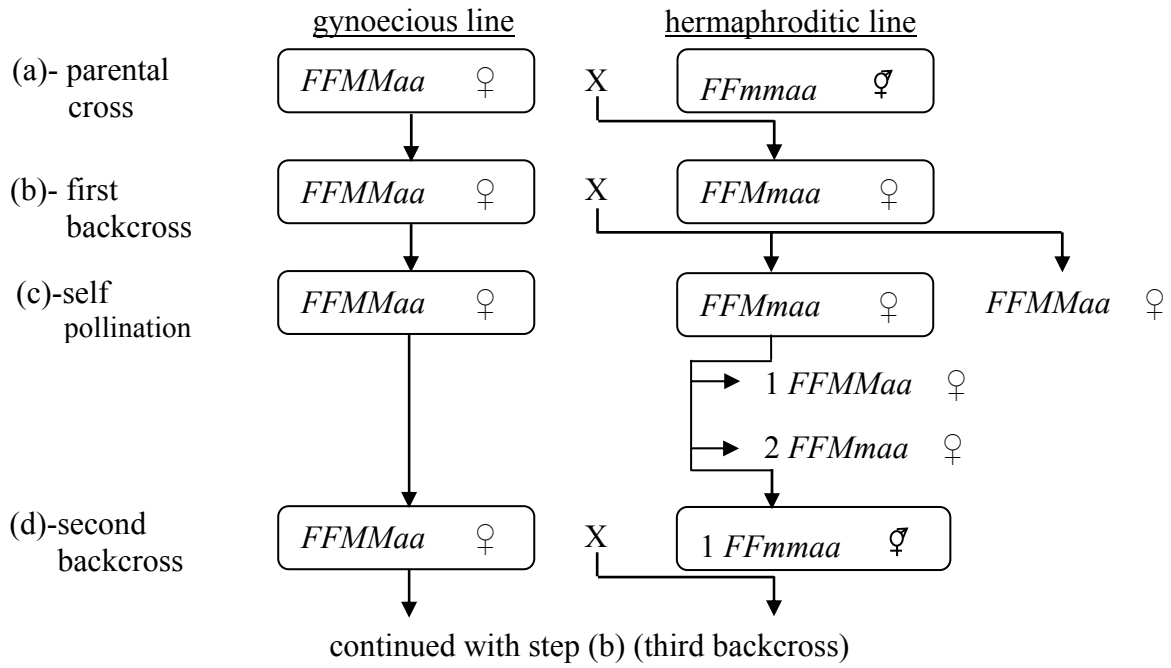

Figure 2.1 Crossing scheme to obtain nearly isogenic lines (NILs) differing in gene *M* with the *MM* genotype as the recurrent parent. (modified from Tatlioglu, 1983)

2.2.2 Growing of experimental plants

Cucumber seeds from monoecious, androecious and the nearly isogenic gynoecious and hermaphrodite plants were sown on a 35 quick-pot-plate filled with Terreau Professional Gepac Einheitserde Typ-T and germinated at a temperature of 28°C. The resulting seedlings were transferred at about 2-3 leaf-stage to clay pot (15 cm in diameter) filled with the same soil type. Cucumber plants used for the chemical application at 4 leaf-stage (Section 2.2.3) were transferred at about 2 leaf-stage to plastic pots (12cm in diameter). Plants were grown at day/night temperatures of 24°/18°C and 16h of assimilation light with day light supplemented by 400W fluorescent lamps (approx. 3500-4000 lux) (Son-T Agro 400, Philips Licht GmbH

Hamburg). They were supplied with 2% liquid fertilizer (N:P:K:Mg) on weekly basis alternating between (15:10:15:2) and (8:12:24:4).

2.2.3 Chemical application at 4 leaf-stage on gynoecious and hermaphrodite cucumbers

$AgNO_3$, $CuSO_4$, 2-chloroethylphosphonic acid (ethephon) and a mixture of $CuSO_4$ and ethephon were used in the experiment to study ethylene receptor and signal transduction-related genes. 300ppm $AgNO_3$, 15mM $CuSO_4$ and 20ppm ethephon (Mibus et al., 2000) containing 0.1% (v/v) Tween 20 were separately applied to the shoot apices of nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber lines of the WrD genetic background. Control plants from each line were applied with water containing 0.1% (v/v) Tween 20. Each of the chemical treatment was performed in separate cabins of the Institute's glasshouse. The applications were performed as described in Yamasaki et al. (2000). Three applications were done on three consecutive days using hand-spray-bottle starting at about 4 leaf-stage. 12h after the last (third) application samples were harvested from the shoot apices, frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Reagents used:

- $CuSO_4$ (Merck)
- $AgNO_3$ (Merck)
- Ethephon (2-chloroethylphosphonic acid) (Rhone-Poulenc)
- Tween 20

2.2.4 RNA isolation

Total RNA was isolated from different parts of the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) as well as monoecious (*ffMMA*-) and androecious (*ffMMaa*) cucumber lines of the three genetic backgrounds, WrD, ED and ECD. Additionally, monoecious cucumber line, Shimshon (*ffMMAA*) and androecious line, Erez (*ffMMaa*) were also used for RNA isolation. Flowers, newly emerged leaves, shoot-tips, stamen-primordia and roots were parts of cucumber lines used to isolate total RNA. In the procedure of RNA isolation from flower, leaf and shoot-tip, samples were cut from the main cucumber lines and

immediately frozen in liquid nitrogen. To get the root sample for RNA isolation, cucumber roots were carefully removed from the pot, cleaned by washing on sieve, fast dried by using tissue paper and frozen in liquid nitrogen. To get sample for RNA isolation from normal stamen-primordia (hermaphrodite) and arrested stamen-primordia (gynoecious), young flowers were first collected at their early growth stage on ice. Then the stamen-primordia were dissected from the flower by using razor blade and immediately frozen in liquid nitrogen. The samples were then milled by using an oscillating mill (Retsch) under liquid nitrogen. 30mg of the powdered sample was used for one time RNA isolation. Total RNA was isolated by using RNA isolation kit (Machery and Nagel). Incubation with the integrated DNAseI and subsequent washing steps allowed the digestion and removal of DNA and other contamination. The concentration of total RNA isolated was measured by using spectrophotometer (Pharmacia). Estimation of the concentration of the isolated total RNA was also done by using λ-DNA on 1.5% agarose gel with ethidiumbromide (1µg/ml) visualized by transilluminator (UV-light, 302nm). RNA of cucumber plants treated with chemicals (section 2.2.3) was isolated using the shoot-tip of the plants. RNA isolation from the normal and arrested stamen-primordia was not performed for monoecious and androecious cucumber plants, instead, RNA was isolated from flowers, leaves and roots of monoecious and androecious cucumber plants.

Reagents used:

- RNA Kit (Machery and Nagel)
- Ethidiumbromide
- 100bp ladder (Gibco)
- Agarose
- Orange G loading buffer (30% Glycerine, 0.25% Orange G)
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)
- λ-DNA (Gibco)

2.2.5 DNA isolation

For genomic DNA isolation, fresh leaves were harvested from the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) as well as monoecious (*ffMMA-*) and androecious

(*ffMMaa*) cucumber plants of the three genetic backgrounds, WrD, ED and ECD. Additional samples from monoecious cucumber line, Shimshon (*ffMMAA*) and androecious line, Erez (*ffMMaa*) were also used for the DNA isolation. The freshly harvested leaves were immediately frozen in liquid nitrogen and samples were then milled by using an oscillating mill (Retsch) under liquid nitrogen. DNA isolation was performed by using serum separation tubes (SST) (Becton-Dickinson) according to a protocol from Bentzen et al. (1990) modified by Engelke and Tatlioglu (2000). About 250mg of the powdered sample was poured into SST tubes and homogenized in a 1.5 ml of 1.3 × CTAB extraction buffer (67 mM Tris/HCl pH 8.0, 13.4 mM EDTA pH 8.0, 0.93M NaCl, 1.33% (w/v) CTAB and 1% (w/v) PVP-40). Samples were incubated in a water bath for 90 min at 65°C under shaking condition. They were then treated with 1.5ml phenol, incubated under shaking condition for 10 min and centrifuged for 15 min at 5000xg (4°C). At this step different phases of solution were visible separated by the serum separator. The upper phase (supernatant) of the solution was transferred to a new SST-tube and treated with 1.5ml phenol:chloroform:isoamylalcohol (25:24:1). They were incubated for 10 min under shaking condition and centrifuged for 15 min as above. RNA digestion was achieved by treating the samples with 0.15 units RNase (Gibco BRL) and incubation for 15 min at 37°C. After an additional step of chloroform extraction (similar to phenol:chloroform extraction), the supernatant was transferred to a new tube. They were then treated with 1.5 ml ice cold isopropanol and 150µl 3M Na-acetate (pH 5.2) and kept at -20°C overnight to precipitate DNA. The DNA was pelleted by centrifugation for 15 min at 15000xg (4°C) and the supernatant discarded. The pellets obtained were washed by 500µl ice cold 70% ethanol. The DNA samples were finally transferred together with the ethanol to 1.5 ml eppendorf tube and centrifuged for 15 min at 15000xg (4°C). The supernatant were discarded and the pellet dried by incubation on a heat block at 60°C for about 30 min. The pellets were then redissolved using 100µl double deionized water (ddH_2O). DNA concentration was

determined using spectrophotometer (Pharmacia). Estimation of the isolated total DNA was also done by using λ-DNA on 1.5% agarose gel with ethidiumbromide (1µg/ml) visualized by transilluminator (UV-light, 302nm). The isolated DNA was stored at -20°C for further use.

Reagents used:

- 1.33% CTAB buffer:
  67mM Tris/HCl;
  13.4mM EDTA; pH 8.0
  0.93M NaCl;
  1% (w/v) PVP-40.
  1.33% (w/v) CTAB
- RNase (Gibco BRL)
- Phenol, Chloroform, Isoamylalcohol
- Isopropanol, 3M Na-Acetate,
- 70% (v/v) Ethanol

2.2.6 Preparation of ethylene receptor and signal transduction-related genes

Preparation of ethylene receptor-related genes

The sequence information of the three already isolated cucumber ethylene receptor-related genes was directly obtained from GenBank. *CsETR1* (*Cucumis sativus* ethylene receptor 1)-GenBank accession number, AB026498; *CsETR2* (*Cucumis sativus* ethylene receptor 2)-GenBank accession number, AB026500 and *CsERS* (*Cucumis sativus* ethylene response sensor)-GenBank accession number, AB026499 (Yamasaki et al., 2000). From these sequences, primer pairs were designed and the obtained fragments were cloned by Mibus (2003). These already available primer pairs (Table 2.1) and the cloned fragments obtained from Mibus (2003) were used in the subsequent northern blot hybridization (section 2.2.7), semi-quantitative RT-PCR (section 2.2.8) and Southern blot hybridization (section 2.2.9).

Table 2.1 Primer pairs used to amplify ethylene receptor-related genes in cucumber, their annealing temperatures and corresponding PCR product size.

Gene name	GenBank accession number	Primer sequences	Anneal. temp.	Size
CsETR1	AB026498	5' TTGCATACTTCTCGATCCCA '3 5'TGCTACCTGATCAGCAACAA '3	55°C	830bp
CsETR2	AB026500	5' GGCATTGCCATCTGGGTTTC '3 5' CACGTTCATCAGAGTCGCCAC '3	55°C	782bp
CsERS	AB026499	5' AGCTGAACAACTTGACAGGGAG '3 5'ACCAACAGCACAGATCGGTAGA '3	55°C	995bp

Preparation of ethylene signal transduction-related genes

The primer pairs (Table 2.2) and the cloned fragments for the cucumber ethylene signal transduction-related genes; constitutive triple response 2 (*CsCTR2*), ethylene insensitive 3 (*CsEIN3),* ethylene insensitive 3 like 1 (*CsEIL1),* ethylene insensitive 3 like 2 (*CsEIL2),* ethylene-responsive element binding factor 1 *(CsERF1)* and ethylene-responsive element binding protein (Cs*EREBP),* were isolated by Mibus (2003) and made available for this study.

The sequence for constitutive triple response 1 (*CsCTR1)* was directly taken from the GenBank accession number: AF529239. Oligonucleotide primer pair was designed by using Primer Premier program (PREMIER Biosoft International, Palo, Alto, CA) (Table 2.2). The designed primer pair was synthesized by MWG Biotech AG. The annealing temperature for the primer pair was obtained in a preliminary PCR by using eight different temperature gradients between 45 and 65°C in a PCR machine (TGradient Block, Biometra). The primer pair was used in RT-PCR on total RNA isolated from cucumber shoot apices. RT-PCR was performed using 40ng of total RNA according to the Qiagen OneStep RT-PCR Kit (Qiagen). The RT-PCR program used was, reverse transcription at 50°C for 30 min, inactivation of reverse transcriptase and activation of DNA polymerase at 95°C for 15 min, followed by 40 PCR cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at

72°C for 2 min, final extension of 72°C for 10 min. The obtained RT-PCR product was separated on flatbed electrophoresis using 1.5% agarose gels. The RT-PCR product was cut from the gel and transferred to 1% low melting agarose gel in 1xTAE buffer using a voltage supply of 3V/cm gel length. The cDNA fragment was further cut from the low melting gel and transferred to pre-weighed 1.5ml eppendorf tube. The newly cut cDNA fragment was weighed and digestion of the low melting gel performed as follow: 25x Agarase buffer (Roche) was added into the cut cDNA in 1.5ml eppendorf tube, melted at 70°C for 3 min and allowed to cool to 45°C, at which the enzyme Agarase (Roche) was added and digestion of the low melting gel for 1h at the 45°C according to the manufacturer's instruction (Roche). The concentration of the purified cDNA was estimated by using λ-DNA (Gibco). The purified cDNA fragment was then cloned using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instruction. Plasmids were recovered using QiaPrep-Mini kit (Qiagen) and sequencing was done by MWG Biotech AG. The isolated cDNA sequences were analyzed using the CLUSTAL W program, European Bioinformatics Institute (EMBL; Thompson et al., 1994) and the BLUSTN program, National Center for Biotechnology Information (NCBI; Altschul et al., 1997). The amplified cDNA fragment was confirmed to be the target *CsCTR1* gene (AF529239).

The newly cloned *CsCTR1* and the already available ethylene signal transduction-related genes (*CsCTR2, CsEIN3, CsEIL1, CsEIL2, CsERF1* and *CsEREBP*) were used in subsequent northern blot hybridization (section 2.2.7), semi-quantitative RT-PCR (section 2.2.8) and Southern blot hybridization (section 2.2.9).

Table 2.2 List of oligonucleotide primer pairs for ethylene signal transduction-related genes in cucumber, their respective annealing temperatures and fragment size.

Gene name	Obtained oligonucleotide primer pairs	Ann. temp.	Fragment size
CsCTR1[a]	5'CGGAAGTTGCTGTGAAGA'3	51°C	510bp
	5'ATTCCCACAAAATGACCC'3		
CsCTR2	5'TCCTCCAAACTTGTCCATT'3	51°C	216bp
	5'GTGTACTTCTTATCAACCAAA'3		
CsEIN3	5' AGGAGGAAGATGTCG '3	53°C	343bp
	5' TTTGAGGAGGGTCACAGT '3		
CsEIL1	5'GATAATTTGAGGGAATGG'3	47°C	371bp
	5'GCAGTTAAAACACCGACT'3		
CsEIL2	5' CGGGCTCAAGATGGGATC '3	56°C	542bp
	5' TTGGACTGGCGAACAAGC '3		
CsERF1	5'GGGAAAGCATTATAGAGGAG'3	49°C	119bp
	5'CCGCAGCCTCGGCAGTGTCA'3		
CsEREBP	5' ATGTGCGGTGGTGCCATTATCT '3	55°C	311bp
	5'GCGGTGTTGAAAGTGCCGAGCC '3		

[a]GenBank accession number: AF529239

Reagents used:

- Ethidiumbromide
- 100bp ladder (Gibco BRL)
- Agarose
- Orange G loading buffer (30% Glycerine, 0.25% Orange G)
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)
- Agarase (0,2 U/µl) with buffer (Roche)
- λ-DNA (Gibco)
- TOPO TA Cloning Kit (Invitrogen)

Reagents used (RT-PCR):

- 10µl 5x Qiagen OneStep RT-PCR Buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 12.5mM $Mgcl_2$, dithiothreitol-DTT (Ph 8.7))
- 2µl dNTP mix, 10mM each of dATP, dCTP, dGTP and dTTP
- 2µl OneStep RT-PCR Enzyme mix (1mM dithiothreitol (DTT), 0.1mM EDTA, 0.5% (v/v) Nonidet P-40, 0.5% (v/v) Tween 20, 50% glycerol (v/v), stabilizer (pH 9.0))
- RNase free water (variable volume)
- 0.6 µM sense-Primer (MWG)
- 0.6 µM antisense-Primer (MWG)

Reagents used (PCR-for the determination of annealing temperature):

- Buffer: 10 mM Tris-HCl; pH 8,8; 50 mM KCl 0,1% Triton X-100, 1,5 mM $MgCl_2$ (Finnzymes)
- 150 µM of each dNTP (Eurogenetech)
- 1 unit Taq-Polymerase DyNAzyme™ II DNA Polymerase (2 Units/µl; Finnzymes)
- 2,5 µM sense-Primer (MWG)
- 2,5 µM antisense-Primer (MWG)

PCR conditions (for the determination of annealing temperature):

- 5 min at 94°C
- for 40 cycles:
  1 min at 94°C (denaturation)
  1 min at 45-65°C (annealing)
  2 min at 72°C (extension)
- 5 min at 72°C (final extension)

2.2.7 Northern blot hybridization analyses using ethylene receptor and signal transduction-related genes

DIG labeling of the probes for northern blot hybridization

The ethylene receptor and ethylene signal transduction-related genes, *CsETR1, CsETR2, CsERS, CsCTR1, CsCTR2, CsEIN3, CsEIL1, CsEIL2, CsERF1* and *CsEREBP* were labeled with DIG (digoxigenin) (Roche) for non-radioactive hybridization. DIG labeling was performed by PCR using 10pg of the plasmid DNA containing each gene of interest, 2.5µM of each standard T_3 (5'ATTAACCCTCACTAAAGGGA'3) and T_7 (5'TAATACGACTCAC-TATAGGG'3) primers, 10x reaction buffer, 10x DIG DNA labeling mix, 1 unit Dynazyme filled to a final volume of 20µl with ddH_2O. PCR was performed as follows: 94°C for 2 min initial denaturation followed by 40 cycles of 94°C denaturation for 30 sec, 50°C annealing for 1 min and 72 °C extension for 2 min and a final extension of 72°C for 5 min. As a control PCR additional reactions were set for every probe labeling by using normal dNTPs without DIG. 5µl of the DIG labeled PCR product and the control were checked by flat bed electrophoresis on 1.5% agarose gel using 5V/cm gel length. Size of the fragments were estimated using 100bp ladder (Gibco). Due to the presence of DIG, the DIG-labeled probe migrate slower and appears to be larger than PCR fragments obtained using the normal dNTPs. The DIG-labeled probes were denatured at 95°C for 10 min and immediately chilled on ice for denaturation. The denatured probe was then used for northern blot hybridization and Southern blot hybridization (section 2.2.9).

Reagents used:

- Ethidiumbromide
- 100bp ladder (Gibco BRL)
- Agarose
- Orange G loading buffer (30% Glycerine, 0.25% Orange G)
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)

Reagents used (PCR) :

- Buffer: 10 mM Tris-HCl; pH 8,8; 50 mM KCl 0,1% Triton X-100, 1,5 mM $MgCl_2$ (Finnzymes)
- DIG DNA labeling mix (1 mM dATP, 1mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM DIG-dUTP, pH 7.5) (Roche)
- 150 µM of each dNTP (Eurogenetech)
- 1 unit Taq-Polymerase DyNAzyme^{TM} II DNA Polymerase (2 Units/µl; Finnzymes)
- 2,5 µM sense-Primer T_3 (Invitrogen)
- 2,5 µM antisense-Primer T_7 (Invitroben)

Electrophoresis, blotting, hybridization and detection

Ten micrograms of total RNA isolated from arrested stamen-primordia of gynoecious (*FFMMaa*) and normal stamen-primordia of hermaphrodite (*FFmmaa*) cucumber plants of the three genetic backgrounds were denatured in freshly prepared loading buffer (2.2 M formaldehyde, 50% formamide, 0.5x MOPS, 0.05% bromophenol blue) in a final volume of 30 µl for 15 min at 65°C. The denatured RNA was then fractionated by electrophoresis in 1% agarose-gels containing 0.7 M formaldehyde in 1x MOPS-buffer for 4–5h (at a constant voltage of 5V/cm gel length). The profiles were checked under UV light, and the gels were washed first in water for 15 min and then twice in 10x SSC for 15 min each. RNA was then transferred to Hybond-N^+ nylon membranes (Amersham) in 10xSSC using a vacuum-system (Pharmacia; 60 mbar, 90 min). After crosslinking by irradiation with UV light, the membranes were rinsed with water and stored until hybridization. The membranes were pre-hybridized in hybridization tubes (Bachofer) containing 15 ml of Dig Easy Hyb (Roche) at 50°C for 30 min. Main hybridization took place overnight under the same conditions with 100ng of denatured DIG-labeled probe in 7 ml of Dig Easy Hyb. Post-hybridization washes were performed twice for 5 min each in 2xSSC/0.1%SDS at room temperature, twice for 15 min each in 0.5x SSC/0.1% SDS at 50°C. The membrane was then agitated in 1x blocking solution (Roche) for 30 min to eliminate nonspecific backgrounds and for another 30 min in an Anti-Dig-AP (Roche) solution (2.5µl (0.75u /µl) Anti-Dig-AP in 50ml 1x blocking solution). Detection of the DIG-labeled probes was performed according to the instructions of the supplier, using CDP-Star (Amersham) as substrate. X-ray films were exposed to the membranes for about 1 h. The transcript sizes were estimated by comparison with the RNA Molecular Weight Marker I (DIG-labeled, 0.3–6.9 kb; Roche, 100ng).

Following the same procedures northern blot hybridization analyses were also performed using ten micrograms of total RNA isolated from flowers, leaves and roots of monoecious

(*ffMMAA*) and androecious (*ffMMaa*) cucumber plants in combination with the ethylene receptor and ethylene signal transduction-related genes, *CsETR1, CsERS* and *CsEIL1*.

Reagents used:

- Ethidiumbromide
- Agarose
- Hybridization buffer: Dig Easy Hyb (Roche)
- Anti-Digoxigenin-AP, FAB Fragment (Roche)
- CDP-Star (Amersham)
- DIG-labeled RNA Molecular-Weight Marker I (0.3-6.9kb) (Roche)
- 20 X SSC: 3 M NaCl; 0,3 M Na-citrate; pH 7,0
- 10 % SDS , pH 7,2
- Tween 20
- Maleic acid buffer: 0.1 M maleic acid; 0.15 M NaCL; 0.185 MNaOH; pH 7,5
- substrate dilution buffer: 100 mM diethanolamine pH 9,5; 1 mM $MgCl_2$
- Low stringency buffer: 2xSSC/0.1%SDS
- High stringency buffer: 0.5x SSC/0.1% SDS
- 10x blocking stock solution(Roche)
- Detection buffer: 0.1 M Tris-HCl pH 9,5; 0.1 M NaCl
- Diethylpyrocarbonate (DEPC) (Fluka)
- MOPS buffer: 200mM 3-(N-Morpholino)-propanesulfonic acid (MOPS); 80mM Sodium acetate 3-hydrate; 12.5mM EDTA; pH 7
- Bromophenol blue loading buffer: 0.25% (w/v) Bromophenol blue; 30% (v/v) Glycerin
- Formaldehyde
- Formamide
- Rnase AWAY (Carl Roth)
- X-ray film (Amersham)
- Hybond-N^+ nylon membranes (Amersham)

2.2.8 Semi-quantitative RT-PCR analyses using ethylene receptor and signal transduction-related genes

Expression analysis of the ethylene signal transduction-related genes, *CsERF1* and *CsEREBP*, were performed by using semi quantitative RT-PCR (Marone et al., 2001). Because northern hybridization yielded insufficient hybridization signal for these genes, semi-quantitative RT-PCR was used to compare their transcript level in arrested and normal stamen-primordia of the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants, respectively. About 1ng of total RNA obtained from arrested stamen of gynoecious and normal stamen of hermaphrodite cucumber plants and 28 PCR-cycles were used in the RT-PCR. Semi-quantitative RT-PCR was performed using Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instruction. For the RT-PCR mixtures and reaction conditions as well as primer annealing temperatures see section 2.2.6. A primer pair (sense, 5'GGCAGTGGTGG-TGAACAT'3; antisense, 5'CTGGTATCGTGCTGGATT'3) obtained

from cucumber mRNA for actin (accession number AB010922) was used as control in the semi-quantitative RT-PCR.

The expression level of *CsETR2, CsEIN3, CsEIL2, CsEREBP* genes were also investigated by semi-quantitative RT-PCR using 1ng total RNA isolated from flowers, leaves and roots of monoecious (Shimshon) and androecious (Erez) cucumber plants following the above procedures.

Following similar procedures, Semi-quantitative RT-PCR was also done for *CsETR1, CsETR2, CsERS, CsEIN3, CsEIL2* and *CsEREBP* using 1ng of total RNA isolated from shoot-apices of $AgNO_3$, $CuSO_4$, ethephon, and a mixture of $CuSO_4$ and ethephon treated nearly isogenic gynoecious and hermaphrodite cucumber plants of the WrD genetic background.

2.2.9 Southern blot hybridization analyses using ethylene receptor and signal transduction-related genes

Twenty micrograms of DNA from monoecious (Shimshon) and androecious (Erez) cucumber plants were digested using 30 U of 30 different restriction enzymes: *Alu*I, *Apa*I, *BamH*I, *Bcl*I, *Bcn*I, *Bfa*I, *Bgl*II, *BsaJ*I, *BseG*I, *Dra*I, *EcoR*I, *EcoR*V, *Hae*III, *Hin6*I, *Hind*III, *HinF*I, *Mbo*I, *Msp*I, *Mva*I, *Nco*I, *Pae*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Sal*I, *Taa*I, *Taq*I, *Xba*I and *Xho*I (MBI Fermentas) in a restriction buffer provided by the manufacturer. Each reaction mixture was kept at the appropriate reaction temperature (mostly 37°C) for 15hr. The restricted fragments were mixed with 3µl bromophenol blue-loading buffer and then separated by flatbed electrophoresis, using 0.8% agarose gels in 1xTAE buffer for 16h (2 V/cm length of the gel). The DNA was cracked by soaking the gel in 0.25 M HCl for 10 min and denatured for 2x 15 min in denaturation solution (0.5M NaOH, 1.5M NaCl). Following 2x 15 min agitation in neutralization solution (0.5M Tris-HCl, pH 7.5; 1.5M NaCl), the DNA was transferred to Hybond-N^+ nylon membranes (Amersham) in 20xSSC, using a vacuum-system (Pharmacia:

60 mbar, for 60 min). After cross-linking by irradiation with UV light, the membranes were rinsed with water and stored at 4°C until hybridization.

Each of the ethylene receptor-related genes (*CsETR1, CsETR2* and *CsERS*), Ethylene signal transduction-related genes (*CsCTR1*, *CsCTR2, CsEIN3, CsEIL1*, *CsEIL2, CsERF1* and *CsEREBP*) were labeled with DIG by using PCR following the procedure described in section 2.2.7. The membranes were pre-hybridized in hybridization tubes (Bachofer) containing 15 ml of Dig Easy Hyb (Roche) at 42°C for 30 min. Main hybridization took place overnight under the same conditions with 100ng of denatured DIG-labeled probes in 7 ml of Dig Easy Hyb. Post-hybridization washes were performed twice for 5 min each in 2xSSC/0.1%SDS at room temperature, twice for 15 min each in 0.5x SSC/0.1% SDS at 65°C. The membrane was then agitated in 1x blocking solution (Roche) for 30 min to eliminate nonspecific backgrounds and for another 30 min in an Anti-Dig-AP (Roche) solution (2.5µl (0.75u /µl) Anti-Dig-AP in 50ml 1x blocking solution). Detection of the DIG-labeled probes was performed according to the instructions of the supplier, using CDP-Star (Amersham) as substrate. X-ray films were exposed to the membranes for about 1 h. The obtained fragment sizes were estimated by comparison with the DNA Molecular Weight Marker III (DIG-labeled, 0.56–21.2 kb; Roche, 1µg).

