Investigation of interspecific genome-plastome incompatibility in *Oenothera* and *Passiflora*

Dissertation zur Erlangung des Doktorgrades der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

vorgelegt von
Jaroslav Mráček
aus Bedihošť, Tschechische Republik

München
2005

1. Gutachter: Prof. Dr. R. G. Herrmann

2. Gutachter: Prof. Dr. G. Heubl

Datum der mündlichen Prüfung: 24.03.2006

CONTENT

ABBREVIATIONS

AA-I	*Oenothera* species with genome AA and plastome I
AA-III	*Oenothera* cybrid with genome AA and plastome III
AB-I	*Oenothera* cybrid with genome AB and plastome I
AFLP	amplified fragment length polymorphism
AP-PCR	arbitrarily primed PCR
APS	ammonium persulfat
BB-III	*Oenothera* species with genome BB and plastome III
CAB	chlorophyll *a/b* binding proteins
CAPS	cleaved amplified polymorphic sequence
cDNA	complementary DNA
CDS	coding sequence
chl	chlorophyll
cpDNA	plastid (chloroplast) DNA
DAF	DNA amplification fingerprinting
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol bis(2-aminoehtylether)-N,N,N',N' tetraacetic acid
EST	expressed sequence tag
LDS	lithium dodecyl sulfate
LHCII	light harvesting complex II
MOPS	4-morpholinopropansulfonacid
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

Ponceau S	3-hydroxy-4-[2-sulfo-4-(sulfophenylazo) phenylazo]-2,7-naphtalene disulfonic acid
PSII	photosystem II
PVP	polyvinyl pyrrolidone
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecylsulfate
SNP	single nucleotide polymorphism
SSR	single sequence repeat marker
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylenesorbitanmonolaurat

1 INTRODUCTION

Development and function of the plant cell depend on the interaction of three genetic compartments, the nuclear genome, plastome and chondriome. The compartmentalized genetic system of the plant cell, as of the eukaryotic cell in general, is the result of endosymbioses. The post-endocytobiotic rearrangements of the genetic potentials of partner cells were accompanied by fundamental changes in expression signals in the entire system at almost all regulatory levels (dePamphilis et al. 1997; Herrmann 1997; Van der Kooij et al. 2000; Herrmann et al. 2003). The genetic mechanisms like lateral gene transfer, gene duplication, genome reduction, gene elimination and mutations in combination with natural selection mainly determined evolution and speciation processes of plants (Darwin and Wallace 1858; Mayr 1988, 1991; Herrmann 1997; Herrmann and Westhoff 2001; Herrmann et al. 2003). The dynamics of evolutional and speciation processes between the three genetic compartments in a cell becomes apparent in interspecific nuclear-plastid cybrids or hybrids. Organelle exchanges, even between closely related species, can greatly disturb the intracellular genetic balance (hybrid bleaching) (Renner 1934; Stubbe 1959, 1989; Kirk and Tilney-Bassett 1978; Medgyesy 1994). Plastome-genome incompatibility was first described from hybrids of *Oenothera* (Renner 1934) and then in hybrids of *Trifolium* (Pandey et al. 1987), *Pelargonium* (Metzlaff et al.1982), *Impatiens* (Arisumi 1985), *Zantedeschia* (Yao et al. 1994) and in cybrids of *Solanaceae* (Kushnir et al. 1991; Babiychuk et al. 1995). Incompatible plants show altered biogenesis of the chlorophyll *a/b* binding proteins (CAB) of the light harvesting complex II (LHCII) (Babiychuk et al. 1995), a lower PSII activity and disorganization of thylakoid membranes (Glick and Sears 1994), plastid retardation of development at an early developmental stage (Yao and Cohen 2000). Also the development of the embryo sac and pollen, can be inhibited (Stubbe 1963). These phenomena are widely observed, but the mechanisms responsible are poorly understood (Yao and Cohen 2000).