Reagents used:

- Ethidiumbromide
- Agarose
- Hybridization buffer: Dig Easy Hyb (Roche)
- Anti-Digoxigenin-AP, FAB Fragment (Roche)
- CDP-Star (Amersham)
- 20 X SSC: 3 M NaCl; 0,3 M Na-citrate; pH 7,0
- 10 % SDS , pH 7,2
- Tween 20
- Maleic acid buffer: 0.1 M maleic acid; 0.15 M NaCL; 0.185M NaOH; pH 7,5
- substrate dilution buffer: 100 mM diethanolamine pH 9,5; 1 mM $MgCl_2$
- Low stringency buffer: 2xSSC/0.1%SDS
- High stringency buffer: 0.5x SSC/0.1% SDS
- 10x blocking stock solution (Roche)
- Detection buffer: 0.1 M Tris-HCl pH 9,5; 0.1 M NaCl
- Bromophenol blue loading buffer: 0.25% (w/v) Bromophenol blue; 30% (v/v) Glycerin
- X-ray film (Amersham)
- Hybond-N^+ nylon membranes (Amersham)
- DIG-labeled DNA Molecular Weight Marker III (0.56–21.2 kb, Roche)
- Restriction enzymes and buffer (MBI Fermentas)

2.3 Results

2.3.1 Influence of the sex-determining *M/m* gene on the expression level of ethylene receptor and signal transduction-related genes

The transcript levels of ethylene receptor and ethylene signal transduction-related genes were compared by using northern blot hybridization. Recent investigations indicated that the sex determination in cucumber is limited to specific floral whorls (Kater et al., 2001; Hao et al., 2003). Based on this finding it was decided to use specific floral tissue in the expression analyses. Thus, total RNA obtained from arrested stamen-primordia of the nearly isogenic gynoecious (*FFMMaa*) and developed stamen-primordia of hermaphrodite (*FFmmaa*) cucumber plants were compared. The comparison was made on all of the three genetic backgrounds; WrD, ECD and ED. Increased accumulation of the transcript level for the ethylene receptor genes, *CsETR1, CsETR2* and *CsERS* were found in developed stamen-primordia in hermaphrodite plants compared to arrested stamen-primordia in gynoecious plants (Fig. 2.2).

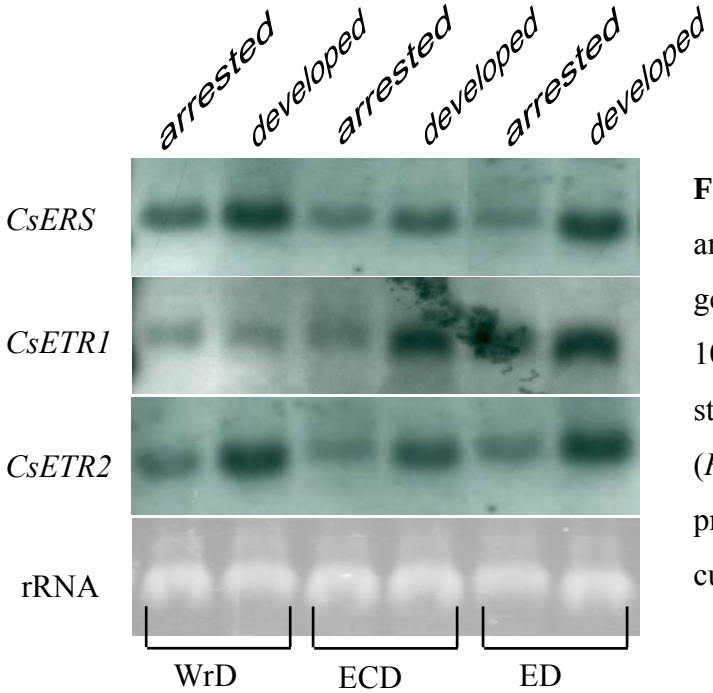

Figure 2.2 Northern blot hybridization analyses of ethylene receptor-related genes, *CsETR1, CsETR2* and *CsERS* on 10µg total RNA obtained from arrested stamen-primordia of gynoecious (*FFMMaa*) and developed stamen-primordia of hermaphrodite (*FFmmaa*) cucumber plants.

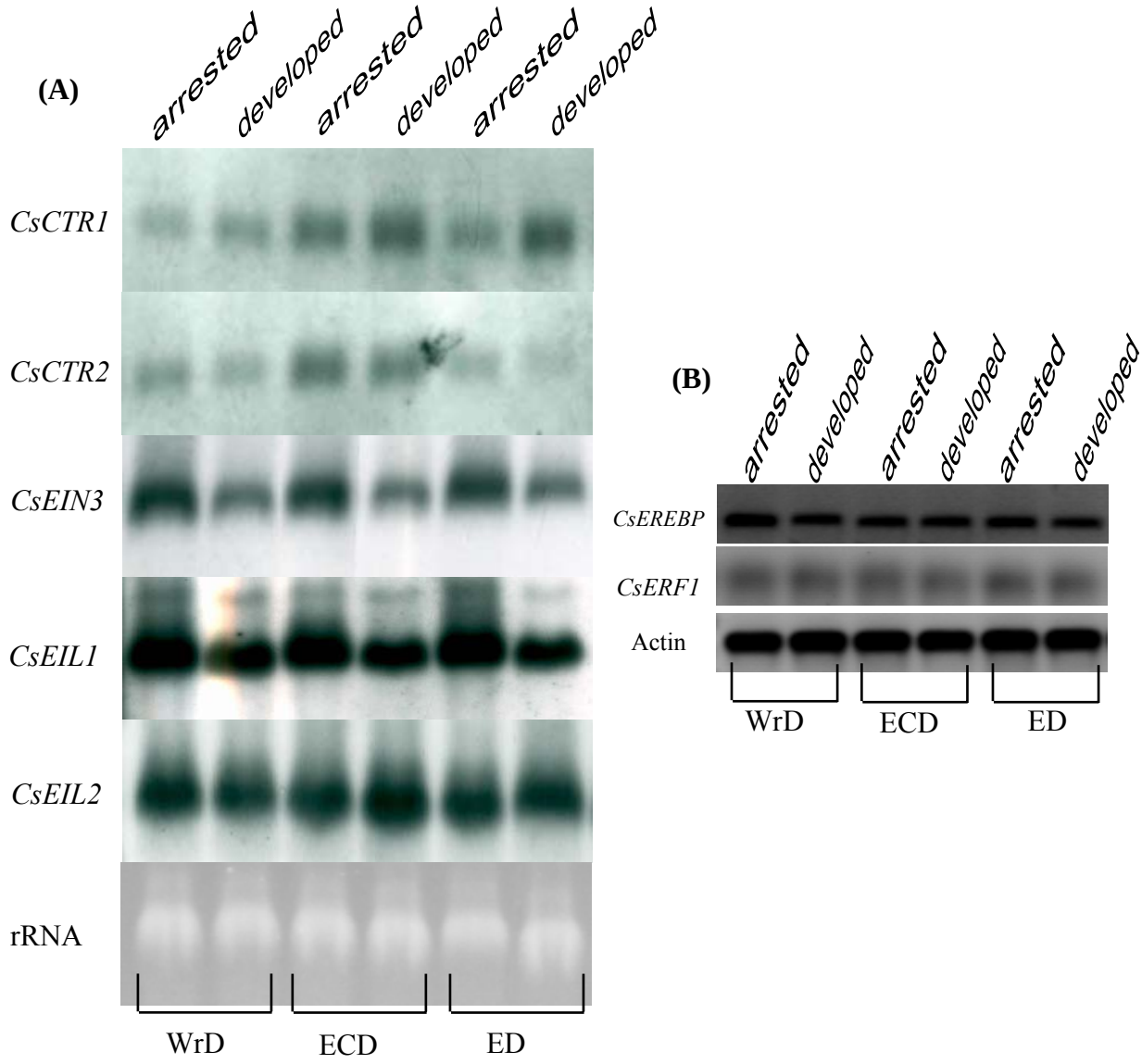

Figure 2.3 Expression analyses of ethylene signal transduction-related genes.
(A) Northern blot hybridization analyses of *CsCTR1, CsCTR2, CsEIN3, CsEIL1* and *CsEIL2* on total RNA obtained from arrested stamen-primordia of gynoecious (*FFMMaa*) and developed stamen-primordia of hermaphrodite (*FFmmaa*) cucumber plants.
(B) Semi-quantitative RT-PCR analysis of *CsERF1* and *CsEREBP* on total RNA obtained from arrested stamen-primordia of gynoecious and developed stamen-primordia of hermaphrodite cucumber plants.

Increased level of expression was detected by northern blot hybridization for *CsEIN3* and *CsEIL1* in arrested stamen-primordia of gynoecious than in the developed stamen-primordia

of hermaphrodite cucumber plants (Fig. 2.3A). Among the ethylene signal transduction-related genes studied, *CsCTR1*, *CsCTR2* and *CsEIL2* yielded identical expression level in both arrested and developed stamen-primordia of the gynoecious and hermaphrodite cucumber plants, respectively (Fig. 2.3A). It was not possible to detect a northern hybridization signal for *CsERF1* and *CsEREBP* genes by using 10µg of total RNA obtained from stamen-primordia. Thus semi-quantitative RT-PCR was utilized to compare the expression level of these two ethylene signal transduction-related genes (*CsERF1* and *CsEREBP*) between stamen-primordia of gynoecious and hermaphrodite cucumber plants. However, identical expression level was observed in the stamen-primordia of both gynoecious and hermaphrodite cucumber plants (Fig. 2.3B)

2.3.2 Influence of the sex-determining *M/m* gene on the expression level of ethylene receptor and signal transduction-related genes after chemical applications

$AgNO_3$, $CuSO_4$, ethephon, and a mixture of $CuSO_4$ and ethephon were applied at about 4-leaf stage to the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants. The effect of these sex-influencing chemicals on the transcript levels of ethylene receptor and ethylene signal transduction-related genes were studied. The interaction of the *M* gene in gynoecious and *m* gene in hermaphrodite plants in relation to the external chemical application was noted. The increase or decrease in the expression pattern of ethylene receptor-related genes was not uniform in gynoecious and hermaphrodite cucumber plants (Fig. 2.4; Table 2.3). For instance, $CuSO_4$ application at the 4-leaf stage increased the transcript levels of *CsETR1, CsETR2* and *CsERS* in gynoecious (*FFMMaa*) cucumber plants, but not in the hermaphrodite (*FFmmaa*) one (Table 2.3). Ethephon application on the other hand increased the expression level of *CsETR1, CsETR2* and *CsERS* in both gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants. The combined application of $CuSO_4$ and ethephon increased the expression level of *CsETR1, CsETR2* and *CsERS* in hermaphrodite

(*FFmmaa*) but not in the gynoecious (*FFMMaa*) cucumber plants. There was no influence from $AgNO_3$ application on the expression level of *CsETR1, CsETR2* and *CsERS,* both in gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants. In Fig 2.4, the increased expression level of *CsETR1* observed in hermaphrodite cucumber after $CuSO_4$ application and the decreased expression level of *CsERS* observed in gynoecious after a combination of $CuSO_4$ and ethephon application lacks consistency when repeated.

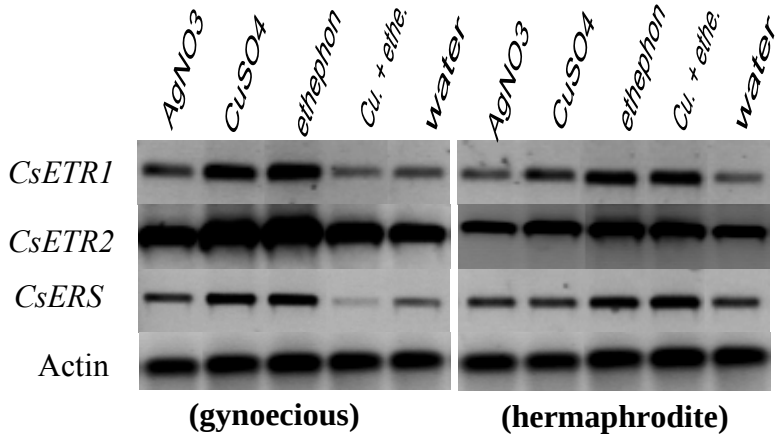

Figure 2.4 Semi-quantitative RT-PCR analyses of ethylene receptor-related genes (*CsETR1, CsETR2* and *CsERS*) after $AgNO_3$, $CuSO_4$, ethephon and a combination of $CuSO_4$ and ethephon application at 4-leaf stage on the nearly isogenic gynoecious and hermaphrodite cucumber plants. Actin was used as a control for the semi-quantitative RT-PCR.

Similarly the patterns in the expression level of ethylene signal transduction-related genes after chemical application was not uniform (Fig. 2.5; Table 2.3). $CuSO_4$ application at the 4-leaf stage decreased the expression level of *CsEIN3, CsEIL2* and *CsEREBP* in gynoecious (*FFMMaa*) however the same application increased the expression level of *CsEIN3, CsEIL2* and *CsEREBP* genes in hermaphrodite (*FFmmaa*) cucumber plants. Ethephon application caused no change on the expression level of *CsEIN3, CsEIL2* and *CsEREBP* genes in gynoecious cucumber, but increased the expression level of those genes in hermaphrodite cucumber plants. The combined application of $CuSO_4$ and ethephon reduced the expression level of *CsEIN3, CsEIL2* and *CsEREBP* genes in gynoecious, but no effect on the expression

level of *CsEIN3, CsEIL2* genes and increased the expression level of *CsEREBP* gene in hermaphrodite cucumber plants. No influence was detected from $AgNO_3$ application on the expression level of *CsEIN3, CsEIL2* and *CsEREBP* genes in both gynoecious and hermaphrodite cucumber plants. Among the ethylene signal transduction-related genes, *CsCTR1, CsCTR2, CsEIL1* and *CsERF1* yielded identical expression levels after $AgNO_3$, $CuSO_4$, ethephon, and a mixture of $CuSO_4$ and ethephon application at 4-leaf stage (Fig. 2.5).

Interestingly treatment of cucumber plants with $CuSO_4$ (source of Cu(I)- cofactor for ethylene receptor) increased the expression level of ethylene receptor-related genes and decreased the expression level of some ethylene signal transduction-related genes in gynoecious cucumber plants. In hermaphrodite cucumber plants however, the treatment caused no effect on the expression level of the receptor-related genes but increased the expression level of some of the signal transduction-related genes. Treatment with ethephon increased the expression level of ethylene receptor-related genes in both gynoecious and hermaphrodite cucumber plants and increased the expression level of some of the ethylene signal transduction-related genes only in hermaphrodite cucumber plants (Table 2.3).

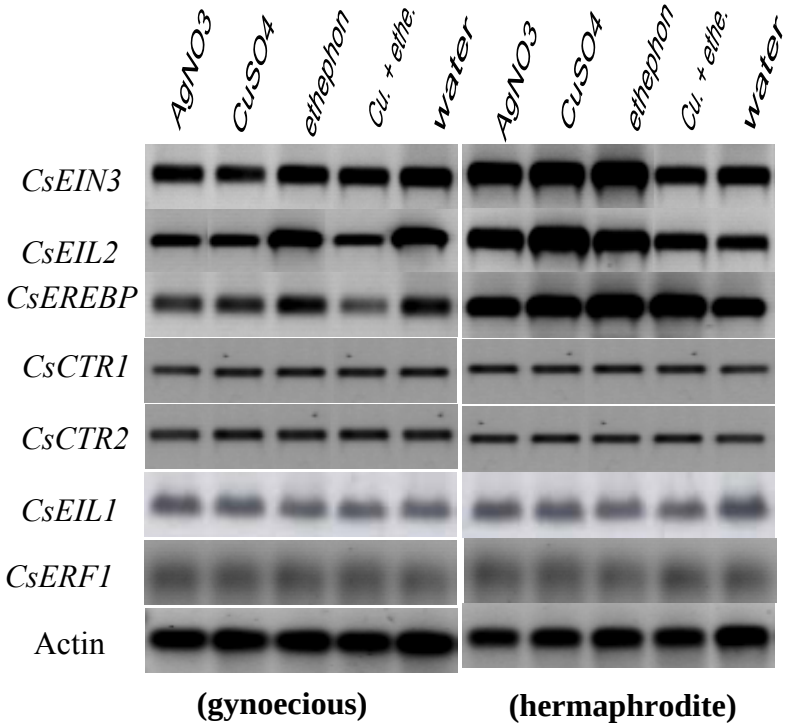

Figure 2.5 Semi-quantitative RT-PCR analyses of ethylene signal transduction-related genes after $AgNO_3$, $CuSO_4$, ethephon and a combination of $CuSO_4$ and ethephon application at 4-leaf stage on the nearly isogenic gynoecious and hermaphrodite cucumber plants of the genetic background WrD. Actin was used as a control for the semi-quantitative RT-PCR. The picture for *CsEIL1* is from northern blot hybridization result.

Table 2.3 Summary representation of the effect of $AgNO_3$, $CuSO_4$, ethephon and a combination of $CuSO_4$ and ethephon application on the expression level of ethylene receptor and some ethylene signal transduction-related genes.

Chemicals applied	The expression level of the genes in gynoecious (**G**) and hermaphrodite (**H**) cucumber plants[b]											
	CsETR1		*CsETR2*		*CsERS*		*CsEIN3*		*CsEIL2*		*CsEREBP*	
	G	**H**	**G**	**H**	**G**	**H**	**G**	**H**	**G**	**H**	**G**	**H**
$AgNO_3$												
$CuSO_4$	†		†		†		–	†	–	†	–	†
Ethephon	†	†	†	†	†	†		†		†		†
$CuSO_4$ + Ethephon		†		†		†	–		–		–	†

[b]†= increase the expression level of the gene; – = decrease the expression level of the gene

2.3.3 Influence of the sex-determining *A/a* gene on the expression level of ethylene receptor and signal transduction-related genes

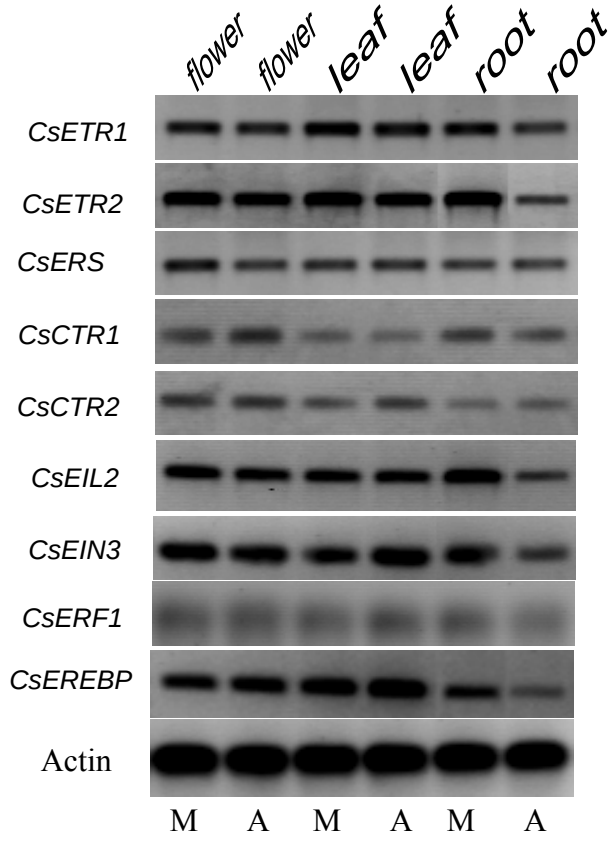

Figure 2.6 Comparison of the expression levels for *CsETR1, CsETR2, CsERS, CsCTR1, CsCTR2, CsEIL2, CsEIN3, CsERF1* and *CsEREBP* in flowers, leaves and roots of monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber by using semi-quantitative RT-PCR. Actin was used as a control for the semi-quantitative RT-PCR. M = monoecious (Shimshon), A = androecious (Erez)

By using semi-quantitative RT-PCR analysis, identical expression level of all the investigated ethylene receptor and signal transduction-related genes were detected in flowers and leaves of monoecious and androecious cucumber plants (Fig. 2.6). However, lower expression level of all the investigated genes was found in the roots of androecious than monoecious cucumber plants (Fig. 2.6).

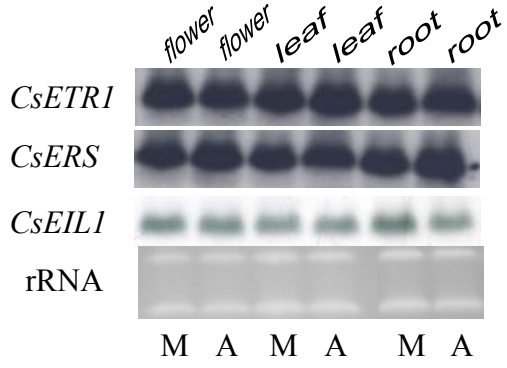

Figure 2.7 Northern blot hybridization analyses of *CsETR1*, *CsERS* and *CsEIL1* as a probe on 10µg total RNA from flowers, leaves and roots of monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants. M= monoecious (Shimshon), A= androecious (Erez)

By using northern blot hybridization analysis identical expression level for *CsETR1, CsERS* and *CsEIL1* genes were detected in flowers, leaves and roots of both monoecious and androecious cucumber plants (Fig. 2.7).

2.3.4 Southern blot hybridization

Southern blot hybridization analyses using ethylene receptor-related genes (*CsETR1*, *CsETR2, CsERS)* and ethylene signal transduction-related genes (*CsCTR1, CsCTR2, CsEIN3, CsEIL1, CsEIL2, CsERF1* and *CsEREBP*) in combination with the 30 randomly selected restriction enzymes produced no polymorphism between monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants. The autoradiogram for some of the gene enzyme combinations are presented in Fig. 2.8. Some of the polymorphisms detected lack consistency when repeated. For example the *CsERS/Msp*I (Fig. 2.8B) yielded one additional faint fragment in one of the monoecious plant, but when repeated it was not detected.

Figure 2.8 (A) *CsETR2*

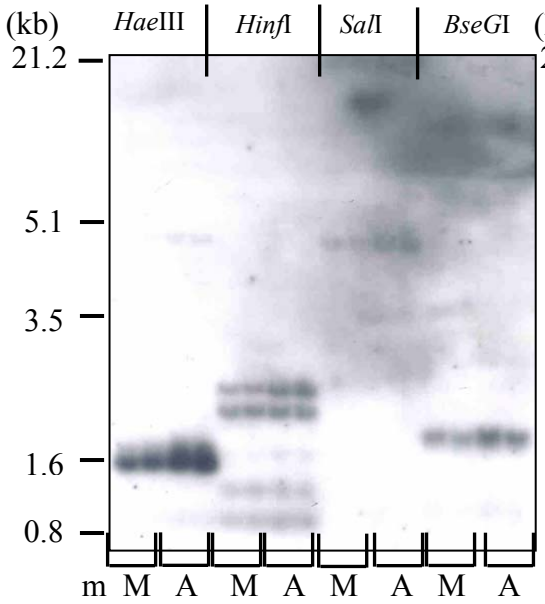

Figure 2.8 (B) *CsERS*

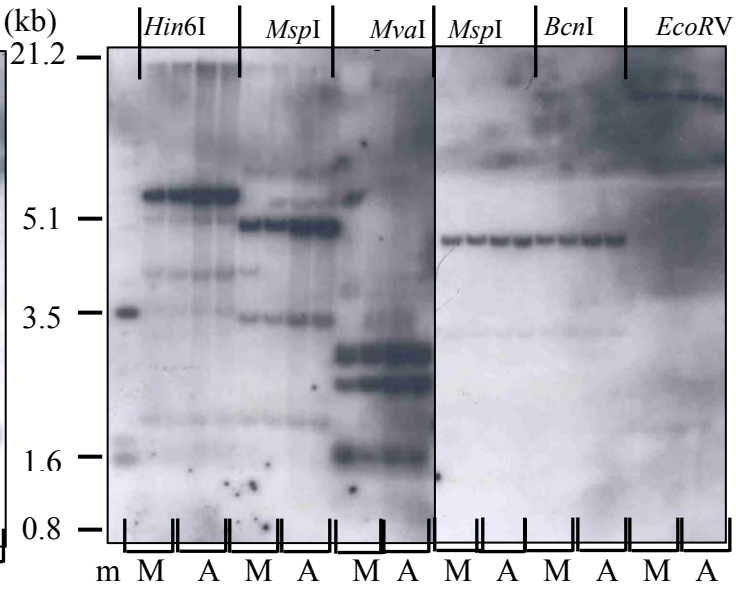

Figure 2.8 (C) *CsCTR1*

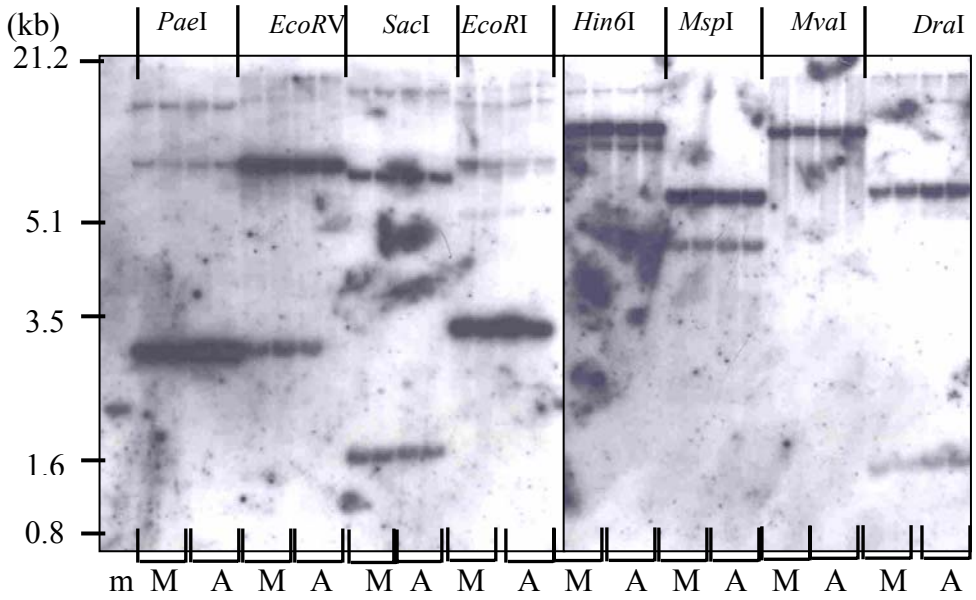

Figure 2.8 (D) *CsCTR2*

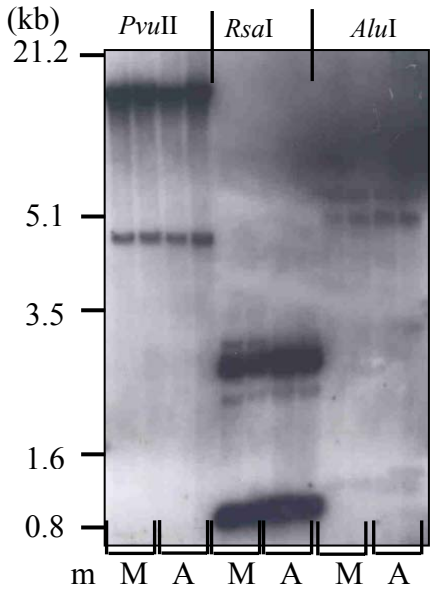

Figure 2.8 (E) *CsEIN3*

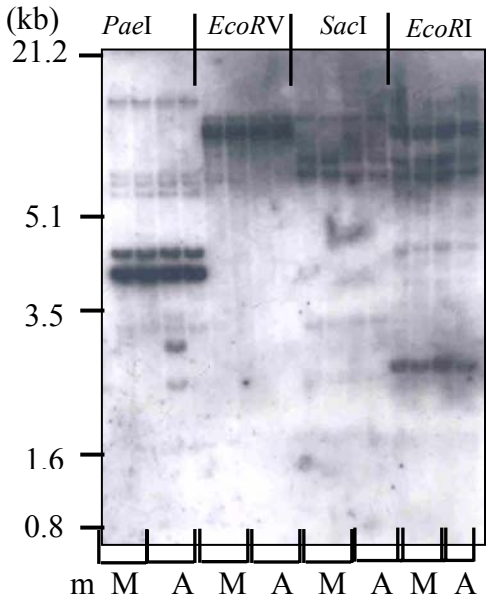

Figure 2.8 Autoradiogram of total cucumber DNA from monoecious and androecious lines restricted by different restriction enzymes and probed with the ethylene receptor and signal transduction-related genes. (**A**) *CsETR2*. (**B**) *CsERS*. (**C**) *CsCTR1*. (**D**) *CsCTR2* and (**E**) *CsEIN3*. m= size marker, M=monoecious (*ffMMAA*), A= androecious (*ffMMaa)*.

2.4 Discussion

The results of Kater et al. (2001) revealed that the arrest of either male or female organ development in cucumber plants is dependent on their positions in the flower and is not related to their sexual identity. Recently, Hao et al. (2003) investigated on the morphological, cellular and molecular changes in the arrested stamen-primordia of female flowers of cucumber and detected DNA damage in cells of the early primordial anther, which might result from anther-specific DNase activation. Based on these foundations, the specific floral part was chosen to study the possible role of the sex-determining *M/m* gene in cucumber under normal condition. Regardless of their sex, all cucumber floral buds are initially hermaphroditic, and it is the arrest of stamen or pistil development that leads to unisexual flowers (reviewed in Perl-Treves, 1999). In the presented investigation the arrested stamen-primordia of gynoecious (*FFMMaa*) and developed stamen-primordia of the nearly isogenic hermaphrodite (*FFmmaa*) cucumber plants were compared. These materials share more than 99.8% common genome and the variation that may arise is expected most probably due to the sex-determining *M/m* gene. Previous characterization indicated no correlation between the *M* gene and ethylene biosynthesis related genes (monoecious and andromonoecious cucumber plants having different forms of the *M/m* gene produced identical amount of ethylene) (Yamasaki et al., 2001). Hence *M* gene may not involve in ethylene production. It has been speculated that the *M* gene or its product may mediate ethylene signaling (Yamasaki et al., 2001) or the sensitivity of the male receptors (Yin and Quinn, 1995). This work also laid a foundation to consider ethylene receptor and signal transduction-related genes as a tool to study the role of the dominant *M* gene in gynoecious and the recessive *m* gene in hermaphrodite plants by using RNA from specific floral tissue.

Under normal condition more transcript level was observed for *CsETR1*, *CsETR2* and *CsERS* in developed stamen-primordia of the nearly isogenic hermaphrodite than arrested stamen-

primordia of gynoecious cucumber plants (Fig. 2.2). On the contrary, increased transcript level was detected for *CsEIN3* and *CsEIL1* in the arrested stamen-primordia of the nearly isogenic gynoecious than developed stamen-primordia of hermaphrodite cucumber plants (Fig. 2.3A). The most probable factor for such difference in the transcript levels of the ethylene receptor and some ethylene signal transduction-related genes between the nearly isogenic hermaphrodite and gynoecious cucumber lines is the *M/m* gene or its products. Thus the recessive *m* gene or its products lead to more increased accumulation of the ethylene receptor-related genes in cucumber stamen-primordia than the dominant *M* gene. The genetic topology of ethylene signal transduction is usually modeled as a linear series of events involving the sensing of ethylene by multiple functionally redundant ethylene receptors, the inhibitory action of the protein kinase *CTR1*, and the putative membrane protein *EIN2* that in turn triggers nuclear events involving the transcriptional activator *EIN3* and structurally related *EIN3*-like (*EIL*) genes that, together with ethylene response factors, control the transcription of ethylene-regulated genes (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Wang et al., 2002). Since ethylene receptors negatively regulate ethylene responses (Hua and Meyerowitz, 1998), the up-regulation of the ethylene receptors in hermaphrodite cucumber plants may be adaptation mechanism that negatively regulate the ethylene response and prevent the inhibition effect of ethylene to stamen arrest. Therefore, in the developed stamen-primordia, the *m* gene might induce increased accumulation of ethylene receptors which in turn negatively regulate the ethylene response in hermaphrodite cucumber plants. In the stamen-primordia of gynoecious cucumbers, the *M* gene may not cause significant increase in the accumulation of the ethylene receptors that could potentially interfere with ethylene response (Fig. 2.9). All of the ethylene receptor-related genes may not be inactivated by the available ethylene in hermaphrodite cucumber floral bud and could lead to activation of the downstream protein kinase *CTR1*, which in turn negatively regulate the downstream

ethylene response pathway (Fig. 2.9). In gynoecious cucumber floral buds, ethylene may bind to all available ethylene receptor-related genes and inactivate them. Such deactivation of the receptors can result in deactivation of *CTR1*, which allows the downstream regulators such as the *EIN3* family to positively regulate the ethylene response (Fig. 2.9). Therefore the lack of *m* gene in gynoecious cucumber leads to the full induction of ethylene responsive gene which might cause the arrest in the stamen development.

In *Arabidopsis EIN3* gene expression is not induced by ethylene (Wang et al., 2002), hence the increase in the accumulation of the mRNA for *CsEIN3* and its family *CsEIL1* in gynoecious compared to the hermaphrodite cucumber plants could be the direct influence of the *M* gene or loss of the *m* gene (Fig. 2.9). Identical hybridization signal is observed for *CsCTR1, CsCTR2*, *CsEIL2, CsERF1* and *CsEREPB* in both gynoecious and hermaphrodite floral buds (Fig. 2.3A, B). Since any of the *EIN3*, *EIL1* or *EIL2* gene can rescue the *ein3* mutant phenotype in *Arabidopsis* (Wang et al., 2002); an increase in one of these genes can also influence ethylene response in cucumber. Therefore an increase in either *CsEIN3* or *CsEIL1* or both is sufficient to positively regulate ethylene response in gynoecious compared to hermaphrodite and the similarity of *CsEIL2* may not influence the full induction of ethylene responsive genes. *CsCTR1* or *CsCTR2* are negative regulator of ethylene response, and should be inactivated by the ethylene receptors upon ethylene binding (Hua and Meyerowitz, 1998). The increase in the accumulation of the mRNA for ethylene receptor genes in hermaphrodite cucumber plants is expected to influence the *CsCTR1* or *CsCTR2* gene expression downstream differently than in gynoecious, however this is not the case in this observation. *EIN3* has been shown to act as a transcriptional activator and bind to the primary ethylene response element present in the promoter of the ethylene responsive *ERF1* gene (Chao et al., 1997; Solano et al., 1998). An increase in the level of *CsEIN3* in the arrested stamen-primordia of gynoecious cucumber is expected to raise the expression level of

the *CsERF1* gene; however, identical expression level for *CsERF1* and *CsEREPB* in both gynoecious and hermaphrodite floral buds was detected. This indicates that there may be additional ethylene signaling pathway (that could be activated by *CsEIN3* downstream of the ethylene signaling pathway) not discovered yet and to clarify this further study is needed.

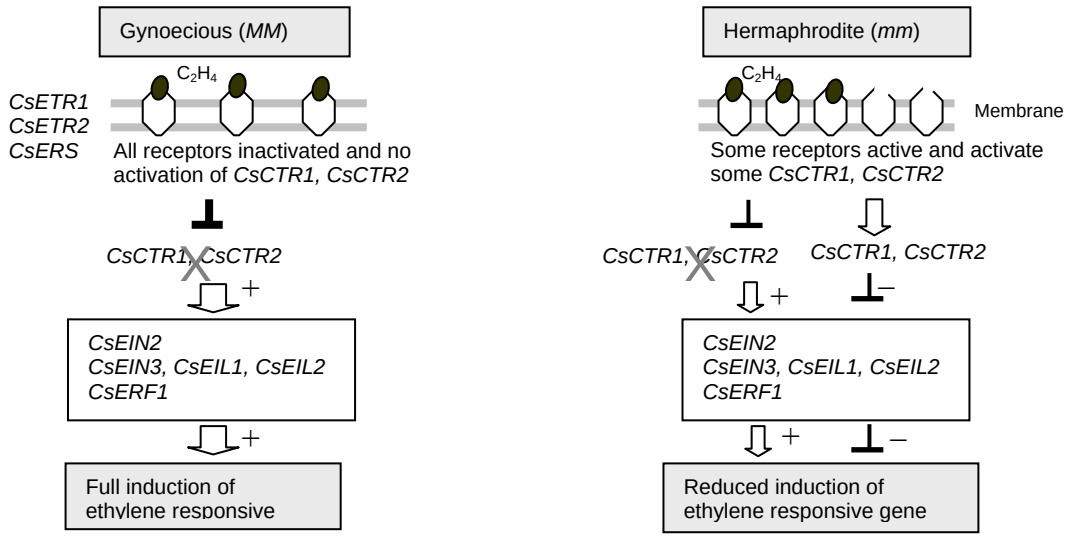

Figure 2.9 Simplified representation of the general ethylene signal transduction pathway and the possible role of *M/m* gene in cucumber in connection with the observation. In gynoecious plants ethylene may bind to all of the receptors, *CsETR1, CsETR2, CsERS* and shut off the negative receptor signaling, hence the *CsCTR1* or *CsCTR2* is inactivated and positive regulation of the downstream signaling which leads to the full induction of the ethylene responsive gene occur. In hermaphrodite plants the *m* gene increases the accumulation of the transcript for the *CsETR1, CsETR2 and CsERS* genes; hence some of the receptors may not bind to ethylene and may activate some *CsCTR1* or *CsCTR2* downstream. The presence of activated *CsCTR1* or *CsCTR2* may negatively regulate the downstream signaling partially and interfere with the full induction of the ethylene responsive gene in hermaphrodite plants. (+ = positive regulation, - = negative regulation, = inactivated receptor, = active receptor)

The *M/m* gene-induced variability in the expression level of ethylene receptor and signal transduction-related genes in cucumber was also confirmed after the chemical applications on the nearly isogenic gynoecious and hermaphrodite cucumber plants (Fig. 2.4 and 2.5, Table 2.3). For example application of $CuSO_4$ increased the expression level of *CsETR1*, *CsETR2*

and *CsERS* in shoot apices of gynoecious but not in that of hermaphrodite cucumber plants. The same application decreased the expression level of *CsEIN3, CsEIL2* and *CsEREBP* in gynoecious, but increased in hermaphrodite. Another interesting fact is that the influence from the *M/m* gene or its downstream product is different under different chemical applications. For example application with ethephon increased the expression level of *CsETR1, CsETR2* and *CsERS* in both gynoecious and hermaphrodite cucumber shoot apices and the same application increased the expression level of *CsEIN3*, *CsEIL2* and *CsEREBP* only in hermaphrodite cucumber shoot apices (Table 2.3). These shows the dominant *M* gene in gynoecious cucumber interact in a different way than the recessive *m* gene in hermaphrodite cucumber plants towards some exogenous chemical application.

Because of the fact that using specific stamen-primordia and shoot tip of cucumber plants revealed the possible influence of the *M/m* gene on the expression level of ethylene receptor and signaling genes, it can be speculated that the *M/m* gene or its product may function in the whole tissues of the cucumber plants.

Plants may reduce ethylene responses or the stress signal due to ethylene response, by increasing the level of receptors or the activity of receptors. Hence the increase in the level of ethylene receptors, *CsETR1*, *CsETR2* and *CsERS* in gynoecious cucumbers after $CuSO_4$ and ethephon application may be regulatory mechanism to reduce ethylene response. Such increase in the accumulation of the ethylene receptors in gynoecious cucumber plants might be due to the *M* allele as reported by Yamasaki et al. (2001) as a possible mechanism to reduce ethylene response. It is very premature to explain the mechanism through which the sex-determining *M/m* gene influences such response in cucumber plants; however speculations can be made as explained in section 5.4.1.

Concerning the sex-determining *A/a* gene, there has been almost no previous investigation conducted to characterize the gene. In one hormone hypothesis (Yin and Quinn, 1995) in

which ethylene is taken as a sex hormone, a fixed female receptor on the ethylene scale, *F/f* gene to control the level of ethylene and *M/m* gene to control the sensitivity level of male receptors, the possible role of the *A/a* gene was described by Mibus et al. (2000) and Vural et al. (2000). Mibus et al. (2000) and Vural et al. (2000) postulated that the *A/a* gene may influence the synthesis or the variation of the sensitivity level of the female receptor. Under the dominant *A* gene, the female receptor may has high sensitivity level and it may be activated at a lower amount of ethylene concentration compared with the condition of recessive *a* gene (Additional explanation in relation to the results of grafting and ethylene production is given in section 5.4.2).

To understand the possible relationship between the *A/a* gene and ethylene signalling genes, similar expression study was conducted as for the *M/m* gene. Semi-quantitative RT-PCR analyses indicated identical expression level for the ethylene receptor and signal transduction-related genes in flowers and leaves of monoecious and androecious cucumber plants (Fig. 2.6). However relatively lower expression level of these genes were detected in the roots of androecious than monoecious cucumber plants. Such difference was not visible after additional northern analysis for some selected genes, *CsETR1*, *CsERS* and *CsEIL1* (Fig. 2.7). RT-PCR is more sensitive to detect a small difference in the expression level of genes, therefore it may be possible that the difference in the expression level of the ethylene receptor and signal transduction-related genes is very small and could not be detected by northern analysis.

The identical expression level of ethylene receptor and signal transduction-related genes in leaves and flowers of monoecious and androecious plants indicate that the *A/a* gene may not involve in the ethylene perception and signalling. Since other plant hormones for example auxins also interact with ethylene (Takahashi and Jaffe, 1984; Trebitsh et al., 1987;), the *A/a*

gene may involve in the regulatory control of these plant hormones and may indirectly interact with ethylene to determine the sex expression in cucumber plants.

The possible effect of the result that shows difference in the expression level of ethylene receptor and ethylene signal transduction-related genes in roots of androecious and monoecious cucumbers was not revealed after grafting monoecious on androecious cucumber as explained in section 5.3.1. Further investigation is recommended to clearly understand the relationship between the weak expression level of ethylene-related genes and the *a* allele in the androecious cucumber plants.

3 Molecular genetic approaches to isolate the sex-determining *M/m* gene in cucumber

3.1 Introduction

Floral induction and differentiation is perhaps the most significant developmental transition in the life cycle of higher plants. It is a step that directly affects the agricultural yield by determining the time of flowering, the number of flowers and fruits, as well as the diversion of resources from vegetative growth (Greyson, 1994). Cucumber plants are originally monoecious but can be androecious, gynoecious, hermaphrodite or andromonoecious. The embryonic flower bud has both stamen and ovary primordia, and all are thus morphologically bisexual at a very early bud stage (Atsmon and Galun, 1960; Goffinet, 1990; Perl-Treves, 1999). Unisexual male flowers develop from the bisexual buds as a result of inhibition of carpel primordia as the stamens develop whereas unisexual female flowers form when stamen-primordia are arrested as the carpels develop. There has been no evidence for programmed cellular death and there has been speculation that the organ primordia may simply cease to grow upon sexual differentiation of the bud (reviewed in Perl-Treves, 1999).

As already described in the general introduction, three major genes are responsible for cucumber sex expression in addition to hormonal and environmental factors. The promoter region of the dominant *F* allele has already been cloned (Mibus and Tatlioglu, 2004) and found to be responsible for ethylene synthesis. Except few characterization studies by using ethylene receptor-related genes, the *M/m* gene still remains unrevealed (Yamasaki et al., 2001).

Because sex determination in cucumber and most other angiosperm species occurs via selective abortion of flower organs, Kater et al. (2001) set out to establish whether this abortion is based on organ identity or positional information within the flower. Using

cucumber homologs of the MADS box ABC homeotic genes (Coen and Meyerowitz, 1991), Kater et al. (2001) show that the sex determination machinery in cucumber selectively aborts sex organs based on their position rather than their identity (i.e., in male flowers, carpels are aborted only in the fourth whorl, and in female flowers, stamens abort only in the third whorl). In addition, because non reproductive organs that develop in the inner whorls of a C-class homeotic mutant are not aborted, Kater et al. (2001) speculated that C-class gene products might be targets of the sex-determining process.

Based on this foundation the study was commenced using floral buds (1-2mm) from nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber lines in the approach to isolate the sex-determining *M/m* gene. In this investigation suppression subtractive hybridization (SSH) technique has been used to generate differentially-expressed cDNAs from the floral buds of gynoecious and hermaphrodite cucumber plants. Some of the obtained cDNA clones were further analyzed in relation to the *M/m* gene and the possible role of some of the isolated cDNA clones are presented. In addition randomly amplified polymorphic DNA (RAPD) analysis was performed using the genomic DNA obtained from the nearly isogenic gynoecious and hermaphrodite cucumber plants. The obtained RAPD fragments were also analyzed in relation to the *M/m* gene and their possible role is discussed.

3.2 Materials and Methods

3.2.1 Plant materials

The nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) as well as monoecious (*ffMMA-*) and androecious (*ffMMaa*) cucumber plants from the three genetic backgrounds, WrD, ECD and ED, were used in this investigation. F_2 population from a cross between gynoecious and hermaphrodite cucumber of the genetic background, WrD, were also used for the segregation study. Growing of the cucumber plants was performed as described in section 2.2.2.

3.2.2 RNA and messenger-RNA isolation

Flower buds of about 1-2mm size were harvested from the nearly isogenic gynoecious and hermaphrodite as well as monoecious and androecious cucumber plants. Total RNA was isolated using 30mg of the ground sample with the help of RNA isolation Kit (Machery and Nagel). The concentration of total RNA isolated was measured by using spectrophotometer (Pharmacia). Estimation of the isolated total RNA was also done by using λ-DNA on 1.5% agarose gel with ethidiumbromide (1µg/ml), visualized by transilluminator (UV-light, 302nm). About 250µg of the isolated RNA was used for the mRNA isolation by using Oligotex mRNA Kits (Qiagen). Isolation of poly A^+ mRNA from total RNA usually provides a slightly greater enrichment than direct isolation from cells or tissues since there is no risk of interference by other cellular components. The mRNA isolation was performed by Oligotex procedure. The principle of Oligotex is that Poly A^+ mRNA can be purified by hybridizing the poly-A tail to a dT oligomer coupled to a solid-phase matrix; the rRNA and tRNA species without a poly-A tail do not bind to the oligo-dT and are easily washed away. Since hybridization requires high-salt conditions, the poly A^+ mRNA can then easily be released by lowering the ionic strength and destabilizing the dT:A hybrids. After isolation the mRNA

concentration was also measured by using spectrophotometer (Pharmacia) and the integrity of the mRNA was examined by gel electrophoresis using 1.5% agarose gel.

Reagents used:

- RNA Kit (Machery and Nagel)
- Oligotex mRNA Kits (Qiagen)
- Ethidiumbromide
- Agarose
- Orange G loading buffer (30% Glycerine, 0.25% Orange G)
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)
- λ-DNA (Gibco)

3.2.3 DNA isolation

DNA isolation was performed by using shoot-tip of the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants of the three genetic backgrounds, WrD, ECD and ED, following the procedures described in section 2.2.5. Additional DNA was also isolated from F_2 (gynoecious x hermaphrodite) cucumber plant population of the WrD genetic background.

Reagents used: see section 2.2.5

3.2.4 Randomly amplified polymorphic DNA (RAPD) analysis

A total of 360 random 10-base oligonucleotide primers obtained from Operon Technologies Inc. (Alameda, CA) were used to screen for DNA markers linked with the sex-determining *M/m* gene. The sequences of the 10-bp primers have 60% to 70% (G+C) content and have no self complementary ends. For each primers, DNA from nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants were used for polymerase chain reaction (PCR). The amplification reactions were carried out according to Williams et al. (1990) with minor modifications: 25µl reaction mixture containing 25ng genomic template DNA, 25ng of primer, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.5 units of Dynazyme DNA polymerase in the reaction buffer as recommended by the supplier (Finnzymes). The PCR cycling profile was: 1 min at 94°C, 1 min at 36°C and 2 min at 72°C for 45 cycles using thermocycler (TGradient Block, Biometra). Amplification products were analyzed after electrophoresis in 1.5% agarose gels at 5V/cm gel length in 1x TAE buffer. The gels were

stained with 1µg/ml ethidiumbromide solution and visualized by transilluminator (UV light, 302nm). The RAPD fragments found interesting were cut from the gel and used for cloning in section 3.2.5.

Reagents used:

- Ethidiumbromide
- 100bp ladder (Gibco BRL)
- Agarose
- Orange G loading buffer (30% Glycerine, 0.25% Orange G)
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)

Reagents used (PCR) :

- Buffer: 10 mM Tris-HCl; pH 8,8; 50 mM KCl 0,1% Triton X-100, 1,5 mM $MgCl_2$ (Finnzymes)
- 100 µM of each dNTP (Eurogenetech)
- 0.5 units Taq-Polymerase DyNAzyme^{TM} II DNA Polymerase (2 Units/µl; Finnzymes)
- 10-bp primers (Operon Technolodies Inc. Alameda, CA)

3.2.5 Cloning and sequencing of the RAPD fragments

The RAPD fragments, found putatively linked to the sex-determining *M/m* gene, were cut from the gel and transferred to 1% low melting agarose gel in 1xTAE buffer using a voltage supply of 3V/cm gel length. The RAPD fragments were further cut from the low melting gel and transferred to pre-weighed 1.5ml eppendorf tube. The newly cut RAPD fragments were weighed and digestion of the low melting gel performed as follows: 25x Agarase buffer (Roche) was added into each cut RAPD fragments in 1.5ml eppendorf tube, melted at 70°C for 3 min and allowed to cool to 45°C, at which the enzyme Agarase (Roche) was added and digestion of the low melting gel performed for 1h at 45°C according to the manufacturer's instruction (Roche). The concentration of the purified RAPD fragment was estimated by using λ-DNA (Gibco). The purified RAPD fragments were then cloned using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instruction. Plasmids were recovered using QiaPrep-Mini kit (Qiagen) and sequencing was done by MWG Biotech AG. The isolated RAPD sequences were analyzed by using the CLUSTAL W program, European Bioinformatics Institute (EMBL; Thompson et al., 1994) and the BLUSTN program, National Center for Biotechnology Information (NCBI; Altschul et al. 1997). From the sequence information primers were designed by using Primer Premier program (PREMIER Biosoft

International, Palo, Alto, CA) and synthesized as Invitrogen Custom Primers (Invitrogen). PCR was performed using additional DNA from some F_2 (gynoecious-WrD x hermaphrodite-WrD) cucumber plants.

Reagents used:

- Agarose
- Agarase (0,2 U/µl) with buffer (Roche)
- Ethidiumbromide
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)
- λ-DNA (Gibco)
- TOPO TA Cloning Kit (Invitrogen)
- QiaPrep-Mini kit (Qiagen)

3.2.6 Suppression subtractive hybridization (SSH) analysis

Messenger RNA from floral buds of the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants were compared using suppression subtractive hybridization techniques (Diatchenko et al., 1996) following the detailed procedures presented below:

First- and second-strand cDNA synthesis

Two micrograms mRNA from the nearly isogenic gynoecious and hermaphrodite floral buds were separately combined with 10µM cDNA synthesis primer (total reaction volume 5µl). The mixture was incubated at 70°C for 2 min. After cooling, 10mM dNTP mix, 20units AMV reverse transcriptase and 2µl 5x first-strand buffer (Clontech) were added to each reaction tube. The final volume was adjusted to 10µl with sterile water. The first-strand cDNA synthesis was performed at 42°C for 1.5h.

For the second-strand cDNA synthesis, 10mM dNTP mix, 16µl 5x second-strand buffer and 4µl 20x second-strand enzyme cocktail (Clontech) were added to the first-strand synthesis reaction tube (containing 10µl). Sterile water was added to a final volume of 80µl. The mixtures were then incubated at 16°C for 2h followed by addition of 6 units T4 DNA polymerase (3U/µl) and incubation for another 30 min at the same temperature condition.

Four microliter 20x EDTA/Glycogen mix was added to terminate second-strand synthesis. The ds cDNA synthesized were then cleaned as follows: 100µl phenol: chloroform: isoamyl alcohol (25:24:1) was added to the second-strand synthesis reaction tubes (containing 86µl) and centrifuged at 14000rpm for 10 min at room temperature. The top aqueous layer was carefully transferred to a new sterile 0.5ml microcentrifuge tube. 100µl chloroform: isoamyl alcohol (24:1) was then added to the aqueous layer and centrifugation step repeated. The top aqueous layer was again carefully transferred to a new 0.5µl microcentrifuge tube. 40µl 4M NH_4OAc and 300µl 95% ethanol was then added to the aqueous layer to precipitate the cDNAs and followed by 14000rpm centrifugation for 20 min at room temperature. The supernatant was carefully discarded and the pellet overlaid with 500µl 80% ethanol. Following another centrifugation at 14000rpm for 10 min at room temperature, the supernatant was carefully discarded and the pellet air-dried for 10 min. The pellet was then dissolved in 50µl of sterile water. The synthesized and purified ds cDNA was then used in the subsequent subtraction steps.

Reagents used:

- dNTP mix (10mM each dATP, dCTP, dGTP, dTTP) (Eurogenetech)
- 20x EDTA/Glycogen (0.2M EDTA; 1mg/ml glycogen) (Clontech)
- 4 M NH_4OAc
- phenol: chloroform: isoamyl alcohol (25:24:1)
- chloroform: isoamyl alcohol (24:1)
- 80% and 95% ethanol
- 10µM cDNA synthesis primer (Clontech)
- 5x first-strand buffer (250mM Tris-HCl, pH 8.5; 40mM $MgCl_2$; 150mM KCl; 5mM Dithiothreitol (DTT)) (Clontech)
- 5x second-strand buffer (500mM KCl; 50mM Ammonium sulfate; 25mM $MgCl_2$; 0.75mM ß-NAD; 100mM Tris-HCl, pH 7.5; 0.25mg/ml BSA) (Clontech)
- 20 units AMV reverse transcriptase (20U/µl) (Clontech)
- 20x second-strand enzyme cocktail (6 units/µl DNA polymerase I; 0.25 units/µl RNase H; 1.2 units/µl *E. coli* DNA ligase) (Clontech)
- 6 units T4 DNA polymerase (3U/µl) (Clontech)

Double-stranded cDNA (ds cDNA) digestion and adapter ligation

*Rsa*I digestion was performed to generate shorter, blunt-ended ds cDNA fragments optimal for subtraction and necessary for adapter-ligation. From the 50µl dissolved pellet of the previous preparation, 43.5µl ds cDNA was subjected to digestion with 15 units *Rsa*I in 10x

*Rsa*I restriction buffer (Clontech) at 37°C for 2h. The restriction reaction was stopped by adding 2µl 20x EDTA/Glycogen mix and followed by cleaning procedures: 50µl phenol: chloroform: isoamyl alcohol (25:24:1) was added to the restriction reaction tube, thoroughly mixed and centrifuged at 14000rpm for 10 min at room temperature. The top aqueous layer was carefully transferred to a clean 0.5ml microcentrifuge tube. 50µl chloroform: isoamyl alcohol (24:1) was then added to the separated top aqueous layer, thoroughly mixed and centrifuged as above. The top aqueous layer was then transferred to a new 0.5ml microcentrifuge tube. 25ml 4M NH_4OAc and 187.5µl 95% ethanol was added to the top aqueous layer to precipitate the cDNA, thoroughly mixed and centrifuged at 14000rpm for 20 min at room temperature. The supernatant was then discarded and the pellets overlaid with 200µl 80% ethanol, thoroughly mixed and centrifuged at 14000rpm for 5 min at room temperature. The supernatant was then discarded and the pellets air-dried for 10 min. The air-dried pellets was then dissolved in 5.5µl sterile water and used for adapter ligation.

The cDNA subtraction was performed for each sample in both directions. The cDNA from gynoecious was used as a tester and the cDNA from hermaphrodite as a driver in the forward subtraction experiment. In the reverse subtraction the cDNA from hermaphrodite was used as a tester and that of gynoecious as a driver. For the forward subtraction, 1µl *Rsa*I-digested ds cDNA from gynoecious was first diluted with 5µl sterile water. Two reaction mixtures were then prepared in two reaction tubes each with 2µl of the diluted *RsaI*-digested ds cDNA from gynoecious, 400 units T4 DNA ligase, in 2µl 5x DNA ligation buffer, and 10µM adapter1 in the first reaction tube and 10µM adapter2R in the second reaction tube (Clontech) (Table 3.1). The reaction mixture in each tube was filled to 10µl with sterile water. The ligation reaction mixtures were then incubated at 16°C for 15h. The ligation reaction was stopped by adding 1µl EDTA/Glycogen mix and the ligase was inactivated by heating the samples at 72°C for 5

min. For the reverse subtraction the same procedure was followed except that the ds cDNA in reverse subtraction was from hermaphrodite cucumber plant.