In this work, two plant models (*Oenothera* and *Passiflora*) have been used to investigate genome-plastome incompatibility. Modern genetic approaches have been applied to the *Oenothera* system in this thesis, an EST project was started to obtain coding sequences from the nuclear genome. Subsequently, AFLP genotyping and linkage analyses have been applied directly to map genome-plastome incompatibility and translocation events of *Oenothera* chromosome arms. In a third part of the work, first evidence of genome-plastome incompatibility in the genus *Passiflora* will be provided.

1.1 *Oenothera* - an ideal system for studying genome-plastome interactions as well as speciation and evolutionary processes

Oenothera is a model plant for almost one century. The purpose of such long interest was in three basic features: biparental transmissions of plastids, the occurrence of stable complex-heterozygosity and fertility of interspecific hybrids, studied predominantly within the subsection (*Eu) Oenothera* (Stubbe 1959). In addition to naturally occurring combinations, a large number of artificial combinations between genomes and plastomes can be produced by interspecific crosses which show a wide range of incompatibility (Stubbe 1959, 1989; Cleland 1972). The completely sequenced *Oenothera* plastomes (Hupfer et al. 2000, unpublished data), EST sequences and the genetic linkage map (reported in this study) move *Oenothera* forward as a model plant in modern science with the aspect of genome-plastome interaction and their role in plant development and speciation processes.

The genus *Oenothera* is composed of 124 species which are native to the New World and in part naturalized in other continents (Stubbe 1989). The genus is divided into 14 sections. The largest section, *Oenothera*, with approximately 76 species is subdivided into five subsections mainly on the basis of fertility of interspecific hybrids and of relationships between genome and plastome (Stubbe and Raven 1979). The subsections are *Emersonia* (4 species), *Munzia* (45 species), *Nutantigemma* (3 species),

Oenothera (13 species), and *Raimannia* (11 species) (Dietrich 1977; Stubbe and Raven 1979; Dietrich et al. 1985; Dietrich and Wagner 1988).

1.1.1 Genetics and chromosome arrangement of *Oenothera*

Soon after *Oenothera* was introduced into genetics, it was discovered that there were some peculiarities in its genetics behaviour. The laws of Mendelian inheritance seemed not to be applicable to some results observed (summarized in Table 1). In various *Oenothera* species and crosses, the gene set seems to belong to single linkage group. For such observation, sufficient explanation was not found until the chromosomal peculiarities were discovered. In 1922 Cleland published an account of meiosis in *O. franciscana*, a Californian race, in which four of the chromosomes were paired (*O. franciscana* has 2n = 14 as all other *Oenothera* species). The fact that unpaired chromosomes formed a closed circle with a high degree of regularity suggested that other *Oenothera* species, in which the segregation behaviour also seemed to be unusual, could also show a similar pattern. Later studies of other materials showed soon that this suggestion was correct, but it was still necessary to uncover how the pairing of chromosomes had arisen. The explanation was based on reciprocal translocations of chromosome arms (Fig. 1) (Blakeslee and Cleland 1930; Cleland and Blakeslee 1930; Cleland and Blakeslee 1931; Cleland 1972; Harte 1994). As a consequence of these surprising data, chromosome arms and not chromosomes, like in other species, were numbered for formal identification (Cleland 1933; Renner and Cleland 1933). The haploid set was considered to contain 14 individual chromosome arms (or chromosome segments), numbered 1 to 14, which could be combined in different patterns to make seven chromosomes of a haploid plant. The arrangement of these 14 chromosomes arms in a given case is called the chromosomal formula, describing a chromosome complex. When two haploid sets of seven chromosomes are combined, they will arrange in meiosis in bivalents or chromosome circles, as expected from pairing of homologous arms. The figure formed by the chromosomes, as seen in diakinesis and metaphase I, is called the chromosome configuration. Each of these configurations can be made up of different

chromosomes, so the meiotic configuration considering the chromosome formula of the two haploid sets involved is called the chromosome arrangement (Harte 1994).