Reagents used:

- 20x EDTA/Glycogen (0.2M EDTA; 1mg/ml glycogen) (Clontech)
- 4 M NH_4OAc
- phenol: chloroform: isoamyl alcohol (25:24:1)
- chloroform: isoamyl alcohol (24:1)
- 80% and 95% ethanol
- 10x *RsaI* restriction buffer (100mM Bis Tris Propane-HCl, pH 7; 100mM $MgCl_2$; 1mM Dithiothreitol (DTT)) (Clontech)
- 15 units *RsaI* (Clontech)
- 5x DNA ligation buffer (250mM Tris-HCl, pH 7.8; 50mM $MgCl_2$; 10mM DTT; 0.25mg/ml BSA) (Clontech)
- 400 units T4 DNA ligase (Clontech)
- 10µM adapter1 (Clontech)
- 10µM adapter2R (Clontech)

First and second hybridization

For the forward subtraction two hybridization reaction tubes were prepared as follows: In the first tube 1.5µl adapter1-ligated ds cDNA from gynoecious, 1.5µl *Rsa*I-digested ds cDNA from hermaphrodite in 1µl 4x hybridization buffer; in the second reaction tube, 1.5µl adapter2R-ligated ds cDNA from gynoecious, 1.5µl *Rsa*I-digested ds cDNA from hermaphrodite in 1µl 4x hybridization buffer (Clontech). For the reverse subtraction two hybridization reaction tubes were prepared as follows: In the first reaction tube, 1.5µl adapter1-ligated ds cDNA from hermaphrodite, 1.5µl *RsaI*-digested ds cDNA from gynoecious in 1µl 4x hybridization buffer; in the second reaction tube, 1.5µl adapter2R-ligated ds cDNA from hermaphrodite, 1.5µl *Rsa*I-digested ds cDNA from gynoecious in 1µl 4x hybridization buffer (Clontech). The samples were then denatured at 98°C for 2 min and incubated at 68°C for 10h for first hybridization.

During second hybridization the two samples of the forward subtraction were mixed together and freshly denatured ds cDNA from hermaphrodite was added as follows: First, 1µl *Rsa*I-digested ds cDNA from hermaphrodite was diluted with 2 µl sterile water in 1µl 4x hybridization buffer. From this dilution 1.5µl was denatured in 0.5ml microcentrifuge tube at 98°C for 1.5 min, immediately added to the previously mixed sample of the forward subtraction. The reaction mixture (all in a singe tube) was incubated at 68°C for 15h for

second hybridization. For the reverse subtraction, the two first hybridization samples were mixed together and freshly denatured ds cDNA from gynoecious was added and the second hybridization was performed following similar procedure described for the forward subtraction. After the second hybridization, each of the forward and reverse subtraction samples were separately diluted in 200µl dilution buffer (Clontech) and used in the subsequent PCR procedures.

Reagents used:

- 4x hybridization buffer (Clontech)
- dilution buffer, pH 8.3 (20mM HEPES, pH 6.6; 20mM NaCl, 0.2mM EDTA, pH 8.0) (Clontech)

Polymerase chain reaction (PCR) amplification

PCR was performed to selectively amplify differentially expressed cDNAs from the forward and reverse subtracted samples. During the first PCR 1µl forward subtracted and diluted cDNA from gynoecious and 1µl reverse subtracted and diluted cDNA from hermaphrodite were separately taken in two 0.5ml microcentrifuge tubes. The following reaction mixtures were then added into each of the reaction tubes, 10x PCR reaction buffer, 10mM dNTP mix, 10µM PCR primer 1 (Table 3.1), 0.5 units of Dynazyme DNA polymerase (Finnzymes), filled to a final volume of 25µl with sterile water. The reaction mixtures were first incubated at 75°C for 5 min to fill-in the missing strands and followed by 32 cycles of 94°C for 30 sec, 66°C for 30 sec and 72 °C for 1.5 min. From this PCR product, 3µl of each sample were diluted in 27µl sterile water (1:10) and used for the next PCR. The second PCR was then performed using 1µl of each diluted first PCR product and the following reaction mixtures: 10x PCR reaction buffer, 10mM dNTP mix, 10µM nested PCR primer 1, 10µM nested PCR primer 2R (Table 3.1) and 0.5 units of Dynazyme DNA polymerase (Finnzymes), filled with sterile water to a final volume of 25µl. The PCR was performed using the PCR cycling profile: 17 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 1.5 min using TGradient

Block (Biometra). The PCR products were then purified and cloned as explained in section 3.2.7.

Reagents used:

- 10x PCR reaction buffer (10 mM Tris-HCl; pH 8,8; 50 mM KCl 0,1% Triton X-100, 1,5 mM $MgCl_2$) (Finnzymes)
- 0.5 units of Dynazyme DNA polymerase (Finnzymes)
- 10mM PCR primer 1(Clontech)
- 10µM nested PCR primer 1 (Clontech)
- 10µM nested PCR primer 2R (Clontech)
- dNTP mix (10mM each dATP, dCTP, dGTP, dTTP) (Clontech)

Table 3.1 List of primers used in the different steps of the SSH procedures

Primer name	Sequences
cDNA synthesis primer	5'TTTTGTACAAGCTT$_{30}$N$_1$N'3
Adapter 1	5'CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-ACCTGCCCGG'3
Adapter 2R	5'CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-ACCTCGGCCG'3
PCR primer 1	5'CTAATACGACTCACTATAGGGC'3
Nested PCR primer 1	5'TCGAGCGGCCGCCCGGGCAGGT'3
Nested PCR primer 2R	5'AGCGTGGTCGCGGCCGAGGT'3

3.2.7 Cloning and differential screening of the subtracted cDNA library

The selectively amplified differentially expressed cDNAs from gynoecious and hermaphrodite floral buds were purified by High Pure PCR product Purification Kit (Roche). The concentration of the purified cDNAs was estimated by using λ-DNA (Gibco). The entire cDNA pool was then cloned using TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instruction. Plasmids were recovered by using QiaPrep-Mini kit (Qiagen). The obtained clones were screened for differentially expressed cDNAs by using dot blot as follows: cDNA inserts were amplified by using standard T3/T7 primers and precipitated with ethanol. The PCR products were denatured in 0.2M NaOH at 37°C for 15 min and manually dotted onto Hybond-N^+ membrane (Amersham). The driver cDNA probe was labeled by PCR using the DIG system (Roche) for non radioactive hybridization using PCR as described in

section 2.2.7. Hybridization and detection procedures were performed as described in section 2.2.9 for the southern blot hybridization.

Reagents used: (additional reagents see section 2.2.7 and 2.2.9)

- High Pure PCR product Purification Kit (Roche)
- TOPO TA Cloning Kit (Invitrogen)
- 0.2M NaOH

3.2.8 Sequence analysis of the selected cDNA clones

Plasmids for cDNA clones found to be up-regulated were recovered using QiaPrep-Mini kit (Qiagen) and sequencing was done by MWG-Biotech. The obtained cDNA sequences were analyzed by using the CLUSTAL W programme, European Bioinformatics Institute (EMBL; Thompson et al., 1994) and the BLUSTN programme, National Center for Biotechnology Information (NCBI; Altschul et al., 1997).

Reagents used: QiaPrep-Mini kit (Qiagen)

3.2.9 Northern blot hybridization and semi-quantitative RT-PCR analyses using the cl-38 and cl-66 cDNA clones

Northern blot hybridization analysis was performed by using cl-38 (differentially expressed in hermaphrodite floral bud) and cl-66 (putatively up regulated in gynoecious floral bud) cDNA clones as a probe on 10µg total RNA obtained from gynoecious and hermaphrodite cucumber floral buds following the procedure described in section 2.2.7.

The expression level of cl-38 and cl-66 cDNAs were further analyzed by using semi-quantitative RT-PCR (Marone et al., 2001). Oligonucleotide primer pairs (sense, 5'-ATTCA-TTCTCGGTCCATA-3'; antisense, 5'-GCCTCGCCGATCTTCTAC-3' for cl-38 and sense, 5'-CATGTTATTTAACGGAACA-3'; antisense, 5'-ACCTACAGAATTTCAAGG-3' for cl-66) were designed by using Primer Premier program (PREMIER Biosoft International, Palo, Alto, CA) from sequence information of the cDNA clones. The designed primer pairs were synthesized by MWG Biotech AG. The annealing temperature for each primer pairs was

obtained by using eight different temperature gradients between 45 and 65°C in a PCR machine (TGradient Block, Biometra). Semi-quantitative RT-PCR was performed by using 160pg of total RNA obtained from gynoecious and hermaphrodite floral buds. The reaction components were 400µM of each dNTP, 0.6µM of each sense and antisense primers, in 1x QIAGEN OneStep RT-PCR buffer and QIAGEN OneStep RT-PCR enzyme mix as recommended by the manufacturer, filled to a final volume of 50µl with RNase-free water (OneStep RT-PCR Kit, Qiagen). The following RT-PCR condition was used: 50°C for 30 min- reverse transcription, 95°C for 15 min- for activation of the DNA polymerase and deactivation of the reverse transcriptase followed by 30 cycles of 94°C for 30 seconds, 58°C (cl-38) / 47°C (cl-66) for 1 min and 72°C for 2 min, and a final extension of 72°C for 10 min. A primer pair (sense, 5'-GGCAGTGGTGGTGAACAT-'3; antisense, 5'-CTGGTATCGTGC-TGGATT-'3) obtained from cucumber mRNA for actin (accession number AB010922) was used as internal control in the semi-quantitative RT-PCR. The obtained amplicons were fractionated on flatbed electrophoresis using 1.5% agarose gels in 1xTAE buffer and the sizes estimated by comparison to a 100-bp ladder (Amersham Pharmacia Biotech)

In the subsequent investigation it was detected that the cl-38 cDNA was found to be a very important gene for stamen development in cucumber. Therefore further comparison was done for the expression level of cl-38 cDNA on male and female floral buds of monoecious, ethephon-induced female floral buds of androecious and $AgNO_3$-induced male floral buds of gynoecious cucumber plants by using semi-quantitative RT-PCR.

Reagents used:

- Ethidiumbromide
- 100bp ladder (Gibco BRL)
- Agarose
- Orange G loading buffer (30% Glycerine, 0.25% Orange G)
- 1xTAE, pH=8 (40mM Tris-acetate, 1mM EDTA)

Reagents used (RT-PCR):

- 10µl 5x Qiagen OneStep RT-PCR Buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 12.5mM $Mgcl_2$, dithiothreitol-DTT (Ph 8.7))
- 2µl dNTP mix, 10mM each of dATP, dCTP, dGTP and dTTP
- 2µl OneStep RT-PCR Enzyme mix (1mM dithiothreitol (DTT), 0.1mM EDTA, 0.5% (v/v) Nonidet®P-40, 0.5% (v/v) Tween®20, 50% glycerol (v/v), stabilizer (pH 9.0))
- RNase free water (variable volume)
- 0.6 µM sense-Primer (MWG)
- 0.6 µM antisense-Primer (MWG)

Reagents used (northern blot hybridization): see section 2.2.7

3.2.10 Southern blot hybridization analysis using cl-38, cl-66, cl-78, cl-83 and cl-115 cDNA clones

Twenty micrograms DNA from the nearly isogenic gynoecious and hermaphrodite cucumber lines were digested using 30 U of the randomly selected restriction enzymes *BamH*I, *Bgl*II, *Dra*I, *EcoR*I, *EcoR*V, *Hind*III, *Msp*I, *Pst*I, *Xba*I, *Bcn*I, *Hin6*I, *Hinf*I, *Mva*I, *Pae*I, *Sac*I, *Sal*I, *Rsa*I, *Xho*I and *Bfa*I (MBI Fermentas) in a restriction buffer provided by the manufacturer. The restriction reaction was incubated at 37°C for 15h. The restricted fragments were mixed with 3µl bromophenol blue-loading buffer and then separated by flatbed electrophoresis, using 0.8% agarose gels in 1xTAE buffer for 16 h (2 V/cm length of the gel). The DNA blotting, hybridization and detection were performed following the procedures described in section 2.2.9. The cDNA clones, cl-66, cl-78, cl-83 and cl-115 (putatively up regulated in gynoecious) and cl-38 (up regulated in hermaphrodite), were DIG-labeled following the procedure given in section 2.2.7 and used as a probe in the southern blot hybridization. The obtained fragment sizes were estimated by comparison with the DNA Molecular Weight Marker III (DIG-labeled, 0.56–21.2 kb; Roche, 1µg).

Reagents used: see section 2.2.7 and 2.2.9

3.3 Results

3.3.1 Randomly amplified polymorphic DNA (RAPD)

From the 360 arbitrary decamer oligonucleotide primers, OPA08 (5'GTGACGTAGG3') and OPB11 (5'GTAGACCCGT3') yielded polymorphism between the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants. Primer OPA08 produced 677bp RAPD marker specific to gynoecious cucumber (Fig. 3.1A). The primer OPB11 produced two RAPD markers, 654bp specific to gynoecious and 726bp specific to hermaphrodite cucumber plants (Fig. 3.1B). For convenience the obtained RAPD fragments were labeled as follows: The 677bp RAPD fragment was named as $OPA08_{677}$; the 726bp RAPD fragment was named as $OPB11_{726}$ and the 654bp RAPD fragment was named as $OPB11_{654}$.

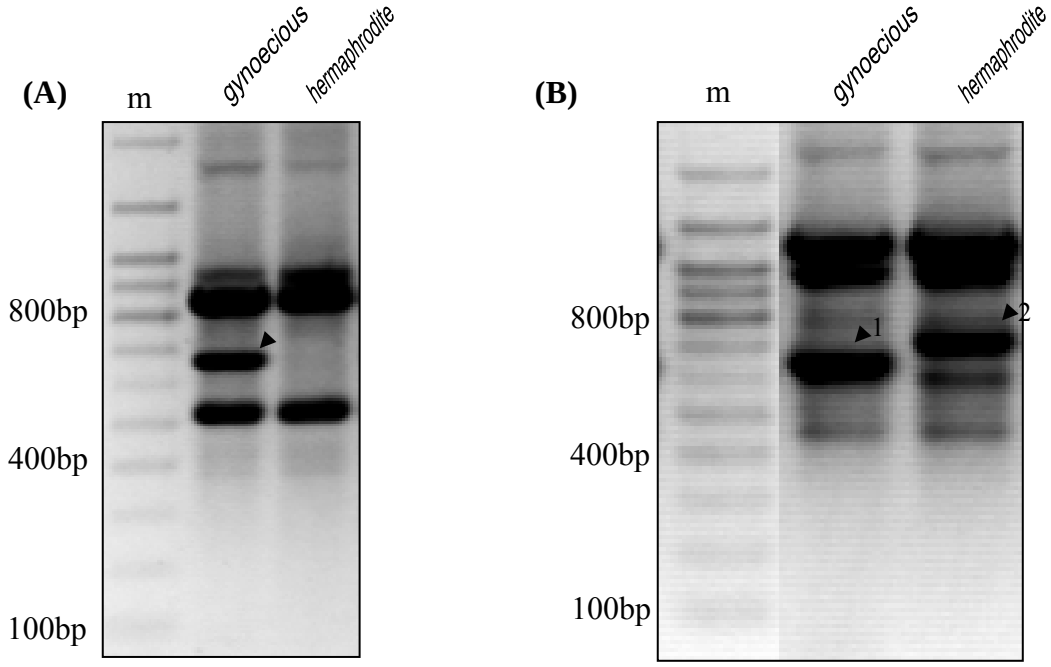

Figure 3.1 Randomly amplified polymorphic DNAs (RAPD). **(A)** Primer OPA08 produced 677bp (*arrowhead*) specific to gynoecious cucumber. **(B)** Primer OPB11 produced 654bp (*arrowhead 1*) specific to gynoecious and 726bp (*arrowhead 2*) specific to hermaphrodite cucumber plants. m= size marker

After sequence comparison the $OPB11_{654}$ and $OPB11_{726}$ were found identical except 72bp additional insert in the $OPB11_{726}$ specific to the hermaphrodite cucumber plant (Fig. 3.2). Sequence analysis indicated that all of the three RAPD fragments obtained are homologous to mitochondrial gene sequences, particularly $OPB11_{654}$ and $OPB11_{726}$ are highly homologous to *Cucumis sativus* cultivar Calypso apocytochrome b (*cob*) gene (GenBank accession number AF288044).

```
OPB11654        GTAGACCCGTATTTCTCCGGACCAGAAACCATAATCAAACTCTGTTCATTTCTTTTCCCT 60
OPB11726        GTAGACCCGTATTTCTCCGGACCAGAAACCATAATCAAACTCTGTTCATTTCTTTTCCCT 60
                ************************************************************

OPB11654        GTCCTAGGAGTTCTTCTTTCATTGCGTTTTCCCGGCTTTTTTGCTTCTAAATAGGACTTT 120
OPB11726        GTCCTAGGAGTTCTTCTTTCATTGCGTTTTCCCGGCTTTTTTGCTTCTAAATAGGACTTT 120
                ************************************************************

OPB11654        TTCTCATATTGATATTTAACGATAAAAGTACTTTTTTGACTGATTTTTTGTCTTTTCTTG 180
OPB11726        TTCTCATATTGATATTTAACGATAAAAGTACTTTTTTGACTGATTTTTTGTCTTTTCTTG 180
                ************************************************************

OPB11654        CTACTTCCTATGGGCAAAACCTAGCAAAATCATGGAATTCACTCCGTCAAAAAGACTAAA 240
OPB11726        CTACTTCCTATGGGCAAAACCTAGCAAAATCATGGAATTCACTCCGTCAAAAAGACTAAA 240
                ************************************************************

OPB11654        ATCGATGTCTGAAATCTCCCATATTCACCTCTTTCTTTTTCATTCCCATTTTTTTCTTCT 300
OPB11726        ATCGATGTCTGAAATCTCCCATATTCACCTCTTTCTTTTTCATTCCCATTTTTTTCTTCT 300
                ************************************************************

OPB11654        TTCATTATCGTTAATGAGCTGTCCTACGTGCCAAAGAAAGGTCCAGGGTCC--------- 351
OPB11726        TTCATTATCGTTAATGAGCTGTCCTACGTGCCAAAGAAAGGTCCAGGGTCCTACGTCCGC 360
                ***************************************************

OPB11654        ----------------------------------GGA---------------------- 354
OPB11726        AGGGCCAAAGAAAGGTCCAGGTGATTTATCCGTAGGACCATCCGTAATTAACCGAAGTAG 420
                                                  ***

OPB11654        ------GTCCGCAGGGCCAAAGAAAGGTCCAGGTGATTTATCCGTAGGACCATCCGTAAT 408
OPB11726        TCCTACGTCCGCAGGGCCAAAGAAAGGTCCAGGTGATTTATCCGTAGGACCATCCGTAAT 480
                      ******************************************************

OPB11654        TAACCGAAGTAGGACCATCCGTAATTAACAGAAGTAGGACCATCCATATGGAAGGAAGGA 468
OPB11726        TAACCGAAGTAGGACCATCCGTAATTAACAGAAGTAGGACCATCCATATGGAAGGAAGGA 540
                ************************************************************

OPB11654        GGACTTTCCGTAATTAACGAAGGGCCCACGGAGCGGGAAGAACTGGAAAGTCTAATCTTT 528
OPB11726        GGACTTTCCGTAATTAACGAAGGGCCCACGGAGCGGGAAGAACTGGAAAGTCTAATCTTT 600
                ************************************************************

OPB11654        CTTTCTCTCAAATTCTCAACGCATATTTCATTGCGCCTTCGGACCTGTCTTATGGCGCCT 588
OPB11726        CTTTCTCTCAAATTCTCAACGCATATTTCATTGCGCCTTCGGACCTGTCTTATGGCGCCT 660
                ************************************************************

OPB11654        TCGGACCAGCTGACTATTCAACCTATTACAAAGGTTAAGCTTGATAAAGAAAAAGGACGG 648
OPB11726        TCGGACCAGCTGACTATTCAACCTATTACAAAGGTTAAGCTTGATAAAGAAAAAGGACGG 720
                ************************************************************

OPB11654        GTCTAC 654
OPB11726        GTCTAC 726
                ******
```

Figure 3.2 Sequence alignment between $OPB11_{654}$ and $OPB11_{726}$ found in gynoecious and hermaphrodite cucumber plants, respectively, using OPB11 primer. Highlighted sequences are additional insert in the hermaphrodite cucumber plants; underlined sequences are the original OPB11 primers and framed sequences are the newly designed $OPB11_{726}$ primers.

From the sequence information primers were designed and PCR performed using additional DNA from F_2 (gynoecious-WrD x hermaphrodite-WrD) cucumber plants. $OPA08_{677}$ primers (Fig. 3.3) and $OPB11_{726}$ primers (Fig. 3.2) were used. By using $OPA08_{677}$ specific primers 482bp fragment was observed in all plants of the F_2 population and the parental plants (Fig. 3.4). By using $OPB11_{726}$ primers, 453bp strong fragment and 380bp faint fragments were observed in all plants of the F_2 population tested and hermaphrodite cucumber (paternal parent). Whereas, strong fragment of 380bp was observed in gynoecious cucumber (maternal parent) (Fig. 3.5).

GTGACGTAGGTAAGACTACCGGAGGCAAGGTTTTGATTCTAATAAGCTACTATTACAAGAATTAGGATGCGAAGC
CAAGCTACTAAGAAATGAGGGGATACTCCTACTACGGGAGCGCACACCGTAGGCAAGGAATGTCCGTAGGACGGA
CCAGACGCATGCGAAGCGCATTTCATAAGCCAAGCAAGGTTTTCGGGAGTAGTTGTCTTCTCTTCTCTCTTTTAA
TAAGTACTATTACTACAAGAATTAGGATGCGAAGAAGAAGAAAGGAAAGGTGAAGATGATTTACAAGATTATTCA
AAACAAGAGAAGAAGAAGACACTCATATTGACTAATTAAGCTACTTCGGACGGAGCGAAGCGACTCTTATATAAG
ATTCAGAAGAAAGGAAGAAAGAAACTCCAAATAAGAAGAACGAATCTTGCATTGAGCCTACTAGGCCCTTGTGTT
AAGCAAATGATAGGGATAGGGAGAAAAGTCTACCTTTCTCACTGAGCTATTAAACTCAAATTCTTTGAGCCTGCT
CTTCGCAAATTCTTTGCTCCTTTGTTGTTTTCTTATACTACGATAATAGTGGTTTTTTGACTGGTCCGGAGTCCG
GAAGATTCTTATCTGGAGTCCGGAGTCCGCAGTCCGGAGTCCGCAGGGCCAAAGAAAGGTCCAGGGTCCTACGTC
AC

Figure 3.3 DNA sequences of the 677bp RAPD fragment obtained using the primer OPA08, specific to gynoecious cucumber. Framed sequences are the original OPA08 primer and highlighted sequences are the newly designed $OPA08_{677}$ specific primers.

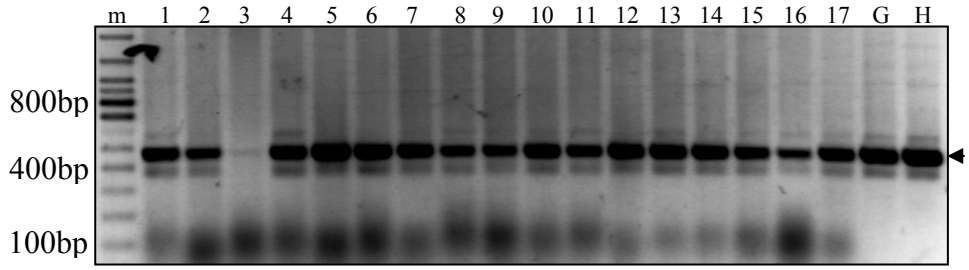

Figure 3.4 PCR using $OPA08_{677}$ specific primers. Lanes 1-13 gynoecious plants from F_2 population, lanes 14-17 hermaphrodite plants from F_2 population. *Arrowhead*= the obtained 482bp fragment, G= gynoecious (maternal parent), H= hermaphrodite (paternal parent); m= size marker

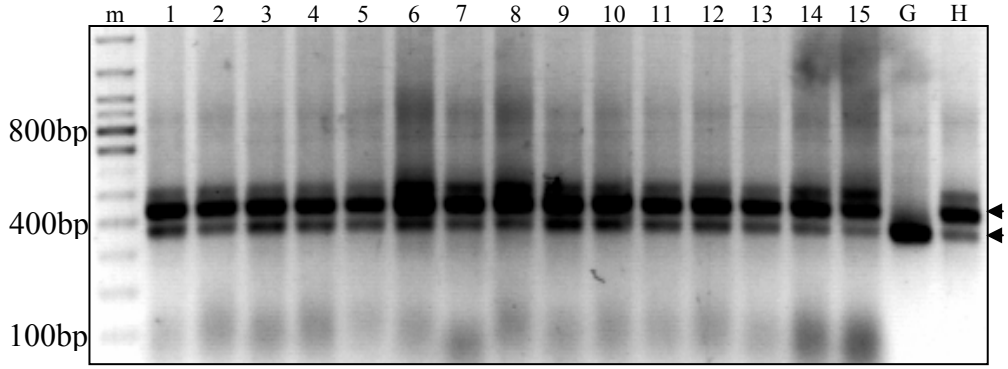

Figure 3.5 PCR using $OPB11_{726}$ specific primers. Lanes 1-11 gynoecious plants of the F_2 population, lanes 12-15 hermaphrodite plants of the F_2 population. G= gynoecious (maternal parent); H= hermaphrodite (paternal parent); m= size marker

3.3.2 Differential screening of the subtracted cDNA library using dot blot analysis

About 178 putative differentially expressed cDNA clones were generated. Out of which, 126 clones were putatively up-regulated in gynoecious and the rest 52 clones in hermaphrodite floral bud. By dot blot screening analysis 10 up-regulated clones from gynoecious and 11 up-regulated clones from hermaphrodite cucumber floral buds were selected (Fig. 3.6, Table 3.2, see also appendix I). Sequence analysis of these cDNA clones indicated some homology to already known genes from the GenBank database (Table 3.2). Some of the clones were found to be identical (Table 3.2).

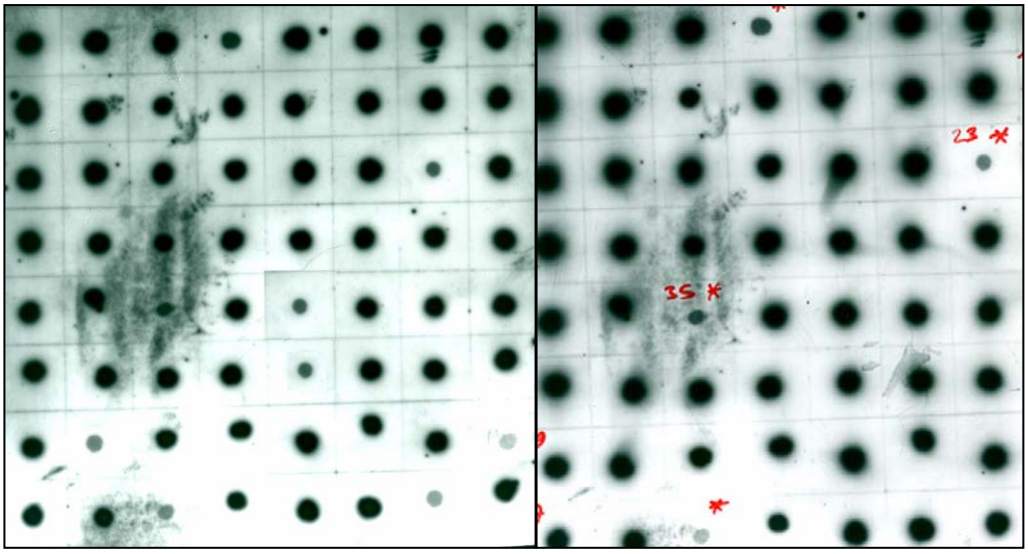

Figure 3.6 Dot blot analysis of subtracted cDNA clones from floral buds of nearly isogenic hermaphrodite cucumber lines after hybridization with driver cDNA from floral buds of nearly isogenic gynoecious cucumber lines

Sequence analysis and database search for cDNA clone, cl-38 (differentially expressed in hermaphrodite floral bud), revealed more than 82 and 90% homology to nucleotide sugar epimerase at nucleotide and amino acid level, respectively. This 416bp cDNA from cl-38 (Fig. 3.7) and homologous to nucleotide sugar epimerase is therefore, hereafter named as cucumber putative nucleotide sugar epimerase[1]

```
G-GATCAGATATACCATTTGGCATGCCCCGCCTCGCCGATCTTCTACAAATACAATCCTGTT
  D  Q  I  Y  H  L  A  C  P  A  S  P  I  F  Y  K  Y  N  P  V
  AAGACAACAAAGACAAATGTCATTGGCACATTGAATATGTTGGGACTTGCCAAGAGAGTT
  K  T  T  K  T  N  V  I  G  T  L  N  M  L  G  L  A  K  R  V
  GGAGCAAGGATTTTGCTTACGTCGACTTCGGAGGTATATGGCGACCCCCTCGTTCATCCT
  G  A  R  I  L  L  T  S  T  S  E  V  Y  G  D  P  L  V  H  P
  CAAGACGAAAGCTACTGGGGAAATGTCAACCCAATCGGTGTTAGAAGTTGCTACGATGAA
  Q  D  E  S  Y  W  G  N  V  N  P  I  G  V  R  S  C  Y  D  E
  GGAAAACGTGTGGCGGAAACATTGATGTTTGATTATCACAGGCAGCATGGGATTGAGATA
  G  K  R  V  A  E  T  L  M  F  D  Y  H  R  Q  H  G  I  E  I
  AGAATTGCTAGGATTTTCAACACATATGGACCGAGAATGAATATTGATGACGGTCGTGTT
  R  I  A  R  I  F  N  T  Y  G  P  R  M  N  I  D  D  G  R  V
  GTTAGCAACTTCCTTGCTCAGGCAATCCGTAGCGAACCATTGACTGTGCAGGCG-C
  V  S  N  F  L  A  Q  A  I  R  S  E  P  L  T  V  Q  A
```

Figure 3.7 cDNA and the corresponding amino acid sequences of the clone cl-38.

[1] The nucleotide sequence for putative nucleotide sugar epimerase of *Cucumis sativus* have been submitted to the GenBank under accession number AY963839

Table 3.2 Up-regulated cDNA clones in flower buds of gynoecious and hermaphrodite cucumber plants with respective size and sequence homology from the GenBank.

Clones	Up-regulated in floral buds	Fragment size	Homology to known sequences from the GenBank (GenBank accession number)	E-value
cl-3	Hermaphrodite	370bp	*Arabidopsis thaliana* sugar transporter family protein (NM_116261)	3E-47
cl-4	Hermaphrodite	390bp	Unknown	
cl-6	Hermaphrodite	276bp	Unknown	
cl-7	Hermaphrodite	353bp	*Betula pendula* glutathione reductase (CAB66332)	9E-32
cl-11	Hermaphrodite	290bp	*Arabidopsis thaliana* putative ubiquitin extension protein (AY059080)	5E-33
cl-12	Hermaphrodite	106bp	Unknown	
cl-21	Hermaphrodite	208bp	*Cucumis sativus* lipoxygenase mRNA (U36339)	2E-10
cl-33	Hermaphrodite	404bp	Unknown	
cl-38	Hermaphrodite	416bp	*Prunus armeniaca* thymidine diphospho-glucose 4-6-dehydratase (U82433)	8E-82
			Arabidopsis thaliana NAD-dependent epimerase/dehydratase family protein (NM_128436)	1E-56
cl-49	Hermaphrodite	192bp	Unknown	
cl-52	Hermaphrodite	589bp	*Cucumis sativus* lipoxygenase mRNA(U36339)	0.0
cl-57[b]	Gynoecious	310bp	Unknown	
cl-66	Gynoecious	127bp	*Arabidopsis thaliana* putative mitochondrial elongation factor (NM_103595)	6E-09
cl-78[c]	Gynoecious	564bp	Unknown	
cl-83[c]	Gynoecious	564bp	Unknown	
cl-90[b]	Gynoecious	313bp	Unknown	
cl-98[b]	Gynoecious	306bp	Unknown	
cl-110	Gynoecious	255bp	*Arabidopsis thaliana* cDNA from flowers and buds (BX819336)	1E-51
cl-115	Gynoecious	422bp	*Arabidopsis thaliana* protein kinase (NM_118607)	6E-21
cl-118[b]	Gynoecious	314bp	Unknown	
cl-128	Gynoecious	456bp	*Arabidopsis thaliana* nucleolar essential protein-related (NP_191259)	6E-20
			Schizosaccharomyces pombe ras-associated protein (T37921)	9E-05

[b]cDNA clones with identical sequences
[c]cDNA clones with identical sequences

3.3.3 Northern blot hybridization and semi-quantitative RT-PCR analyses using the cl-38 and cl-66 cDNA clones

Northern blot hybridization analysis using the cDNA clone, cl-66 yielded weak hybridization signal (Fig. 3.8B) in both gynoecious and hermaphrodite cucumber plants with no difference in the expression level. Semi-quantitative RT-PCR analysis of cl-66 clone resulted in identical expression level in both cucumber plants of the three genetic backgrounds (Fig. 3.8A). In all the cases identical expression level of 121bp fragment was obtained.

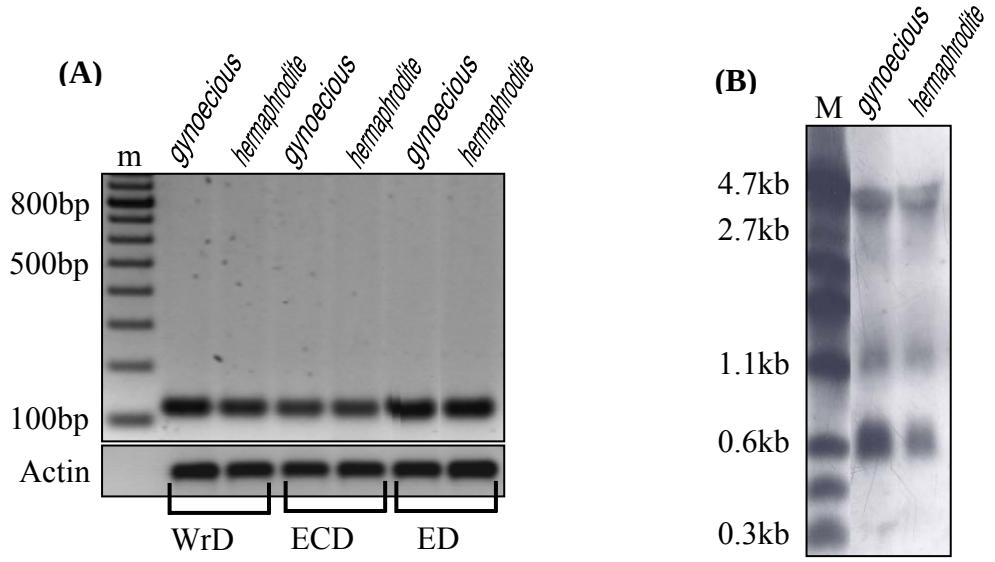

Figure 3.8 Expression analysis of cDNA clone 'cl-66'. (**A**) Semi-quantitative RT-PCR using cl-66 on total RNA obtained from gynoecious and hermaphrodite cucumber plants of the three genetic backgrounds, WrD, ECD and ED. (**B**) Northern blot hybridization analysis of cl-66 clone using 10µg of total RNA from gynoecious and hermaphrodite cucumber plants. m= 100bp ladder, M= RNA molecular weight marker.

When ten microgram total RNA was used to study the level of gene expression by using northern blot hybridization technique, no hybridization signal was detected in all the three genetic backgrounds, indicating that the transcript level of the putative nucleotide sugar epimerase (cl-38) is too weak. The expression level of the selected gene, putative nucleotide sugar epimerase was then analyzed by using semi-quantitative RT-PCR. Primer pair that amplify 315bp was designed from the sequence information and utilized in the semi-

quantitative RT-PCR. The expression level of putative nucleotide sugar epimerase was compared between nearly isogenic gynoecious and hermaphrodite cucumber lines of the three genetic backgrounds, WrD, ECD and ED. Strongly expressed putative nucleotide sugar epimerase was detected in the floral buds of hermaphrodite cucumber, whereas, weak transcript level was detected in gynoecious lines, in all the three genetic backgrounds (Fig. 3.9A). However, identical transcript level of putative nucleotide sugar epimerase was detected in leaves of both gynoecious and hermaphrodite cucumbers (Fig. 3.9B).

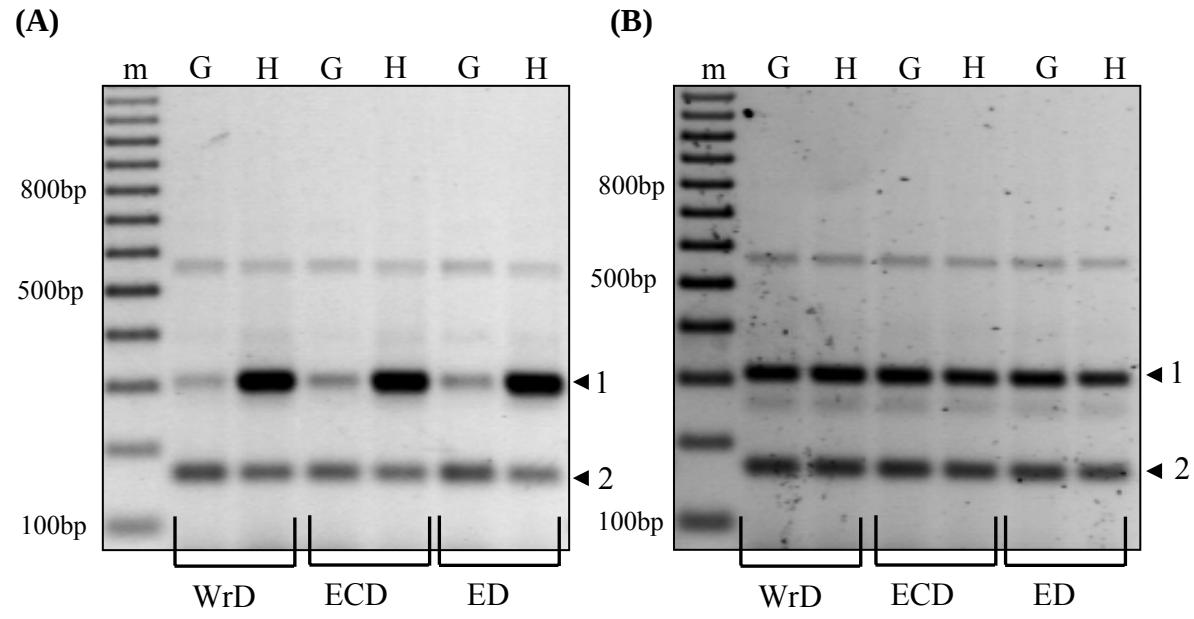

Figure 3.9 Semi-quantitative RT-PCR analysis of putative nucleotide sugar epimerase gene on the floral buds and leaves of gynoecious and hermaphrodite cucumber lines from three genetic backgrounds, WrD, ECD and ED. **(A)** Total RNA obtained from floral buds (1-2mm) of nearly isogenic gynoecious and hermaphrodite cucumber lines. **(B)** Total RNA obtained from leaves of nearly isogenic gynoecious and hermaphrodite cucumber lines. G= gynoecious, H= hermaphrodite, m= 100bp ladder. The *arrowhead* 1 indicates putative nucleotide sugar epimerase (315bp), the *arrowhead* 2 indicates actin (167bp) as internal control

Expression analysis of the putative nucleotide sugar epimerase on female and male floral buds obtained from a single monoecious (*ffMMA*-) cucumber plant showed weak transcript level in

female floral bud and strong expression in male floral bud. This was confirmed in all the three genetic backgrounds (Fig. 3.10A).

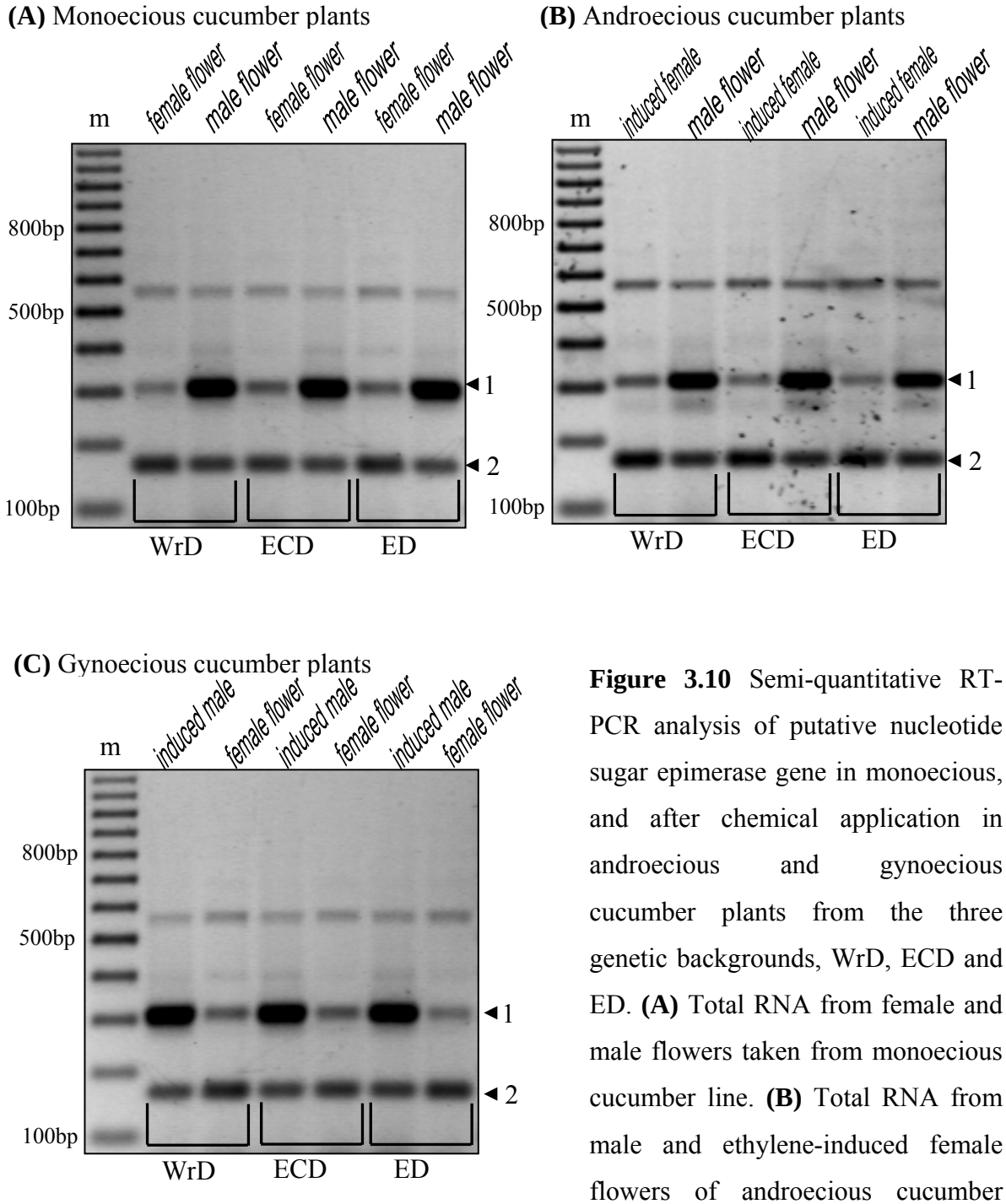

Figure 3.10 Semi-quantitative RT-PCR analysis of putative nucleotide sugar epimerase gene in monoecious, and after chemical application in androecious and gynoecious cucumber plants from the three genetic backgrounds, WrD, ECD and ED. **(A)** Total RNA from female and male flowers taken from monoecious cucumber line. **(B)** Total RNA from male and ethylene-induced female flowers of androecious cucumber **(C)** Total RNA from female and $AgNO_3$-induced male flowers of gynoecious cucumber plants. m= 100bp ladder. The *arrowhead* 1 indicates putative nucleotide sugar epimerase (315bp), the *arrowhead* 2 indicates actin (167bp) as internal control

A comparison was also made between ethephon-induced female flower buds on androecious cucumber and male flower buds of the same androecious cucumber plant for the transcript level of putative nucleotide sugar epimerase. Ethephon-induced female flower buds showed weak expression level of the putative nucleotide sugar epimerase, whereas, male flower buds showed strong expression level (Fig. 3.10B). After ethephon application some male flowers were still available and used as a control. A strong expression level of putative nucleotide sugar epimerase was still detected in the male flowers developed together with ethephon-induced female flowers (Fig. 3.10B). Similarly $AgNO_3$-induced male flower buds on gynoecious cucumber plants were compared with female flower buds of the same plant. The $AgNO_3$-induced male flower buds showed strong expression level of putative nucleotide sugar epimerase (Fig. 3.10C). After $AgNO_3$ application some female flowers were still available and used as a control. A weak expression level of putative nucleotide sugar epimerase was detected in the female flowers developed together with the male flowers formed after $AgNO_3$ application (Fig. 3.10C).

3.3.4 Southern blot hybridization analysis using cl-38, cl-66, cl-78, cl-83 and cl-115 cDNA clones

Southern blot hybridization analysis for cl-66, cl-78, cl-83 and cl-115 cDNA clones showed identical fragments in both cucumber plants. Polymorphisms were not detected between gynoecious and hermaphrodite cucumber plants (Fig. 3.11A-D).

Figure 3.11 (A)

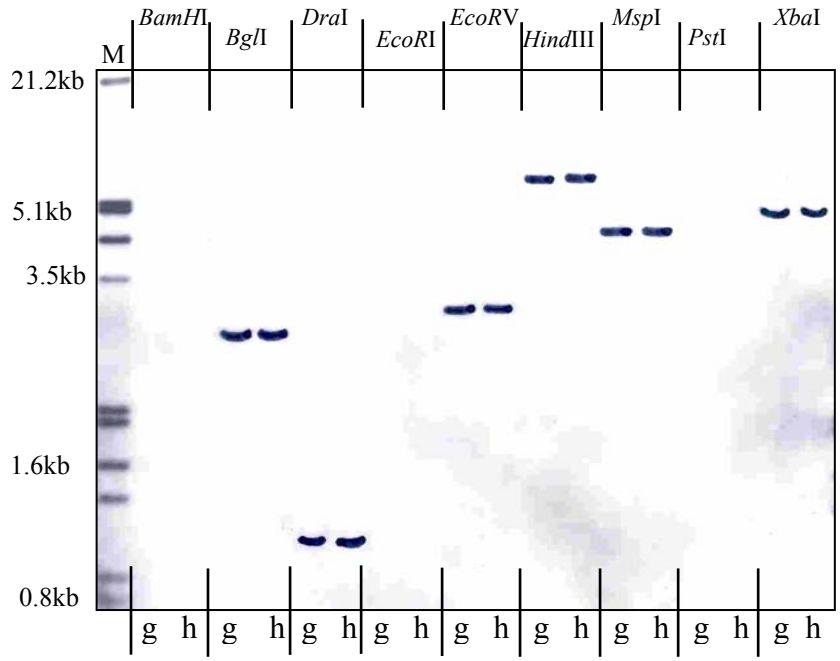

Figure 3.11 (B)

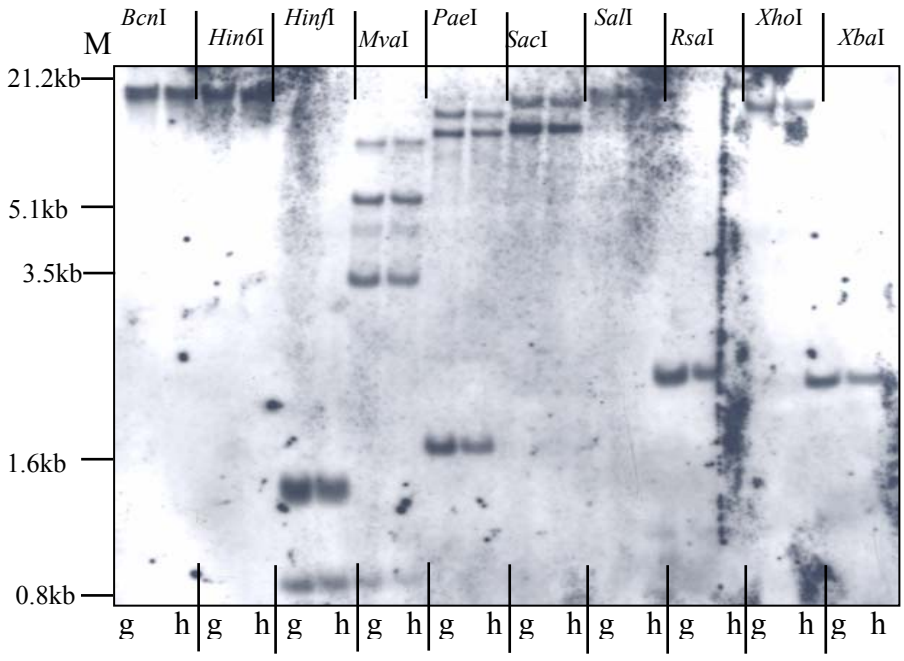

Figure 3.11 (C)

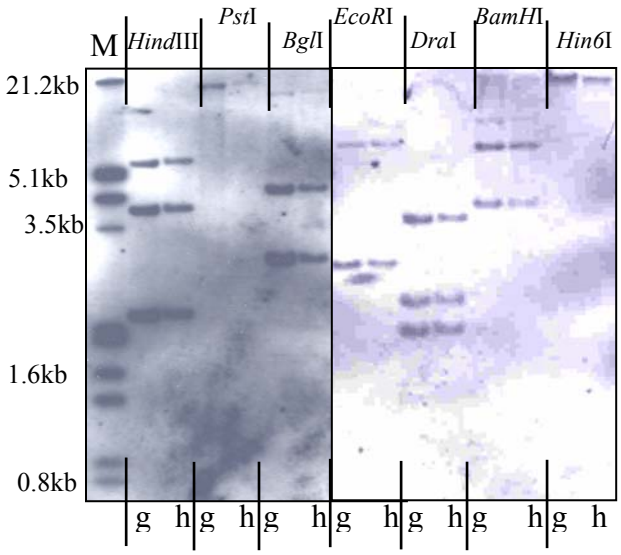

Figure 3.11 (D)

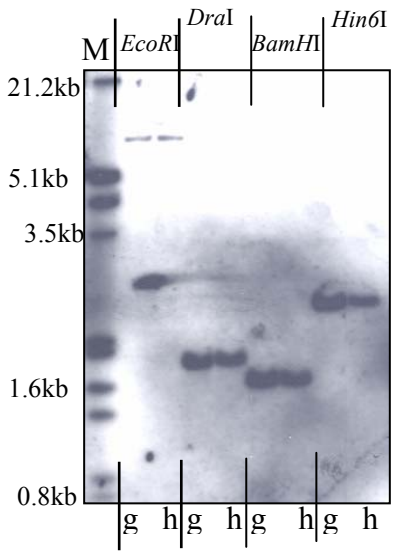

Figure 3.11 Southern blot hybridization analysis of randomly selected cDNA clones. Each lane contains ca. 20µg genomic DNA isolated from nearly isogenic gynoecious and hermaphrodite cucumber plants subjected to digestion with randomly selected restriction enzymes. **(A)** cl-66. **(B)** cl-78. **(C)** cl-83. **(D)** cl-115. g= gynoecious; h= hermaphrodite; M= DNA molecular weight marker III.

No polymorphism was also detected between the nearly isogenic gynoecious and hermaphrodite cucumber lines by using the 416bp cDNA for putative nucleotide sugar epimerase (Fig. 3.12). Some restriction enzymes, for example, *Bgl*II, *Mva*I, *Xho*I, *Dra*I, *Hind*III, *Msp*I, *Bcn*I, *Hinf*I, *Sac*I *and Bfa*I, showed the existence of multiple copies of the putative nucleotide sugar epimerase in both nearly isogenic gynoecious and hermaphrodite cucumber lines (Fig. 3.12).

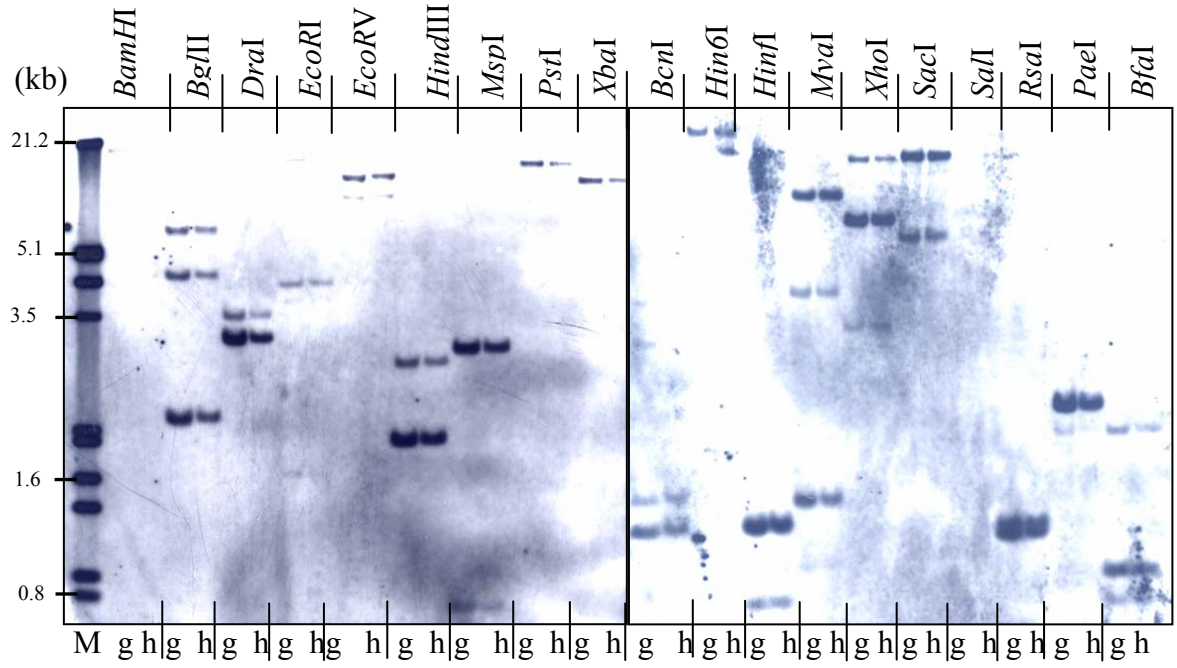

Figure 3.12 Southern blot hybridization analysis of cucumber putative nucleotide sugar epimerase gene. Each lane contains ca. 20µg genomic DNA isolated from nearly isogenic gynoecious and hermaphrodite cucumber lines subjected to digestion with 19 different randomly selected restriction enzymes. M= DNA molecular weight marker III, g = gynoecious, h = hermaphrodite.

3.4 Discussion

3.4.1 Implications of the randomly amplified polymorphic DNA (RAPD) fragments

Randomly amplified polymorphic DNA was conducted between the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants to exploit possible polymorphism which could arise from the *M/m* gene. Because the plant materials are NILs, it is expected that the gynoecious and hermaphrodite lines share 99.8% common genome and their difference is on the sex-determining *M/m* gene. The RAPD analysis revealed 677bp fragment specific to the nearly isogenic gynoecious and missing in the hermaphrodite cucumber (Fig. 3.1A). The other RAPD primer produced two RAPD markers, 654bp specific to the nearly isogenic gynoecious and 726bp specific to hermaphrodite cucumber plants (Fig. 3.1B). Sequence homology search revealed that the sequences of the three fragments are partly or totally homologous to the mitochondrial sequences and have no identity to the nuclear genes. Cucumber mitochondrial genomes are paternally inherited (Havey, 1997) and in the nearly isogenic lines used in these study, the hermaphrodite cucumber was the paternal parent. Under normal paternal transmission, identical mitochondrial genome sequence is expected in both gynoecious and hermaphrodite cucumbers.

After PCR using the $OPA08_{677}$ specific primers, identical 482bp fragment was observed in the F_2 plants and paternal parents (Fig. 3.4). When the primer was designed from the $OPA08_{677}$, the original OPA08 primer sequence of the 5' region was not included in the new primer (Fig. 3.3). So the part of the sequence towards the 5'region was the one missing in hermaphrodite, otherwise the remaining sequences exist in the F_2 plants and both parents. For the RAPD fragments obtained using the primer OPB11, new primers were designed, one from the common 5' region and the other from the region partly missing in the gynoecious cucumber. The new primer produced both fragments in the F_2 plants. But the fragment specific to

gynoecious (found to be missing the additional 72bp) was shown as a very weak amplicon, indicating that it exist as a sublimon (rearranged mtDNA molecules present at a very low levels (Small et al., 1987)). In all the case the obtained fragments were not found to be linked either to the *M/m* gene or to the sex type of cucumber. So the RAPD fragments generated are most probably due to mutational changes spontaneously occurring in the mitochondrial genome as reported by Lilly et al. (2001).

3.4.2 The possible roles of some putatively up-regulated cDNA clones

The cDNA clone, cl-115, putatively up-regulated in gynoecious cucumbers is homologous to protein kinases (Table 3.2). Protein kinases include many growth factor receptors and are important regulators of cellular responses (Sessa et al., 1996). They also play a significant role in signal transduction within the cell. Sessa et al. (1996) identified ethylene-induced cDNA clone, PK12, encoding a protein kinase. The apparent PK12 activity is rapidly increased when plants are treated with ethylene (Sessa et al., 1996). The up-regulated cDNA clone cl-115 in cucumber and homologous to protein kinases may also play some role in the course of cucumber sex expression by interacting with ethylene.

The cDNA clone, cl-128 which is putatively up-regulated in gynoecious cucumber is found homologous to ras-associated protein (Table 3.2). Ras-related protein is a small GTP-binding protein belonging to the ras superfamily that is essential for the translocation of RNA and proteins through the nuclear pore complex (Ren et al., 1993). Ras-related protein is also involved in control of DNA synthesis and of cell cycle progression. Because of its many functions, it is likely that ras-related protein interacts with several other proteins. Ras-related protein also plays a key role in the nuclear delivery of proteins that suppress auxin action and regulate mitotic progress in root tips (Kim et al., 2001). Since auxin promotes female flower

and suppress male flower in cucumber, the cDNA clone homologous to ras-related protein, cl-128, or the ras-related protein itself may play a role in cucumber sex determination.

One of the obtained cDNA clone, cl-66, is homologous to mitochondrial genome sequences. The up-regulation of this cDNAs is most probably because of the paternal inheritance of the mitochondrial genome (Havey, 1997) and the spontaneous mutations that commonly take place within the mitochondrial genome (Lilly et al., 2001) which could create variation within the different cucumber sex types and plants independent of the sex-determining genes.

Most of the putatively up-regulated cDNA clones, for example cl-7, cl-21 and cl-52 are homologous to different types of enzymes. This shows that the floral bud is the most important site where the enzymatic activities are strong. Such strong enzymatic activities could be a factor that plays a role in cucumber sex determination.

The above mentioned information depict that, the isolated cDNA clones in this study have some significant role directly or indirectly in determining cucumber sex expression. Because of time constraints, it was not possible to further study the expression analysis of each of the putatively up-regulated cDNA clones. A single cDNA clone, cl-38 (putative nucleotide sugar epimerase) up-regulated in hermaphrodite floral bud was selected because of its homology to the most important known gene (epimerase) in the GenBank database. The possible function of this cDNA clone is separately discussed in the next section of this chapter (section 3.4.3).

3.4.3 Sex determination and the possible role of putative nucleotide sugar epimerase in cucumber stamen development

After cDNA subtraction on the nearly isogenic gynoecious and hermaphrodite cucumber plants, a cDNA whose transcript level is high in hermaphrodite floral bud and weak in gynoecious one was found (Fig.3.9A). Sequence analysis of this cDNA indicated more than 90% homology on amino acid level to nucleotide sugar epimerase from *Arabidopsis thaliana* and other plant species (*Prunus armeniaca, Cicer arietinum, Pisum sativum, Phragmites*

australis). The interest was to identify cDNAs in cucumber floral buds that might relate to the known sex-determining gene*, M/m*, in cucumber. It was approached by using nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber lines that share more than 99.8% common genome. Initially it was thought that the expression level of the cDNA homologous to nucleotide sugar epimerase was directly related to the sex-determining *M/m* gene. Such assumption came from the fact that the two cucumber lines used were nearly isogenic and expected to be different by the *M/m* gene only. To understand this, a comparison was made for the expression level of putative nucleotide sugar epimerase between male and female floral buds obtained from a single monoecious (*ffMMA-*) cucumber plant. Semi-quantitative RT-PCR detected strong expression level of putative nucleotide sugar epimerase in male floral bud but weak in the female floral bud, both of them obtained from a single monoecious cucumber plant (Fig. 3.10A). This result revealed that the difference in the transcript level of the putative nucleotide sugar epimerase in cucumber is specific to the flower type (male and bisexual floral buds have strong expression level of putative nucleotide sugar epimerase whereas female floral bud have weak transcript level). In addition to the sex-determining, *F/f, M/m* and *A/a* genes, plant hormone ethylene also influences sex expression in cucumber (McMurray and Miller, 1968; Iwahori et al., 1970; reviewed in Malepszy and Niemirowicz-Szczytt, 1991). To understand possible relationship between ethylene response and the expression level of putative nucleotide sugar epimerase, ethephon was applied on androecious (*ffMMaa*) cucumber plants to obtain ethylene-induced female flowers. The transcript level of putative nucleotide sugar epimerase was strong in male flowers and weak in ethylene-induced female flowers. Here ethylene converted the plant destined to bear male flower to female and reduced the expression level of putative nucleotide sugar epimerase in the induced female flower (Fig. 3.10B). $AgNO_3$ was then applied on gynoecious (*FFMMaa*) cucumber plants. The $AgNO_3$-induced male flowers showed strong expression of putative

nucleotide sugar epimerase. Weak transcript level of putative nucleotide sugar epimerase was detected in the female flowers grown together on the same plant (Fig. 3.10C). There was no change (decrease) in the transcript level of putative nucleotide sugar epimerase in the remaining male floral buds after ethephon application on androecious cucumber plants. On the other hand no change (increase) in the transcript level of putative nucleotide sugar epimerase was detected in the remaining female floral buds after $AgNO_3$ application on gynoecious cucumber plants. Both normally grown and exogenous ethylene-induced female floral buds are characterized by weak transcript level of putative nucleotide sugar epimerase than normally grown male or bisexual and $AgNO_3$-induced male floral buds.

Nucleotide sugar epimerase families are involved in catalytic activity of nucleotide-sugar metabolism. For example the enzyme uridine 5'-diphosphate (UDP)-glucose 4-epimerase (UDP-glucose 4-epimerase) is used in the interconversion of UDP-glucose and UDP-galactose. UDP-galactose is needed for the synthesis of arabinogalactan-proteins (AGPs) and cell wall polysaccharides (reviewed by Reiter and Vanzin, 2001). The AGPs are ubiquitous in plants and involved in controlling cell expansion (Willats and Knox, 1996). The loss of UDP-glucose 4-epimerase function affects the morphology of root epidermal cells by interfering with the synthesis of AGPs (reviewed by Reiter and Vanzin, 2001). Mutation in the UDP-glucose 4-epimerase gene of Arabidopsis cause bulging of the root epidermal cells (Schiefelbein and Sommerville, 1990). Some of the UDP-glucose 4-epimerase isoform play important roles in specific cell types. For example the *ROOT HAIR DEFICIENT1 (RHD1*) mutant of *Arabidopsis* carries a defect in the UDP-glucose 4-epimerase gene (reviewed by Reiter and Vanzin, 2001). The weak transcript level of putative nucleotide sugar epimerase in female floral buds of cucumber plants might be the indication for improper functioning of the gene in this specific part of the cucumber plant. Such improper functioning of the putative nucleotide sugar epimerase gene might affect cell expansion. Thus the stage specific arrest in

the development of stamen-primordia in female flowers of cucumber plants could be due to the weak transcript level of putative nucleotide sugar epimerase. In other words, cucumber flowers which are initially bisexual undergo stage specific arrest of preformed stamen-primordia due to weak transcript level (improper functioning) of putative nucleotide sugar epimerase gene. And the proper functioning (strong expression level) of putative nucleotide sugar epimerase in male and bisexual flowers enable the preformed stamen-primordia continue to develop to sexual maturity. Previous studies have indicated that sex determination in cucumber is limited to specific floral whorl (Kater et al., 2001), yet the specific site is unknown. Recently Hao et al. (2003) detected DNA damage in cells of early primordial anther of female flowers. Such DNA damage could be part of the process in stamen arrest possibly caused by inadequate transcription level (improper functioning) of the putative nucleotide sugar epimerase gene in female flower buds as discussed above.

Interestingly, epimerase belongs to the superfamily of "short-chain" dehydrogenases (Baker and Blasco, 1992; Thodon et al., 1997; Holm et al., 1994). The sex determination gene *TASSELSEED2* (*TS2*) of maize, that cause feminization of the tassel, belongs to the family of short-chain alcohol dehydrogenases (DeLong et al., 1993). Unlike the *TS2* of maize the putative nucleotide sugar epimerase isolated here for the first time from cucumber could cause stamen arrest and lead to female flower. Although the anticipated role of the putative nucleotide sugar epimerase in cucumber is opposite to that of *TS2* in maize, it can be understood that gene families of short-chain dehydrogenase are responsible for stage specific arrest of either stamen (the case in cucumber) or pistil (the case in maize) primordia in plant species.

As suggested by Yin and Quinn (1995) if ethylene is indeed the sex hormone and the *F* allele controls the level of ethylene production, speculation can be made for the possible role of *M/m* gene in controlling the expression level of putative nucleotide sugar epimerase in

cucumber plants. In the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber lines, the *F* allele is dominant in both plants and the expected ethylene level due to the *F* allele is identical. The dominant *M* allele may also involve to some extent in ethylene production (the nearly isogenic gynoecious cucumber plants produce slightly more ethylene than hermaphrodite under normal condition- see section 5.3.4) and the *M* allele may additionally control the level of ethylene response in a specific floral part (some what in accordance to the sensitivity level of male receptor as explained by Yin and Quinn (1995)). Thus through its effect on ethylene response the *M* allele or its downstream products could negatively interfere with the expression level of putative nucleotide sugar epimerase in gynoecious floral bud and lead to the arrest of stamen development. In monoecious cucumber plants in which, commonly, first male flowers develop followed by a mixed male and female and at the later stage female flowers, the strength from the influence of the dominant *M* allele or its downstream products may be plant growth-stage specific. The loss of the dominant *F* allele could lead to lower level of ethylene, hence the expression level of putative nucleotide sugar epimerase remain normal and male flowers continue to form from the beginning. As the monoecious plants continue to grow, the strength of the influence from the *M* allele or its downstream products may increase and may slowly start to interfere with the expression level of putative nucleotide sugar epimerase. This could lead to the formation of mixed male and female flowers. At the later developmental stage of the plant, the strength of the influence from the *M* allele or its downstream products may further increase and completely suppress the expression level of putative nucleotide sugar epimerase. This could then lead to the arrest of the stamen development and the formation of female flowers at the later developmental stage of the monoecious plants.

It is difficult to explain the development of hermaphrodite and androecious cucumber sex-types in relation to the expression level of putative nucleotide sugar epimerase. Although,

these two sex-types show different sex expression patterns, the expression level of the putative nucleotide sugar epimerase is strong and similar in the floral buds of these sex-types. For the occurrence of these sex-types, interactions between the putative nucleotide sugar epimerase and the genes *F/f* and /or *A/a* may be responsible. This speculation is very premature and further experiments are necessary to elucidate the relationship between the sex-determining genes and the expression level of the putative nucleotide sugar epimerase in cucumber plants. It is also recommended to further analyze the specific tissue within the floral bud, at which the expression level of the putative nucleotide sugar epimerase gene may get impaired.

4 Molecular genetic investigations on a relationship between responsive to antagonist1 (*RAN1*) and the sex-determining *A/a* gene.

4.1 Introduction

Responsive to antagonist1 (*RAN1*) was identified by a screen for mutations that alter sensitivity to the hormone ethylene. The sequence of *RAN1* revealed that it is homologous to the *Arabidopsis* Ca^{2+}-sensitive cross-complementer2 (*CCC2*) or Menkes' disease (*MNK*) (Hirayama et al., 1999; Himelblau and Amanso, 2000). The defective gene in human Menkes' disease (*MNK*) and its homolog in yeast, *CCC2*, form part of copper trafficking (Payne and Gitlin, 1998; Himelblau and Amanso, 2000). From sequence homology and from the fact that it can rescue *ccc2* mutant yeasts, *RAN1* is considered as an important protein that involves in specific copper delivery pathways (Hirayama et al., 1999; Himelblau and Amanso, 2000). *RAN1* proteins are localized to a post-Golgi compartment where they function to transport copper ions into the secretary pathway, delivering copper to secreted or membrane bound proteins that require the metal for functionality (Yuan et al., 1997; Hirayama et al., 1999; Himelblau and Amanso, 2000)

The *ran1* mutant is altered in ethylene perception (Hirayama et al., 1999; Woeste and Kieber, 2000). The ethylene receptor, *ETR1*, homodimer surrounds a single copper atom that is required for high affinity ethylene binding (Rodriguez et al., 1999). It has been suggested that the ethylene receptors dimerize and bind copper in the post-Golgi system as they move towards the membrane in which they act (Himelblau and Amanso, 2000). *Ran1* mutants have two possible ethylene-related phenotypes. First the absence of copper from the ethylene-binding site could prevent ethylene binding and cause ethylene insensitivity. Second, the absence of copper could prevent the functioning of the signaling domain possibly by inducing a conformational change in the receptors that target them for degradation. (Himelblau and Amanso, 2000; Woeste and Kieber, 2000; Wang et al., 2002).

Plants use copper as a cofactor for a wide variety of physiological processes such as photosynthesis, mitochondrial respiration, superoxide scavenging, cell wall metabolism and ethylene sensing (Märschner, 1995). Since ethylene is considered as a sex hormone in cucumber plants (Yin and Quinn, 1995), the ethylene signaling role of the copper ion and its transporter, *RAN1* gene may interact with the sex-determining genes.

In cucumber sex determination, ethylene interacts with the three major sex-determining, *F/f*, *M/m* and *A/a* genes. The *F/f* gene regulates the endogenous level of ethylene and the *M/m* gene is thought to regulate the ethylene perception and signaling pathways. Initially it was thought that the *A/a* gene also control the level of ethylene or its perception and signaling (Mibus et al., 2000; Vural et al., 2000). The findings in section 2.3.3 indicate that the *A/a* gene have no influence on the expression level of the ethylene receptor and signal transduction-related genes. Furthermore, the findings in section 5.3.3 revealed that both monoecious and androecious cucumber plants produce identical ethylene level and the influence of the *A/a* gene is similar in dominant or recessive condition. To further characterize the *A/a* gene, a new approach was sought by using copper transporter, *RAN1* genes. The assumption was that the *A/a* gene may involve in some sort of *RAN1* regulation and control the copper transport to ethylene receptors, which then govern ethylene response in cucumber plants. Based on this assumption molecular characterization of the sex-determining *A/a* gene was performed using the copper transporter gene (*RAN1*) isolated from cucumber and the possible outcomes are presented in this section of the study.

4.2 Materials and methods

4.2.1 Plant materials

Monoecious cucumber line, Shimshon (*ffMMAA*) and androecious line, Erez (*ffMMaa*) were grown as explained in section 2.2.2, and used in these investigations. Additionally, F_1 and F_2 population of the cross between Shimshon (*ffMMAA*) and Erez (*ffMMaa*) were also used as a source of DNA in the linkage analysis.

4.2.2 RNA and DNA isolation

RNA was isolated from flowers, leaves and roots of monoecious and androecious cucumber plants following the procedures described in section 2.2.4.

Fresh leaves from monoecious and androecious cucumbers as well as their F_1 and F_2 population were harvested and immediately frozen in liquid nitrogen. Genomic DNA isolation was done according to the procedure described in section 2.2.5

4.2.3 Isolation of responsive to antagonist1 (*RAN1*) gene from cucumber

Cucumis sativus putative copper transporter gene was isolated by using sequence information obtained from the database. Sequence information was taken from *Arabidopsis thaliana* ATP dependent copper transporter (*RAN1*) mRNA, accession number AF082565 and *Arabidopsis thaliana* copper-exporting ATPase mRNA, accession number NM_123847. The obtained sequences from GenBank were analyzed by using the CLUSTAL W programme, European Bioinformatics Institute (EMBL; Thompson et al., 1994). Degenerate oligonucleotide primer pair homologous to the most conserved regions of the two sequences was designed by using Primer Premier Program (PREMIER Biosoft International, Palo, Alto, CA). The designed primer pair was synthesized by MWG Biotech AG (sense 5'TATGGAGATGTGGACCCT'3 and antisense 5'TTAACGTAACTTGAACCC'3). The annealing temperature was obtained by

using eight different temperature gradients between 45 and 65°C in a PCR machine (TGradient Block, Biometra). The primer pair was used in RT-PCR on total RNA isolated from cucumber shoot apices. RT-PCR was performed using 40ng of total RNA according to the Qiagen OneStep RT-PCR Kit (Qiagen). The RT-PCR program used was, reverse transcription at 50°C for 30 min, inactivation of reverse transcriptase and activation of DNA polymerase at 95°C for 15 min, followed by 40 PCR cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 2 min, final extension at 72°C for 10 min. The obtained RT-PCR product was separated on flatbed electrophoresis using 1.5% agarose gels. The isolated partial cDNA product was cut from the gel and transferred to 1% low melting agarose gel in 1xTAE buffer using a voltage supply of 3V/cm gel length. The cDNA fragment was further cut from the low melting gel and transferred to pre-weighed 1.5ml eppendorf tube. The newly cut cDNA fragment was weighed and digestion of the low melting gel performed as follows: 25x Agarase buffer (Roche) was added into the cut cDNA in 1.5ml eppendorf tube, melted at 70°C for 3 min and allowed to cool to 45°C, at which the enzyme Agarase (Roche) was added and digestion of the low melting gel for 1h at the 45°C according to the manufacturer's instruction (Roche). The concentration of the purified cDNA was estimated by using λ-DNA (Gibco). The purified cDNA fragment was then cloned using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instruction. Plasmids were recovered using QiaPrep-Mini kit (Qiagen) and sequencing was done by MWG Biotech AG. The isolated cDNA sequences were analyzed by using the CLUSTAL W program, European Bioinformatics Institute (EMBL; Thompson et al., 1994) and the BLUSTN program, National Center for Biotechnology Information (NCBI; Altschul et al. 1997). The amplified cDNA fragment was designated as *cucumus satuvis* responsive to antagonist1 (*CsRAN1*) and used in the subsequent northern and Southern blot hybridization.

Reagents used: see section 2.2.6

4.2.4 Northern blot hybridization analysis of Cs*RAN1* gene

CsRAN1 gene was investigated by northern blot hybridization. Ten microgram total RNA isolated from flowers, leaves and roots of monoecious (Shimshon) and androecious (Erez) cucumber plants were subjected to electrophoresis and transferred to Hybond-N^+ nylon membranes as described in section 2.2.7. DIG-labeling of the *CsRAN1* probe was done by PCR as described in section 2.2.7. Pre-hybridization and main hybridization was carried out according to the instructions for Hybond-N^+ with a DIG-labeled and denatured *CsRAN1* probe. Post-hybridization washes were performed as described in section 2.2.7. Then the washed membrane was exposed to X-ray film for about 1h. The transcript sizes were estimated by comparison with the RNA Molecular Weight Marker I (DIG-labeled, 0.3–6.9 kb; Roche, 100ng).

Reagents used: see section 2.2.7

4.2.5 Southern blot hybridization analysis of Cs*RAN1* gene

Twenty micrograms DNA from monoecious (Shimshon) and androecious (Erez) cucumber plants were digested using 30 U of 30 different restriction enzymes: *Alu*I, *Apa*I, *BamH*I, *Bcl*I, *Bcn*I, *Bfa*I, *Bgl*II, *BsaJ*I, *BseG*I, *Dra*I, *EcoR*I, *EcoR*V, *Hae*III, *Hin6*I, *Hind*III, *HinF*I, *Mbo*I, *Msp*I, *Mva*I, *Nco*I, *Pae*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Sal*I, *Taa*I, *Taq*I, *Xba*I and *Xho*I (MBI Fermentas) in a restriction buffer provided by the manufacturer. Each reaction mixture was kept at the appropriate reaction temperature (most at 37°C) for 15h. The restricted fragments were mixed with 3µl bromophenol blue-loading buffer and fractionated on 0.8% agarose gels in 1xTAE buffer for 16 h (2 V/cm length of the gel). After series of washing steps, the DNA was transferred to Hybond-N^+ nylon membranes as described in section 2.2.9. The probe, *CsRAN1,* was labeled with DIG by using PCR following the procedure described in section 2.2.7. Pre-hybridization, main hybridization and detection was also performed following the same procedure described in section 2.2.9 (for reagents used see section 2.2.7 and 2.2.9).

4.3. Results

4.3.1 Isolation of the *CsRAN1* gene and northern blot hybridization analysis

A 495bp partial sequence of copper transporting gene was isolated. Sequence homology search revealed 81% identity to *Arabidopsis thaliana* ATP dependent copper transporter (*RAN1*) (accession number AF082565) on nucleic acid level and 85% identity to *Arabidopsis thaliana* ATP dependent copper transporter (*RAN1*) (accession number AAC79141) on amino acid level. The isolated cucumber copper transporting gene was labeled as *CsRAN1* gene (Fig. 4.1).

TATGGAGATGTGGACCCTTCCTCATGGATGATTGGTTAAAGTGGGCATTGGTGACTGTTGTG
CAATTTGTCATTGGAAAGCGCTTTTATGTTGCAGCTGCTAGAGCCCTTCGAAATGGTTCAAC
TAACATGGATGTCTTGGTTGCTTTGGGTACTACGGCCTCTTACGTCTATTCTGTTTGTGCAC
TACTCTATGGTGCAGTCACTGGATTCTGGTCTCCGACGTATTTTGAAACAAGTGCTATGTTG
ATAACTTTTGTATTATTGGGAAAGTATTTGGAGTGTCTTGCCAAGGGGAAAACATCAGATGC
CATCAAGAAGTTGGTAGAACTTGCTCCTGCAACTGCTTTACTGCTTATCCGAGATAAAGGTG
GGAATCTTATAGAAGAAAGGGAGATCGATGCTCTGTTAATTCAACCTGGAGATGTGTTGAAG
GTTCTTCCTGGTACAAAAATTCCAGCGGATGGTGTTGTTGTTTGGGGTTCAAGTTACGTAA

Figure 4.1 Nucleotide sequences of the newly isolated *CsRAN1* gene. Framed sequences represent the degenerate primers.

Northern blot hybridization analysis using the *CsRAN1* gene as a probe detected three distinct transcripts, ca. 0.5, 1.7 and 7.0 kb in all parts of the cucumber plants examined (Fig. 4.2). However, there was no difference in the expression levels of *CsRAN1* gene in all parts (flowers, leaves and roots) of both monoecious and androecious cucumber plants.

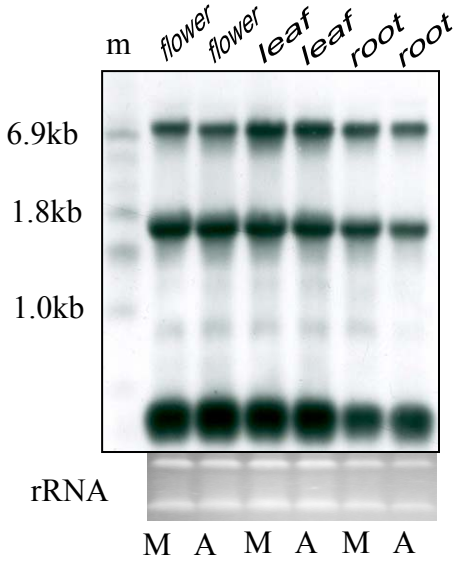

Figure 4.2 Northern blot hybridization analysis of *CsRAN1* (responsive to antagonist 1) on 10µg total RNA from flowers, leaves and roots of monoecious (Shimshon) and androecious (Erez) cucumber plants. M= monoecious (Shimshon), A= androecious (Erez), m= RNA molecular weight marker.

4.3.2 Southern blot hybridization

Southern blot hybridization analyses using copper transporter gene, *CsRAN1* in combination with the restriction enzyme, *Hin*6I yielded polymorphism between monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants. The *CsRAN1/Hin*6I combination produced ca. 15.0kb restriction fragment linked to the *a*-allele in androecious (*ffMMaa*) and ca. 5.0kb fragment linked to the *A*-allele in monoecious (*ffMMAA*) (Fig. 4.3). The F_1 (Shimshon x Erez) cucumber plants showed both restriction fragments linked to *A*- and *a*-alleles. The linkage is confirmed with 11 F_2 cucumber plants (Fig. 4.3).

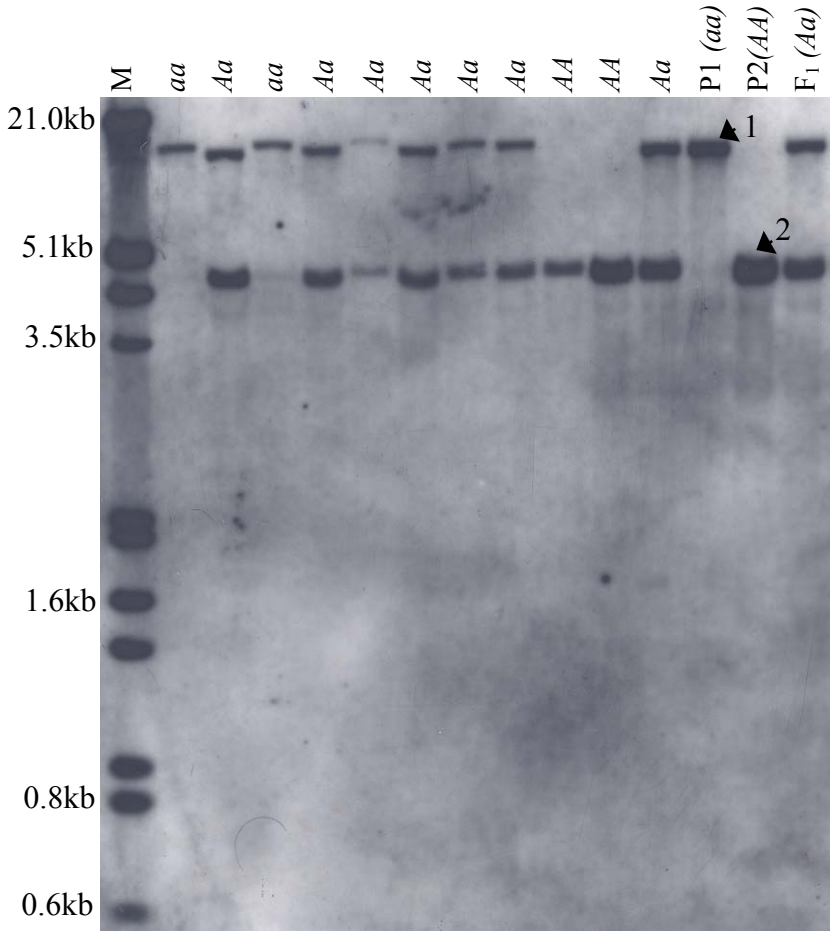

Figure 4.3 Autoradiogram of Southern blot analysis of genomic DNA isolated from monoecious (Shimshon), androecious (Erez), F_1 (Shimshon x Erez) and F_2 cucumber plants. Each lane contains ca. 20µg DNA digested by *Hin6*I and probed with *CsRAN1*. Lanes 2 - 12 (from left to right) shows segregating F_2 population. Lane 13 shows parent 1-androecious (Erez) and lane 14 shows parent 2-monoecious (Shimshon). Lane 15 shows F_1. The *arrowhead* 1 indicates the restriction fragment linked to the recessive *a*-allele in androecious parent and segregates. The *arrowhead* 2 indicates the restriction fragment linked to the dominant *A*-allele in monoecious parent and segregates. M= DNA molecular weight marker

4.4 Discussion

Copper is a co-factor for ethylene receptors (Rodriguez et al., 1999). Positional cloning of responsive to antagonist1 (*RAN1*) revealed that this gene encodes a protein with significant amino acid sequence similarity to the human Menkes/Wilson protein (Hirayama et al., 1999). Human Menkes/Wilson proteins are copper transporting P-type ATPases (Payne and Gitlin, 1998). The high similarity between *RAN1* protein and copper transporter (human Menkes) suggests that *RAN1* functions in plant cells to deliver copper ions into proteins in the secretary pathway (Hiramaya et al., 1999). In this investigation partial sequences of the *CsRAN1* gene was isolated from cucumber and its possible role in cucumber sex expression interacting with the *A/a* gene was studied. Identical expression level of the *CsRAN1* gene was detected in flowers, leaves and roots of androecious (*ffMMaa*) and monoecious (*ffMMAA*) cucumber plants (Fig.4.2). Investigations on *Arabidopsis* revealed that severe alterations in the expression of *RAN1* cause partial or complete activation of ethylene-responsive genes and phenotypes. Furthermore, the phenotypes observed in *RAN1*-cosuppressed plants were indistinguishable from those described for the quadruple ethylene receptor knockout mutants (Hirayama et al., 1999). Since no difference in the expression level of *CsRAN1* gene was detected between the androecious and monoecious cucumber plants, there is identical influence on the ethylene responsive genes in both plants. The possible effect from the *CsRAN1* gene expression level on the sex determination can then be the same in both androecious and monoecious cucumber plants.

RFLP analysis using *CsRAN1* gene as a probe revealed polymorphism between androecious (*ffMMaa*) and monoecious (*ffMMAA*) cucumber plants (Fig.4.3). Preliminary analysis using 11 F_2 (monoecious x androecious) plants of a single population showed complete linkage between the obtained RFLPs and the sex-determining *A/a* gene. Based on this observation it can be speculated that the ca. 5kb fragment is linked to the *A* allele and may be the dominant

A gene itself. On the other hand the ca. 15kb fragment is linked to the *a* allele and may be the recessive *a* gene itself. Because of the fact that the cucumber plant materials used in these studies are not nearly isogenic, the observed polymorphisms may also be due to variation in other plant characteristics rather than the difference due to the *A/a* gene.

The sequences of the *CsRAN1* gene was provided to a partner researcher in Israel and further linkage analysis have been underway.

5 Physiological studies on the sex-determining *M/m* and *A/a* genes in cucumber plants

5.1 Introduction

Cucumber plants exhibit unstable physiological condition with regard to sex expression. Because ethylene is a sex hormone in cucumber (Yin and Quinn, 1995), any factor that alters the endogenous level of ethylene also influences the sex expression of the plant. A variety of chemical compounds can alter sex expression in cucumber through either direct or indirect influence on the level of endogenous ethylene (Veen, 1987; Perl-Treves, 1999). Ethephon treatment increased the number of female flowers in monoecious and andromonoecious cucumber (Robinsen et al., 1969; Rudich et al., 1969). External ethephon application compensate the loss of the function of the dominant *F* allele in these monoecious and andromonoecious cucumber and increased the female flowers. Application of $AgNO_3$ lead to the formation of male and bisexual flowers in gynoecious cucumber plants (Beyer, 1976a; b; Beyer, 1979; Nijs and Visser, 1980). These indicate that by altering the amount of endogenous ethylene it is possible to replace the function of the *F* allele.

It has been postulated that the *M/m* gene regulates the sensitivity level of male receptor to ethylene (Yin and Quinn, 1995). Understanding the stage specific effect of the sex-influencing chemicals or stage specific regulation of the *M/m* and *A/a* genes might help to target isolation of the genes. Recently, Mibus et al. (2000) has studied the influence of different chemical at 2- and 8-leaf stages of nearly isogenic gynoecious and hermaphrodite cucumber and indicated the possible role of the *M/m* gene. Concerning the *A/a* gene, there has been no stage-specific investigation in relation to such external chemical application.

Grafting experiments indicated that endogenous factors that mediate sex expression are diffusible across a graft junction (Friedlander et al., 1977b; Takhashi et al., 1982; Mibus et al.,

2000). Friedlander et al. (1977b) and Mibus et al. (2000) grafted cucumbers of different sex genotypes on each other, and indicated that the *F* allele or the product of the *F* allele is transportable from root to the scion. The passage of such factors may be through xylem transport (Bradford and Yang, 1980). Mibus et al. (2000) also showed that the *M/m* gene or its product is not transportable across a graft connection. However, in the previous grafting studies the role of the *A/a* gene was not investigated.

In Arabidopsis it has been reported that in the course of ethylene perception and signaling a copper co-factor is required to create functional ethylene receptors (Hirayama et al., 1999; Rodriguez et al., 1999). In cucumber, only few physiological studies have been conducted on the role of copper ion (Mibus, 2003). Copper ion may interact with the sex-determining genes in cucumber in its way of creating functional receptors and additional investigations could lead to a further understanding of the sex-determining genes.

In these investigations the stage specific interactions between the sex-determining *A/a* gene and chemicals were studied. Grafting between monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants were performed and the influence of the *A/a* gene is presented. The possible role of exogenous copper application on the amount of ethylene production in the nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) as well as monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber lines was also studied. The results are discussed in connection with the *M* allele in gynoecious and the *m* allele in hermaphrodite as well as the *A* allele in monoecious and the *a* allele in androecious cucumber lines.

5.2 Materials and Methods

5.2.1 Plant materials

Nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants from the genetic background, WrD, were used to study the relationship between the *M/m* gene and ethylene production after chemical application. In order to investigate the sex-determining *A/a* gene by grafting and chemical application, monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants were used. Cucumber seeds were sown on a 35 quick-pot-plate filled with Terreau Professional Gepac Einheitserde Typ T, and germinated at a temperature of 28°C. The resulting seedlings were transferred at about 1.5 leaf-stage to clay pot (15 cm diameter) filled with the same soil type (Terreau Professional Gepac Einheitserde Typ T). Plants were grown at day/night temperatures of 24°/18°C and 16h of assimilation light with day light supplemented by 400W fluorescent lamps (approx. 3500-4000 lux) (Son-T Agro 400, Philips Licht GmbH Hamburg). They were supplied with 2% liquid fertilizer (N:P:K:Mg) on weekly basis alternating between (15:10:15:2) and (8:12:24:4).

5.2.2 Grafting between monoecious and androecious cucumber plants

Cleft grafting, a technique adapted in cucumber cultivation (Lee, 1994) was used for cucumber grafting in this study. Monoecious and androecious cucumber plants at about 2.5 weeks stage bearing 6-7 expanded leaves were used as graft partners. The cucumber plants used as rootstock were cut horizontally just above the second leaf by using a clean sharp blade. A clean vertical cut or a “V” cleft was made to receive the scion. Two true leaves were left on the rootstock which was later removed after the graft union was successful. A scion containing 2-3 leaves was cut off from the top of the cucumber plants and two smooth straight cut were made to form a blunt wedge. The scion was then inserted into the “V” cleft in the end of the rootstock. The graft was then held firm with a plastic clip. The graft unions were

kept under high humidity in a fog-system for 10 days. The older leaves from the rootstock were removed after the graft union was successful (4 days after grafting). Then they were transferred to a growing condition, day/night temperatures 24°C/18°C and 16h of assimilation light with day light supplemented by 400W fluorescent lamps (approx. 3500-4000 lux) (Son-T Agro 400, Philips Licht GmbH Hamburg). They were also supplied with fertilizers as stated in section 5.2.1. Four different graftings were made as follows: monoecious (Shimshon) scion on androecious (Erez) rootstock, monoecious (Shimshon) scion on monoecious (Shimshon) rootstock, androecious (Erez) scion on monoecious (Shimshon) rootstock and androecious scion on androecious (Erez) rootstock (Fig. 5.1). For each grafting a replication of five graft unions were made. In addition to the graft union five plants each from monoecious (Shimshon) and androecious (Erez) cucumber plants were used as a control without grafting. The grafted and non-grafted cucumber plants were allowed to grow and the flower types of each scion were counted on each node until 30 node-stage. The obtained result was analyzed by using χ^2-test.

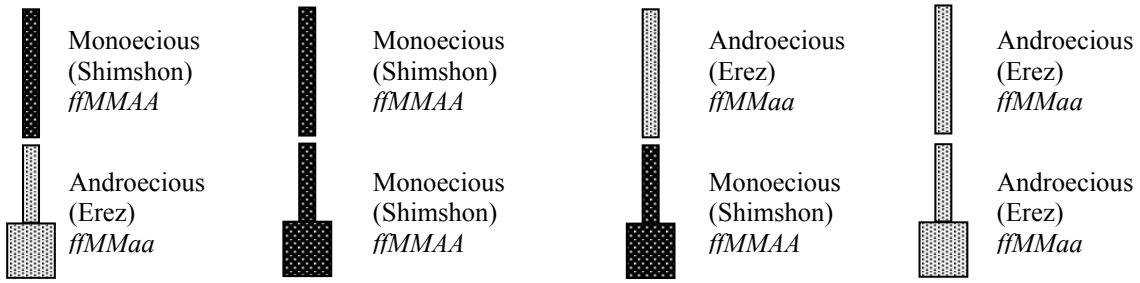

Figure 5.1 Grafting scheme between monoecious (Shimshon) and androecious (Erez) cucumber plants and control graft in which the scion and rootstock originate from the same cucumber sex type.

5.2.3 Chemical application at 2 and 8 leaf-stage and investigation on the sex expression

Chemical application was performed at different growth-stages to understand their stage-specific influence. Monoecious (Shimshon) and androecious (Erez) cucumber seedlings were prepared as explained in section 5.2.1. 15mM $CuSO_4$, 300ppm $AgNO_3$, 20ppm ethephon and

a combination of 15mM $CuSO_4$ and 20ppm ethephon were applied separately on monoecious and androecious cucumber plants at 2 and 8 leaf-stages. The concentration of the chemicals used was according to Mibus et al. (2000). Five monoecious and androecious plants were used in each application and stage of application. For each stage of application five control plants were treated with water from both monoecious and androecious plants. Each of the treatment was mixed with 0.1% (v/v) Tween 20. The chemical applications for each leaf-stage were performed three times. The first application was at the stage of 2 or 8 leaf-stage, the second application was done one week after the first application and the third application was done two weeks after the first application. The plants were allowed to grow under conditions stated in section 5.2.1. The sex expression was investigated and the flower types of each node of the monoecious and androecious cucumber plants were counted until 30 node-stage. The obtained results were analyzed by using χ^2-test

Reagents used:

- 15mM $CuSO_4$ (Merck)
- 300ppm $AgNO_3$ (Merck)
- 20ppm ethephon (2-chloroethylphosphonic acid) (Rhone-Poulenc)
- 0.1% (v/v) Tween 20

5.2.4 Chemical application and ethylene measurement

Gynoecious (*FFMMaa*), hermaphrodite (*FFmmaa*), monoecious (*ffMMAA*) and androecious (*ffMMaa*) cucumber plants were grown to 4 leaf-stage in a single glasshouse cabin. At 4 leaf-stage the plants were separately arranged for three treatments; $CuSO_4$, a combination of $CuSO_4$ and ethephon, and water (control). For each treatment five plants were considered from each gynoecious, hermaphrodite, monoecious and androecious cucumber plants. The plants were separated in three different cabins for the three treatments to avoid contamination. Five plants each from gynoecious, hermaphrodite, monoecious and androecious cucumber plants were treated with 15mM $CuSO_4$ in one cabin, with a combination of 15mM $CuSO_4$ and 20ppm ethephon in the second cabin, and another five plants treated with water (control) in

the third cabin. Each of the treatments was mixed with 0.1% (v/v) Tween 20. Each application was done once per day for three consecutive days starting at 4 leaf-stage. After 12h from the last application the top part of the cucumber plants were cut by razor blade and immediately sealed in a gas-tight 250ml bottle. The gas-tight bottles containing the samples were then incubated at 24°C in a growth chamber. After 12h of incubation, 0.5ml of gas was taken from each bottle using a gas-tight syringe and injected into a Perkin-Elmer portable digital gas chromatograph (GC Voyager FFKG312, Canada) equipped with a photoionization detector. The carrier gas was N_2 at 40 ml min^{-1}, the injection pressure was 69 kPa, the oven temperature was 60°C and the column temperature was 60°C. The gas chromatograph was calibrated with 0.5ml standard ethylene gas before measuring the original samples. After ethylene measurement was completed, the top of the plants were removed from the gas-tight bottles, and weight and volume of the samples were measured. By using the obtained weight and volume of the samples as well as the 12h incubation time in the gas-tight bottles, the ethylene production was calculated in $\mu l.kg^{-1}.h^{-1}$. The volume of the gas was obtained by subtracting the volume of the sample from the volume of the gas-tight bottle. Variance analysis was used for a statistical significance test between the level of ethylene production after chemical applications and among the cucumber lines.

Reagents used:

- 15mM $CuSO_4$
- 20ppm ethephon (2-chloroethylphosphonic acid)
- 0.1% (v/v) Tween 20
- 0.5ml standard ethylene gas

5.3 Results

5.3.1 Effect of grafting on the sex expression pattern in monoecious and androecious cucumber plants

There was no change in the sex expression patterns of androecious cucumber plants grafted on monoecious plants. In all cases only male flowers were developed (Table 5.1) and no female flowers were observed. Even on the side branches all of the flowers observed were only male. Similarly there was no significant difference in the proportion of male to female flowers between the monoecious scion grafted on androecious rootstock, monoecious scion grafted on the same monoecious rootstock and monoecious without grafting at; p=5% (Table 5.2).

Table 5.1 Number of nodes with male or female flower on androecious scion after grafting upon monoecious root stock and controls

Type of grafting	Nr. of nodes with different sex types of flowers (average of five plants)[a]	
	Male	Female
Androecious upon monoecious	30	0
Androecious upon androecious	30	0
Androecious without grafting	30	0

[a]for the value of individual plants see Appendix II-B, C

Table 5.2 Number of nodes with male or female flower on monoecious scion after grafting upon androecious root stock and controls

Type of grafting	Nr. of nodes with different sex types of flowers[b] (average of five plants)[c]	
	Male	Female
Monoecious upon androecious	22.6	7.4
Monoecious upon monoecious	22.6	7.4
Monoecious without grafting	23.8	6.2

[b]χ^2-value for proportion of male:female flower was 0.89 between reciprocal graft, control graft and control without graft, at P=5% non-significant.
[c]for the value of individual plants see Appendix II-A, C

5.3.2 Effect of chemical application at 2 and 8 leaf-stage on the sex-expression

$AgNO_3$ application on monoecious plants at 2 and 8 leaf-stage increased the number of male flowers and decreased the number of female flowers. Male and female as well as male and hermaphrodite flowers together on a single node were also observed after application with $AgNO_3$ at 8 leaf-stage (Tables 5.3 and 5.4). $CuSO_4$ application on monoecious plants at 2 leaf-stage caused the formation of female and male flowers together on a single node and the formation of flowerless node. No change was detected after $CuSO_4$ application on monoecious plants at 8 leaf-stage (Tables 5.3 and 5.4). At both 2 and 8 leaf-stages of application the response from ethephon was identical in monoecious plants. It increased female flowers, decreased male flowers, caused the formation of female and male on a single node, and flowerless node (Tables 5.3 and 5.4). After application with a combination of $CuSO_4$ and ethephon on monoecious plants at 2 leaf-stage, increased number of female flowers and decreased number of male flowers were observed (Tables 5.3). Although no change was detected on the number of male and female flower after application with a combination of $CuSO_4$ and ethephon on monoecious plants at 8 leaf-stage, the application induced flowerless node in both stages (Tables 5.3 and 5.4).

In androecious cucumber plants, application of $AgNO_3$ at both 2 and 8 leaf-stage caused no change to the sex expression pattern (Tables 5.5 and 5.6). Application of $CuSO_4$ on androecious plants at 2 leaf-stage caused the formation female and male flowers on a single node but statistically there is no significant difference. Hence, application of $CuSO_4$ on androecious plants at both 2 and 8 leaf-stages caused no change to the sex expression pattern (Tables 5.5 and 5.6). Application of ethephon on androecious plants at both 2 and 8 leaf-stages increased female flowers, decreased male flowers, caused the formation of female and male as well as male and hermaphrodite flowers on a single node (Tables 5.5 and 5.6). In addition ethephon application on androecious plants at 2 leaf-stage caused the formation of

separate hermaphrodite flowers and flowerless node (Tables 5.5). Application with a combination of $CuSO_4$ and ethephon on androecious plants at 2 leaf-stage also increased female flowers, decreased male flowers, caused the formation of female and male flowers on a single node. It also lead to the formation of flowerless node (Tables 5.5). No influence on the sex expression pattern was detected after $CuSO_4$ and ethephon application on androecious plants at 8 leaf-stage (Tables 5.6)

Table 5.3 The influence of chemical application at 2 leaf-stage on the number of different flower types produced on monoecious cucumber plants.

Treatments (Chemicals)	Nr. of nodes with different sex types of flowers[d] (average of five plants)[e]			
	female	male	female + male	empty node
$AgNO_3$	4.6	25.4	0	0
$CuSO_4$	6.6	21.8	0.6	1
Ethephon	8.4	18.4	1.8	1.4
$CuSO_4$ + Ethephon	9.8	18	0	2.2
H_20	7.8	22.2	0	0

[d]χ^2-value for $AgNO_3$ Vs control 5.20, at P=5% significant; χ^2-value for $CuSO_4$ Vs control 8.52, at P=5% significant; χ^2-value for ethephon Vs control 17.88, at P=1% significant; χ^2-value for $CuSO_4$ + Ethephon Vs control 14.33, at P=1% significant

[e]for the value of individual plants see Appendix III-A

Table 5.4 The influence of chemical application at 8 leaf-stage on the number of different flower types produced on monoecious cucumber plants.

Treatments (Chemicals)	Nr. of nodes with different sex types of flowers[f] (average of five plants)[g]				
	female	male	female + male	male + hermaphrodite	empty node
$AgNO_3$	1.4	25.8	2.2	0.6	0
$CuSO_4$	6.8	23.2	0	0	0
Ethephon	6.8	15.2	6.6	0	1.4
$CuSO_4$ + Ethephon	5.2	22.8	0	0	2
H_20	6.4	23.6	0	0	0

[f]χ^2-value for $AgNO_3$ Vs control 30.5, at P=1% significant; χ^2-value for $CuSO_4$ Vs control 0.07, at P=5% non-significant; χ^2-value for ethephon Vs control 49.1, at P=1% significant; χ^2-value for $CuSO_4$ + Ethephon Vs control 10.68, at P=1% significant

[g]for the value of individual plants see Appendix III-B

Table 5.5 The influence of chemical application at 2 leaf-stage on the number of different flowers produced on androecious cucumber plants.

Treatments (Chemicals)	Nr. of nodes with different sex types of flowers[h] (average of five plants)[i]					
	female	male	hermaph	female + male	Male +hermaph.	Empty node
$AgNO_3$	0	30	0	0	0	0
$CuSO_4$	0	29.8	0	0.2	0	0
Ethephon	1.2	25.2	0.4	1.4	0.8	1
$CuSO_4$ + Ethephon	2	26.8	0	0.4	0	0.8
H_20	0	30	0	0	0	0

[h] χ^2-value for $CuSO_4$ Vs control 1.00, at P=5% non-significant; χ^2-value for ethephon Vs control 25.44, at P=1% significant; χ^2-value for $CuSO_4$ + Ethephon Vs control 20.0, at P=1% significant

[i] for the value of individual plants see Appendix III-A

Table 5.6 The influence of chemical application at 8 leaf-stage on the number of different flowers produced on androecious cucumber plants.

Treatments (Chemicals)	Nr. of nodes with different sex types of flowers[J] (average of five plants)[k]					
	female	male	hermaph	female + male	male + hermaph.	Empty node
$AgNO_3$	0	30	0	0	0	0
$CuSO_4$	0	30	0	0	0	0
Ethephon	2.8	23	0	4	0.2	0
$CuSO_4$ + Ethephon	0	30	0	0	0	0
H_20	0	30	0	0	0	0

[J] χ^2-value for ethephon vs control 27.08, at P=1% significant

[k] for the value of individual plants see Appendix III-B

5.3.3 Influence of *A/a* gene on the level of ethylene production as affected by chemical application

In the control under the application with water 1.38 and 1.33 $\mu l.kg^{-1}.h^{-1}$ ethylene gas was detected in monoecious and androecious plants, respectively. After $CuSO_4$ application the amount of ethylene production was significantly increased to 4.32 and 4.02 $\mu l.kg^{-1}.h^{-1}$ in monoecious and androecious plants, respectively (P=1%). After a combination of $CuSO_4$ and

ethephon application the amount was further significantly increased to 23.23 and 23.55 µl.kg^{-1}.h^{-1} in monoecious and androecious plants, respectively (P=1%) (Table 5.7). However, there was no significant difference between monoecious and androecious cucumber plants in the level of ethylene with application as well as without chemical application (p=5%) (Table 5.7)

Table 5.7 Ethylene production after treatment with $CuSO_4$, a combination of $CuSO_4$ and ethephon, and water in monoecious and androecious cucumber plants.

Cucumber plant	Ethylene production in µl.kg^{-1}.h^{-1}±SE after treatment with chemicals[l] (Average of five plants)[m]		
	Control (water)	$CuSO_4$	$CuSO_4$ + ethephon
Monoecious	1.38±0.19	4.32±0.37	23.23±1.64
Androecious	1.33±0.14	4.02±0.32	23.55±1.57

[l]$CuSO_4$ Vs Control and $CuSO_4$ + ethephon Vs Control, at P= 1% significant for both monoecious and androecious plants; monoecious Vs androecious, at P=5% non-significant under all the three treatments.

[m]for the value of individual plants see Appendix IV

5.3.4 Influence of *M/m* gene on the level of ethylene production as affected by chemical application

In the control treatment, gynoecious and hermaphrodite cucumber plants produced about 3.57 and 2.89µl.kg^{-1}.h^{-1} ethylene, respectively. After $CuSO_4$ application about 4.62µl.kg^{-1}.h^{-1} ethylene in gynoecious and 5.75µl.kg^{-1}.h^{-1} ethylene in hermaphrodite plants were produced. After application with a combination of $CuSO_4$ and ethephon the amount of ethylene production was further increased to about 14.75µl.kg^{-1}.h^{-1} in gynoecious and 25.88µl.kg^{-1}.h^{-1} in hermaphrodite plants (Table 5.8). Under normal condition without chemical application, gynoecious cucumbers produce slightly more ethylene than hermaphrodite, however the difference was not significant (P=5%). Application with $CuSO_4$ and a combination of $CuSO_4$ and ethephon significantly increased the level of ethylene production in both gynoecious and hermaphrodite (P=1%). The level of ethylene production was significantly higher in hermaphrodite than in gynoecious after $CuSO_4$ application (P=5%) and was even further

significantly increased after a combination of $CuSO_4$ and ethephon application (P=1%) (Table 5.8).

Table 5.8 Ethylene production after treatment with $CuSO_4$, a combination of $CuSO_4$ and ethephon in gynoecious and hermaphrodite cucumber plants.

Cucumber plant	Ethylene production in $\mu l.kg^{-1}.h^{-1}$±SE after treatment with chemicals[n] (Average of five plants)[o]		
	Control (water)	$CuSO_4$	$CuSO_4$ + ethephon
Gynoecious	3.572±0.079	4.620±0.395	14.747±0.886
Hermaphrodite	2.890±0.325	5.745±0.485	25.878±1.219

[n]$CuSO_4$ Vs Control and $CuSO_4$ + ethephon Vs Control, at P= 1% significant for both gynoecious and hermaphrodite plants; gynoecious Vs hermaphrodite after $CuSO_4$, at P=5% significant; gynoecious Vs hermaphrodite after $CuSO_4$ + ethephon, at P=1% significant; gynoecious Vs hermaphrodite after water, at P=5% non-significant

[o]for the value of individual plants see Appendix IV

5.4 Discussion

5.4.1 Influence of the *M/m* and *A/a* genes on the level of ethylene production as affected by chemical application.

External application of $CuSO_4$ and ethephon increased the production of ethylene gas in both gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants (Table 5.8). It is clear that ethephon releases ethylene gas upon decomposition and leads to increased level of ethylene gas after its application. The mechanism through which $CuSO_4$ application increases the ethylene level is not clear; however speculations can be made as follows. Different stress conditions stimulate ethylene biosynthesis and promote ethylene formation (Yang and Hoffman, 1984; Barker and Corey, 1988; Abeles et al., 1992; Dolan, 1997; Morgan and Drew, 1997; Mathooko et al., 1998; Knoester et al., 1998; Mathooko et al., 1999; Nakamura, 1999). The increase in the production of ethylene gas after $CuSO_4$ application might be triggered by a possible stress condition from phytotoxicity effect of copper compound. In Arabidopsis it has been reported that Cu (I) create functional ethylene receptors and facilitate the binding of ethylene to the receptors (Hirayama et al., 1999; Rodriguez et al., 1999). The copper ion from the applied $CuSO_4$ may facilitate the binding of endogenous ethylene gas to receptors through creation of functional receptors. Ethylene receptors are negative regulators of ethylene response (Hua and Meyerowitz, 1998). Therefore the Cu (I) induced activation of receptors might lead to a strong ethylene and its receptor binding activity, which may interfere with ethylene and ethylene response balance. Because ethylene biosynthesis can be controlled by autoinhibition in vegetative tissues such as leaves (Philosoph-Hadas et al., 1985) and pelargonium cuttings (Kadner and Druege, 2004), the observed increase in ethylene production after $CuSO_4$ application in the current investigations could be due to autoinhibition to maintain a kind of balance between ethylene and ethylene response.

The increase in the level of ethylene gas after $CuSO_4$ and ethephon treatment is not uniform in the two cucumber sex types. Before the treatments the level of ethylene production is slightly higher in gynoecious (*FFMMaa*) plants (statistically not significant), but after the treatments significantly higher ethylene level is detected in hermaphrodite (*FFmmaa*) than in gynoecious (*FFMMaa*) (Table 5.8). Under normal conditions higher level of ethylene production is correlated with *F* allele in gynoecious than with *f* allele in monoecious plants (Rudich et al., 1972a; Rudich et al., 1972b; Takahashi and Suge, 1982; Trebitsh et al., 1987; Trebitsh et al., 1997; Kamachi et al., 2000; Yamasaki et al., 2000; Yamasaki et al., 2001). In a comparison between monoecious (*ffM-*) and andromonoecious (*ffmm*) cucumber plants, no difference in the level of ethylene is observed (Yamasaki et al., 2001). This shows that unlike the *F/f* gene, the *M/m* gene is not involved in ethylene production. In the gynoecious and hermaphrodite materials investigated herein there is no difference in their sex-determining genotype except for the *M/m* gene. The small variation in the level of ethylene production (not statistically significant) between the two sex types without the treatment might be from a possible interaction between the two dominant loci in gynoecious (*F* and *M*).

After treatment with $CuSO_4$ and a mixture of $CuSO_4$ and ethephon, the level of increase in ethylene production is significantly higher in hermaphrodite (*FFmmaa*) than in gynoecious (*FFMMaa*) (Table 5.8). For instance after $CuSO_4$ application the level of ethylene is increased by 23% and 50% in gynoecious and hermaphrodite cucumber plants, respectively. Under such identical treatments and use of nearly isogenic material, the obtained difference in the level of ethylene production is most likely from the difference in the *M/m* gene. So the *m* allele in hermaphrodite cucumber triggers the formation of ethylene at higher rate than the *M* allele in gynoecious cucumbers under a condition that favors ethylene production. It might also possible that the *M*-allele involves in controlling ethylene level (under conditions promoting ethylene production) to limit at a certain threshold level. In this case, the higher

level of increase in ethylene production after the treatments in hermaphrodite cucumber might be due to the loss of *M* allele to control the ethylene production.

Because for these investigations the top part of cucumber plants are used, if the variation in ethylene level is indeed due to the *M/m* gene, the results support the speculation made in section 2.4. Hence the function of the *M/m* gene may not only be specific to floral whorls but also on the entire parts of the cucumber plants. This speculation supports the results of Yamasaki et al. (2001) in which external ethylene application significantly reduced the hypocotyl elongation of the gynoecious cucumber (*MM*) than the andromonoecious cucumber plants (*mm*). From Yamasaki et al. (2001), it can be understood that the *M/m* gene may control the hypocotyl elongation under the influence of ethylene and its function may not necessarily be limited to flower tissue.

Application with $CuSO_4$ and the combination of $CuSO_4$ and ethephon significantly increased the level of ethylene production in both androecious (*ffMMaa*) and monoecious (*ffMMAA*) cucumber plants (Table 5.7). Interestingly in both androecious and monoecious cucumber plants the level of increase is similar and the amount of ethylene gas produced is identical before application and also after application. There was no significant difference between the two different sex types in the level of ethylene production after the different chemical applications. This shows that there might be no regulatory control from the *A/a* gene on the level of ethylene production after chemical application as speculated above for the *M/m* gene. It also supports the assumption indicated for the *M/m* gene, because both androecious and monoecious cucumber plants contained the dominant *M* gene and the influence from this gene is identical in both plants.

5.4.2 Effect of grafting and chemical application in androecious and monoecious cucumber plants.

Grafting between androecious and monoecious cucumber plants revealed no significant difference in the flower distribution of the scion (Tables 5.1 and 5.2). There is no influence from the monoecious root to the androecious scion and vice versa. Unlike the *F/f* gene (Friedlander et al., 1977b; Mibus et al., 2000), the product of the *A/a* gene is not transportable across the graft junction from root to the scion. Similar result was reported for the *M/m* gene by Engelke et al. (1999) and Mibus et al. (2000), in which grafting between nearly isogenic gynoecious (*FFMMaa*) and hermaphrodite (*FFmmaa*) cucumber plants showed no influence from the product of the *M/m* gene on the sex expression of the scion.

In one hormone hypothesis (Yin and Quinn, 1995), there is no statement about the formation of the androecious plant type and about the role of the gene *A/a*. Within the one hormone model two assumptions were made by Mibus et al. (2000) and Vural et al. (2000) concerning the formation of the androecious plants in connection with the gene *A/a.* First assumption was that gene *A/a* influences the synthesis or the variation of the sensitivity level of the female receptor. If the *A/a* gene is dominant (*ffMMAA*) the female receptor has high sensitivity level and it can be activated at a lower amount of ethylene concentration compared with the case when *A/a* gene is recessive (*ffMMaa*). Second assumption, gene *A/a* influences the amount of ethylene. If the *A/a* gene is dominant (*ffMMAA*) plants can produce more ethylene than androecious genotypes which are homozygous recessive at the *A/a*- locus (*ffMMaa*). The absence of any significant change after grafting between androecious and monoecious (Tables 5.1 and 5.2) and the detection of similar ethylene level in both androecious (*ffMMaa*) and monoecious (*ffMMAA*) cucumber plants (Table 5.7) indicate that the second assumption may not be true. The presence of identical expression levels of the ethylene receptor and signal transduction-related genes in leaves and flowers of both monoecious and androecious

cucumber plants (section 2.3.3) show that the *A/a* gene may not involve in ethylene perception or signaling. The relationship between the ethylene receptor and the female receptor proposed by Yin and Quinn (1995) is not known. If the ethylene receptor and female receptors of the one hormone hypothesis are understood to be the same, then the first assumption that relates the *A/a* gene with the sensitivity level of the female receptors of the one hormone model may not also be true.

The presented results indicate that the effect of chemical application particularly $CuSO_4$ and the combination of $CuSO_4$ and ethephon are plant growth stage specific. Application of $CuSO_4$ at 2 leaf-stage in monoecious plants caused a significant change in sex expression; however, the same application at 8 leaf-stage yielded no significant change in the flower distribution (Tables 5.3 and 5.4). Poor response from the $CuSO_4$ application was observed in androecious cucumber plants at both stages of application. Similar result was reported by Mibus et al. (2000) in which monoecious plants showed better tendency towards femaleness after $CuSO_4$ application and the hermaphrodite cucumbers failed to respond to the $CuSO_4$ application.

6 Summary and Outlook

In these investigations, the action mechanisms of the sex-determining *M/m* and *A/a* genes of cucumber plants were studied in relation to the sex-hormone ethylene. Furthermore, different approaches were implemented to isolate the *M/m* and *A/a* genes. The overall outcomes are summarized as follows:

- The sex-determining *M/m* gene in cucumber plants controls the expression levels of ethylene receptors (*CsETR1*, *CsETR2* and *CsERS*) and ethylene signal transduction-related genes (*CsEIN3* and *CsEIL1*).
- 21 up-regulated cDNA clones have been isolated from gynoecious and hermaphrodite cucumber plants. The clone'cl-38' (up-regulated in hermaphrodite cucumber plants) is found to be homologous to nucleotide sugar epimerase.
- Strong expression level of the putative nucleotide sugar epimerase is detected in male and hermaphrodite cucumber floral buds but not in female one. Such difference in the expression level of the putative nucleotide sugar epimerase most probably determines the growth or arrest of stamen development in cucumber plants. Furthermore, the function of the putative nucleotide sugar epimerase may be controlled by the *M/m* gene or its downstream products.
- Copper transporter gene, responsive to antagonist1 (*CsRAN1*), is isolated in cucumber plants by using sequence information from *Arabidopsis thaliana*.
- Preliminary investigation indicated that the *CsRAN1* gene is linked to the sex-determining *A/a* gene in cucumber plants.

- The sex-determining *A/a* gene has no influence on the expression level of ethylene receptor and signal transduction-related genes in leaves and flowers of cucumber plants.
- Grafting between monoecious and androecious cucumber plants revealed that the products of the *A/a* gene are not transportable across a graft junction.
- Ethylene receptor and signal transduction-related gene expression analyses, grafting and ethylene measurement indicated that the function of the *A/a* gene or its products are independent of ethylene biosynthesis, perception and signal transduction.

To better understand the scientific questions lying behind these studies:

- Definitive isolation of the sex-determining *M/m* and *A/a* genes and further investigations are necessary to clearly show the action of these genes.
- Further analysis of the isolated cDNA clones up-regulated in floral buds of either gynoecious or hermaphrodite cucumber plants could provide additional hint for a better approach in the isolation of the sex-determining genes.
- Isolation of the complete sequences of the putative nucleotide sugar epimerase and further investigations, for instance, by over-expression or gene silencing, could provide additional information on the role of the gene and its relation to the *M/m* gene.
- To clearly elucidate the linkage between the sex-determining *A/a* gene and the copper transporter, *CsRAN1* gene, further investigations are recommended by isolating the complete sequences of the *CsRAN1* gene and using additional F_2 cucumber populations. This possibly allows targeting the isolation of the *A/a* gene.

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8 Appendix

Appendix I: Sequences of the putatively up-regulated cDNAs in gynoecious and hermaphrodite cucumber floral buds (from cl-3 to cl-128)

Putatively up-regulated in hermaphrodite floral buds

Cl-3-370bp

GTTTGATAATCCCAACAAGACTCCAGCATATCGCGGTCCAATGTCTTGGTGATTG
GAGTAGAGTCCGGATTGTGAAAATGCATCAGATCCCTGACTGCATGCCATGCACA
ATACTGCCATAGCAGGTGTTCTAACATGGCTCAGCTGAGTAAGGAAAAATGCTGG
TCCCAGAAATCCAATAGATTGCATGATCTTTCGAACCGTAGTGATGGAAAACCCT
CTGCTCACTAGTGTGTCTGCAATCCATCCACCGATGTTTGCAAAAACAGCCATGG
TCAACCATGGCAGAACACAGAAGAGTCCAGATTCGGTGAGGTTGAACTTCAGAA
CCTGATTATAGTATGTGGGCATCCAAGTCAACAGAATGAAG

Cl-4 -390bp

AGCACTGCAGAAATGATCCGATCTGCTGCGGCAAGAGTCGGTGGATCTCGAGTA
GCAAGTGCTTGTATTGCATTACAAGAGCATTTGAATAACAATAACCTTAATGGTT
CAAAGAAGCTTACAAGAAACTCTCCTGGGAATACTATGTCATACGTGATTCTTTC
CACCACATGCTTCAAGCTGAGAAGACAGTGGAGGTTTAAGAGAGGAGATTAATG
AAGGACGAAGCAAGATTTGGTGATAAATAAAGAGCTTGTTTAGTTAGTTTCATTT
TTTGGATTTGTAATATATATTTTTTAAACAAGCTTGTTTGGACAAACATATATACT
CTACAAGAATACTAATAGAATTGACTTCGAAAAAAAAAAAAAAAAAAAAAAAAA
AAGCTTGT

Cl-6- 276bp

CAAACAATAATACTACTAATCCAACACTAAACTATGGTTCAAACTTTAAAGGAAA
GAGACACACCTATCCTTTTACAGCAATAACTATAAGCTTTTTACTTATTGATCAAT
CACGACCATAATAAAACATTAAAAAAAAAAAAAAAAAAACACTTCCATAATTGA
ACACACACTTCCTCTAGCAATTTGGAGACTTAGAAATGTTGCAACGCTTAGGTAG
CTCCATTGCAAGTTCAGGGTTAATGCCAAAAGAAGAAAGAGCTCCCGAGTTCCGG
T

Continuation of Appendix I

Cl-7- 353bp

ACAGTCGTTTGTGAATACTCATTAACCTTCACAGCACCATGTTTTATCAACTTCGA
CACCAACTGCATTCAAATTTAATCTTTTTGAATTAGGAGCACGACCAGTCGCAAA
CAACACAGCATCTGCCAACAACTCCTCGCCATGATCTGTGATAACTTTTATTCCGT
TCTCTGTTTTTATCAATTCTGTCAAATTTGTTCTTGGGTGCATATTAATTCCTCTGG
CTTCCAGGTTCCTTGCAACCACTGCTCTCATTTCATCATCAAAACCTCTCAGTGGA
AGTTCCCTTCTAAAACATAGGTCAACTTTGGCACCCATACCATTCCAGATCGAAG
CAAATCCAACGGCAATGT

CL-11- 290bp

ACTCATTTCAAAACAAAATATTCCATTACTCGAGAATATTTAAAAGCAAAGAACATAACA
ATTCAAACTCCAAATGAGTATAAAGCAGATGGTCCTAGGCATAAGCAATCCAGAAAATA
AATCAATGCATAAACTTTTGTATAGATTTCCTCTCTCAAAATCAACAGTCTACTTGATTTT
CTTCTTTGGCCTCAACTGGTTGCTGTGTCCACACTTCTTTTTCCTGCAGTTGACAGCGCGA
GGGTGCAGGCGGGCATAGCACTTGCGGCAGATCATCTTGTCCTGATTGT

Cl-12-106bp

AAAAAAAAAAAAAAAAAAAAAACCAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAGCTTGACAAAAATTATATTCCAATATTCAGATTAAAGTTTCGGAGTATAGT

Cl-21- 208bp

ACTGAAGGTCTACAGGTAGAGGAATTCCAAGCAGTATCTCTATTTGAAGGGGCAC
TTTGAGCATGTGGTTGGATTAGTAATATGTCGTTCCTTTATGTTCTATTGTAACTCT
CCTATGTTTAATGTTTGAAAAAAAAAAAAAAAAGCTACTTTCTTTGGCTAGTCAT
GAAATAAAATAAAAGAAAAGAGTGCTTTAGAGTAGCTTTGT

Cl-33-404bp

ACATTGCATGAGTAGTATAGAGAGCTCACTCTCTCTTTTATTTTCCCCCTAAACAA
ATTATTGTTTTCTTCATAGATCTTCTTGACTCTCAGCTTGAAGCCTTCTGATCATTT
ATAAACTTCTAAAATGAAACTACGATAAAGCTTAAGGTTACTACATGTGTTTTTA
TTCCATTGCATTATAAGATTGAGCATACTTTCAAGGATGGCTGGGGAGCACTGGT
GGACGATATGGTTTAGGAAATGGCTTCTTGATGATTGGTTGACGAGGAATTGTCT
TGAAAATGGGCTTCTTGTAGAATGGTATGTGGTCGGGAAGAAACTTGAATTTCTC
TTCTTTGGCTTCTTCTCCATTTTTGGTGATACCAGCTCCTTGAGCAGGCAGAAAGG
CATCTTCATTTTCTT

Continuation of Appendix I

Cl-38-416bp

GGATCAGATATACCATTTGGCATGCCCCGCCTCGCCGATCTTCTACAAATACAAT
CCTGTTAAGACAACAAAGACAAATGTCATTGGCACATTGAATATGTTGGGACTTG
CCAAGAGAGTTGGAGCAAGGATTTTGCTTACGTCGACTTCGGAGGTATATGGCGA
CCCCCTCGTTCATCCTCAAGACGAAAGCTACTGGGGAAATGTCAACCCAATCGGT
GTTAGAAGTTGCTACGATGAAGGAAAACGTGTGGCGGAAACATTGATGTTTGATT
ATCACAGGCAGCATGGGATTGAGATAAGAATTGCTAGGATTTTCAACACATATGG
ACCGAGAATGAATATTGATGACGGTCGTGTTGTTAGCAACTTCCTTGCTCAGGCA
ATCCGTAGCGAACCATTGACTGTGCAGGCGC

Cl-49-192bp

ACGAGAACAGAAGTGTTGCTGCGTAAAGACCATGTGAAGTTTTGTCCTTTGCTAT
TTTTTTAAAATATTTTATTCTGTTTTTATTTTTATGTATTGGATGATGAGACTCTTT
GAAAAAATAAAAATAGAAATTCGAGACCTTAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAA

Cl-52-589

GTGTTTCAGAACTTCTTAGAACCGAAAGTGACCAACGCTTTCTCAAATTTTCACCC
CCACAAGTTGTTAAACACGACAAGTCTGCTTGGCTAACCGACGAAGAATTTGCAA
GAGAAATGCTAGCTGGAGTTAACCCTCTAATCATTCGTGGTCTTGAGGAATTTCC
TCCTAAGAGCAAACTTGACCCAAAATTGTATGGTGATCAACATAGCAAGATTAGT
GAAGAAGACATAAAGTCCGGCTTAGAAGGTCTTACAGTTGCTGAGGCATTGAATC
AGAAGAAACTATACATATTGGATCACCATGATGCATTAATGCCATATCTTAGAAA
AATAAATTCAACAAAAACAAAAGCATATGCCACAAGAACTTTGCTACTTTTGAAA
GATGATGGAACTTTGAAGCCATTGGTTATTGAGTCGAGTCTGCCACACCCTCAAG
GTGATCAGTTTGGTGTAAATAGCAAACAATATTTTCCAGCTGAAGAAGGAGTTCA
AAAGTCAATATGGCAATTGGCTAAGGCTTATGTGGTTGTCAATGATGCTGGTTAC
CATCAACTTATCAGCCATTGGTTGAATACTCATGCAGT

Continuation of Appendix I

Putatively up-regulated in gynoecious floral buds

Cl-57-310bp

ACATCTTGCTGACAAGTAGGATTAACAGGTTGGAGCTAGTCTGGTTCCCTCCCTTC
GGCAACCAACTTCAACCTTCTTCTATATTTCAAAATTTGTAACTCTTGACGGTCAT
TGTTTTCTTGAAAGATCAAAGTTGAAGCTTTTGGTTTCCCTTCTTCAAGTGCTCCA
ATTGTCGTACATGAACATTTGCATTACCCATGTTGATTCATGTTAATAGTTGATGC
ACAAGTTTGAACCGGGTCTCCCTTCAACTGCGAACTAAGATTTGTGGATGAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAGCTTGT

Cl-66-127bp

ACCAAACATGTTATTTAACGGAACATGTGCCGTAATTATAGAGTCGTCTCCATCC
TGGTCATTTCCAACAATAATACCTTTTCGCTTGTTGATGTCACCACCAACAGTGCC
TTGAAATTCTGTAGGT

Cl-78-564bp

GAACATTCTCTCACACGGCTGGATTTGTCAAAGAATCAGTTAACAGGATCCATAC
CAGATAGTTTAGCATCTCGAAATTTGCAGCTTGTGATATTAAATGACAACTTATTG
GAAGGAAGGGTGCCCGAAGAACTTTATTCTATTGGTGTGCATGGTGGAGCCATTG
ACCTATCTAGCAACAAGGGTTTATGTGGTGTGCCTTCCTTGCCAACATGCCCTCTA
TTTTGGGAACATGGGCGGTTGTCTAAAACTGGTAAAATTGCAATAGCTTTATCAT
CCACATTCCTTTTCTGTGCACTGCTGGCAATTATCTACATATGCTGTATCAGAAGA
AGTAGAAACGATTACGATTTCGGCTTGCCTAGTGAATTATTGTCTCTTGCTGCAAA
AAGGAACAGGTATCAGAGGCAGAAATCCTTAATGCTGTTGGAAATGGAGAGTCA
ACATGCCAAGGGATTGTCACCATTCACCCCCCTCAACTGAATTATGCTCCAAAAT
GGAAATAATCCTTGAGAGTTTTGAAACTGCAGCGAACCTTTAGAATGCTAACTTA
AATACGACCGT

Continuation of Appendix I

Cl-83-564bp

GAACATTCTCTCACACGGCTGGATTTGTCAAAGAATCAGTTAACAGGATCCATAC
CAGATAGTTTAGCATCTCGAAATTTGCAGCTTGTGATATTAAATGACAACTTATTG
GAAGGAAGGGTGCCCGAAGAACTTTATTCTATTGGTGTGCATGGTGGAGCCATTG
ACCTATCTAGCAACAAGGGTTTATGTGGTGTGCCTTCCTTGCCAACATGCCCTCTA
TTTTGGGAACATGGGCGGTTGTCTAAAACTGGTAAAATTGCAATAGCTTTATCAT
CCACATTCCTTTTCTGTGCACTGCTGGCAATTATCTACATATGCTGTATCAGAAGA
AGTAGAAACGATTACGATTTCGGCTTGCCTAGTGAATTATTGTCTCTTGCTGCAAA
AAGGAACAGGTATCAGAGGCAGAAATCCTTAATGCTGTTGGAAATGGAGAGTCA
ACATGCCAAGGGATTGTCACCATTCACCCCCCTCAACTGAATTATGCTCCAAAAT
GGAAATAATCCTTGAGAGTTTTGAAACTGCAGCGAACCTTTAGAATGCTAACTTA
AATACGACCGT

Cl-90-313bp

ACATCTTGCTGACAAGTAGGATTAACAGGTTGGAGCTAGTCTGGTTCCCCCCCTT
CGGCAACCAACTTCAACCTTCTTCTATATTTCAAAGTTTGTAACTCTTGACGGTCA
TTGTTTTCTTGAAAGATCAAAGTTGAAGCTTTTGGTTTCCCTTCTTCAAGTGCTCC
AATTGTCGTACATGAACATTTGCATTACCCATGTTGATTCATGTTAATAGTTGATG
CACAAGTTTGAACCGGGTCTCCCTTCAACTGCGAACTAAGATTTGTGGATGAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAGCTTGT

Cl-98-306bp

TTGCTGACAAGTAGGATTAACAGGTTGGAGCTAGTCTGGTTCCCTCCCTTCGGCA
ACCAACTTCAACCTTCATCTATATTTCAAAATTTGTGACTCTTGACGGTCATTGTT
TTCTTGAAAGATCAAAGTTGAAGCTTTTGGTTTCCCTTCTTCAAGTGCTCCAATTG
TCGTACATGAACATTTGCATTACCCATGTTGATTCATGTTAATAGTTGATGCGCAA
GTTTGAACCGGGTCTCCCTTCAACTGCGAACTAAGATTTGTGGATGAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAGCTTGT

Cl-110- 225bp

CTCTTCTGCATCTTCACCAGCACATCCAACTCCGGCGGTGCAACTTCGATTCCATG
GCGCCAAAGAAGGATAAGGCTCCTCCACCGTCTTCCAAGCCTGCCAAATCTGGCG
GAGGAAAGCAGAAGAAGAAGAAGTGGAGTAAGGGAAAGCAAAAGGAGAAGGTC
AACAACATGGTTTTGTTTGATCAAGGAACTTATGACAAGCTCCTTGTTGAAGTTCC
CAAGT

Continuation of Appendix I

Cl-115-422bp

ATTTTCCTTCTGCCCAATTATATTTCTTCTCCTCTCCTTCTCCTTCCCCAAACCCCA
ACGTAGTCTGCTTCTTCAATCCTTCTTCCTTCTGCGAGATCATGGAGATGGAACGC
ATGATCGAGTTTCCTCACACACATCTTGATCGGAGGCCCCGTAAGAGGGCTCGTC
TGGGCTGGGACGTTGTCCCTGAGGCCCCTAAGGCTCAGGTAGGAATATGTTGTGG
ACAAGAGATTGCGAATATTCCAAGCTTTGCATCTACAAGAGCACCATCAGATCAT
TCTTCTAATCCACTATTTGTTAAGGGAGTGGCACGAAATGGTTCTCCCCCCTGGCG
AGAAGATGACAAAGATGGGCATTACATGTTTGCGCTTGGAGAAAATTTAACTTCT
CGCTATAAAATACATAGCAAAATGGGAGAAGGT

Cl-118-314bp

ACATCTTGCTGACAAGTAGGATTAACAGGTTGGAGCTAGTCTGGTTCCCTCCCTTC
GGCAACCAACTTCAACCTTCTTCTATATTTCAAAATTTGTAACTCTTGACGGTCAT
TGTTTTCTTGAAAGATCAAAGTTGAAGCTTTTGGTTTCCCTTCTTCAAGTGCTCCA
ATTGTCGTACATGAACATTTGCATTACCCATGTTGATTCATGTTAATAGTTGATGC
ACAAGTTTGAACCGGGTCTCCCTTCAACTGCGAACTAAGATTTGTGGATGAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAGCTTGT

Cl-128-456bp

ACAATCACTTCTTACAGCTAAAAGTTCTATGCTTTCAATTAAATTTAGAAGAGGT
ATTAATATTTTAATTTAAAAAATGGAATATTGATCTATTATGGTTCCACTAATAAA
CCTAAAAACTCTGCTGGATCATATTTTCTGAAATAATAACTAAACATATGAAACA
AGATAGTTGAGGCACTGATTTTGATGGAGTTGTAAGCAGAGTTCACCCTACATTC
CATTTCTCTGCCAAATCTTTGCAAATATCCGCAATACAGCAACTAGCACTAAGAG
GATATTCAGAAATTGCTAACAGATCATCCGTGTAATCAGTCTCAATTTTCCCATGA
GCCATTGCACCCACCACAAAAACAAGGTCTACATCATCTTTGACAGCATCCAGAT
AATTTCGCACCTTGACCAACTTATCCGAACTATGCGAGAAGCCCATTTTACGACA
ATTGGCAGGTAAGT

Appendix II The effect of grafting monoecious upon androecious and vice versa on the sex expression of the scion.

Appendix II-A Monoecious upon androecious and monoecious on monoecious grafting

Plant number (replication)	Type of grafting and obtained flower types			
	Grafting monoecious on androecious		Grafting monoecious on monoecious	
	Nr. of nodes with male flower	Nr. of nodes with female flower	Nr. of nodes with male flower	Nr. of nodes with female flower
1	22	8	22	8
2	23	7	23	7
3	22	8	23	7
4	23	7	23	7
5	23	7	22	8
Average	**22.6**	**7.4**	**22.6**	**7.4**

Appendix II-B Androecious on monoecious and androecious on androecious grafting

Plant number (replication)	Type of grafting and obtained flower types			
	Grafting androecious on monoecious		Grafting androecious on androecious	
	Nr. of nodes with male flower	Nr. of nodes with female flower	Nr. of nodes with male flower	Nr. of nodes with female flower
1	30	0	30	0
2	30	0	30	0
3	30	0	30	0
4	30	0	30	0
5	30	0	30	0
Average	**30**	**0**	**30**	**0**

Appendix II-C Monoecious and androecious plants without grafting

Plant number (replication)	Control without grafting and obtained flower types			
	Monoecious		Androecious	
	Nr. of nodes with male flower	Nr. of nodes with female flower	Nr. of nodes with male flower	Nr. of nodes with female flower
1	24	6	30	0
2	24	6	30	0
3	24	6	30	0
4	24	6	30	0
5	23	7	30	0
Average	**23.8**	**6.2**	**30**	**0**

Appendix III The influence of chemical application on the sex expression of monoecious and androecious cucumber plants

Appendix III-A The influence of chemical application at 2 leaf-stage

Treatments[b]	Nr. of nodes with different sex types in monoecious plants[a]					Nr. of nodes with different sex types in androecious plants[a]				
	F	M	H	F+M	H+M	F	M	H	F+M	H+M
AgNO3	5	25	0	0	0	0	30	0	0	0
	4	26	0	0	0	0	30	0	0	0
	5	25	0	0	0	0	30	0	0	0
	4	26	0	0	0	0	30	0	0	0
	5	25	0	0	0	0	30	0	0	0
Average	**4.6**	**25.4**	**0**	**0**	**0**	**0**	**30**	**0**	**0**	**0**
$CuSO_4$	7	20	0	2	0	0	29	0	1	0
	7	21	0	1	0	0	30	0	0	0
	6	23	0	0	0	0	30	0	0	0
	7	22	0	0	0	0	30	0	0	0
	6	23	0	0	0	0	30	0	0	0
Average	**6.6**	**21.8**	**0**	**0.6**	**0**	**0**	**29.8**	**0**	**0.2**	**0**
Ethephon	11	17	0	1	0	1	24	1	1	2
	9	19	0	0	0	1	24	0	1	2
	5	21	0	2	0	1	24	0	4	0
	7	19	0	3	0	2	27	1	0	0
	10	16	0	3	0	1	27	0	1	0
Average	**8.4**	**18.4**	**0**	**1.8**	**0**	**1.2**	**25.2**	**0.4**	**1.4**	**0.8**
CuSO4 +	12	17	0	0	0	0	28	0	1	0
Ethephon	9	19	0	0	0	4	24	0	0	0
	8	18	0	0	0	3	26	0	1	0
	11	16	0	0	0	0	29	0	0	0
	9	20	0	0	0	3	27	0	0	0
Average	**9.8**	**18**	**0**	**0**	**0**	**2**	**26.8**	**0**	**0.4**	**0**
Control	7	23	0	0	0	0	30	0	0	0
	8	22	0	0	0	0	30	0	0	0
	7	23	0	0	0	0	30	0	0	0
	8	22	0	0	0	0	30	0	0	0
	9	21	0	0	0	0	30	0	0	0
Average	**7.8**	**22.2**	**0**	**0**	**0**	**0**	**30**	**0**	**0**	**0**

[a]F= female flower, M= male flower, H= hermaphrodite flower, F+M= female and male flowers together on a node, H+M= hermaphrodite and male flowers together on a node

[b]Each of the treatments were used on five plants (replications) as shown in five rows

Appendix III-B The influence of chemical application at 8 leaf-stage

Treatments[d]	Nr. of nodes with different sex types in monoecious plants[c]					Nr. of nodes with different sex types in androecious plants[c]				
	F	M	H	F+M	H+M	F	M	H	F+M	H+M
AgNO3	4	25	0	1	0	0	30	0	0	0
	0	25	0	4	1	0	30	0	0	0
	2	25	0	3	0	0	30	0	0	0
	1	27	0	1	1	0	30	0	0	0
	0	27	0	2	1	0	30	0	0	0
Average	**1.4**	**25.8**	**0**	**2.2**	**0.6**	**0**	**30**	**0**	**0**	**0**
$CuSO_4$	8	22	0	0	0	0	30	0	0	0
	6	24	0	0	0	0	30	0	0	0
	6	24	0	0	0	0	30	0	0	0
	7	23	0	0	0	0	30	0	0	0
	7	23	0	0	0	0	30	0	0	0
Average	**6.8**	**23.2**	**0**	**0**	**0**	**0**	**30**	**0**	**0**	**0**
Ethephon	7	15	0	6	0	2	28	0	0	0
	6	16	0	7	0	1	24	0	5	0
	7	14	0	7	0	0	30	0	0	0
	7	16	0	6	0	3	19	0	7	1
	7	15	0	7	0	8	14	0	8	0
Average	**6.8**	**15.2**	**0**	**6.6**	**0**	**2.8**	**23**	**0**	**4**	**0.2**
CuSO4 +	4	24	0	0	0	0	30	0	0	0
Ethephon	5	23	0	0	0	0	30	0	0	0
	6	22	0	0	0	0	30	0	0	0
	4	24	0	0	0	0	30	0	0	0
	7	21	0	0	0	0	30	0	0	0
Average	**5.2**	**22.8**	**0**	**0**	**0**	**0**	**30**	**0**	**0**	**0**
Control	6	24	0	0	0	0	30	0	0	0
	5	25	0	0	0	0	30	0	0	0
	8	22	0	0	0	0	30	0	0	0
	7	23	0	0	0	0	30	0	0	0
	6	24	0	0	0	0	30	0	0	0
Average	**6.4**	**23.6**	**0**	**0**	**0**	**0**	**30**	**0**	**0**	**0**

[c]F= female flower, M= male flower, H= hermaphrodite flower, F+M= female and male flowers together on a node, H+M= hermaphrodite and male flowers together on a node

[d]Each of the treatments were used on five plants (replications) as shown in five rows

Appendix IV The level of ethylene production in monoecious, androecious, gynoecious and hermaphrodite cucumber plants after chemical application at 4 leaf-stage.

Cucumber plant	Replication	Amount of ethylene produced in $\mu l^{-1}.kg^{-1}.h^{-1}$ after treatment		
		control	$CuSO_4$	$CuSO_4$ + ethephon
Monoecious	1	1.89	4.89	20.58
	2	1.10	3.56	27.26
	3	0.81	5.46	22.64
	4	1.64	3.93	26.67
	5	1.45	3.74	18.98
Average		**1.38**	**4.32**	**23.23**
Androecious	1	1.51	3.64	25.59
	2	1.41	4.88	27.05
	3	0.77	3.09	25.49
	4	1.58	4.54	19.02
	5	1.38	3.97	20.58
Average		**1.33**	**4.02**	**23.55**
Gynoecious	1	3.527	4.470	15.654
	2	3.389	4.215	17.650
	3	3.823	3.467	12.460
	4	3.447	5.201	13.825
	5	3.675	5.749	14.144
Average		**3.57**	**4.62**	**14.75**
Hermaphrodite	1	2.949	5.816	22.052
	2	2.550	5.851	26.077
	3	2.073	4.033	28.653
	4	4.041	5.970	24.436
	5	2.837	7.054	28.171
Average		**2.89**	**5.74**	**25.88**

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Curriculum Vitae

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Education

2002-2005	PhD. in Horticulture, University of Hannover, Germany
2000-2002	MSc. in Horticulture, University of Hannover, Germany (*Graduated with summa cum laude*)
1994-1998	BSc. in Plant Sciences from Awassa College of Agriculture, Ethiopia (*Graduated with very great distinction and winner of gold medal*)
1990-1994	Successfully completed secondary school in Gedo (Shoa), Ethiopia

Additional trainings

1999 Training of trainers on agricultural training process, SNNPR, Ethiopia

2001 Practical training on classical and molecular genetic analyses at BAZ, Institute of Agricultural crops, Groß Lüsewitz, Germany

Employment

1998-2000 Graduate Assistant in the Department of Plant Sciences, Debub University, Ethiopia