Table 1 Differences between Mendelian genetics and genetics of *Oenothera* (Harte 1994)

	Mendelian genetics	***Oenothera***
F1:	Uniformity	Multiple hybrid phenotypes
	Reciprocal identity	Differences between hybrids from reciprocal crosses
	Dominance or intermediary phenotype	New phenotypes
F2:	Segregation	Constancy of F1-phenotype
	Between two loci, either free recombination of alleles or linkage and crossing-over	Absolute linkage or variable linkage relations
	Random variability of crossover frequencies	Variable crossover frequencies

From the chromosome configuration and genetic linkage data the new assumption was made that if all chromosomes of paternal and maternal origin alternate in a circle then paternal chromosomes of the circle will be distributed to one pole, all maternal chromosomes to the other in any cell with the regular zigzag arrangement. If a circle of 14 chromosomes is present, all paternal genes will enter one generative cell, all maternal genes another. Only two kinds of germ cells will be formed and these will be genetically identical with gametes that unite to form the plants (Fig. 2) and all genes will be in a single linkage group. If, on the other hand, smaller circles with less chromosomes are present, or more than one circle, one may expect separation of all paternal from all maternal chromosomes within a given circle, but a different circle, or different circles and chromosome pairs will segregate independently. Instead of a single linkage group, there will then be as many groups as there are chromosome groups (Cleland 1972).

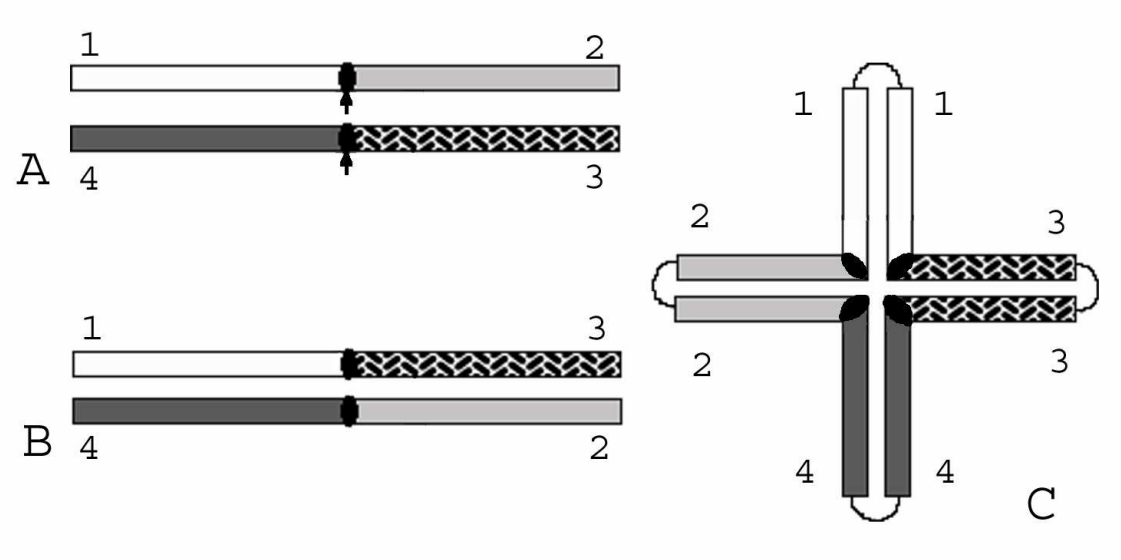

Fig. 1 Schematic presentation of a reciprocal translocation between two chromosomes as an example for the chromosome configuration in meiosis of a heterozygote with one reciprocal translocation involving two chromosomes, combined with two chromosomes with the original arrangement of the same chromosome parts. Movement of the chromosomes to the poles in anaphase will lead to a separation of the chromosome complexes of two chromosomes each in such a way that at one end of the spindle the two translocated and at the other the two original chromosomes will be included in the dyad cell. **A** Structure of the original chromosomes; breakage points for the reciprocal translocations indicated by arrows. **B** Structure of the translocated chromosomes with interstitial segments between the centromere and translocation point. **C** Cross figure in pachytene as a result of pairing of homologous chromosome parts between two translocated chromosomes and two chromosomes with the original arrangement of the chromosome arms (Harte 1994)

Fig. 2 Diagram to show that alternation of paternal and maternal chromosomes in the circle, coupled with disjunction of adjacent chromosomes, result in the formation of only two kinds of gametes, which are identical with those that united to form the plant (Cleland 1972).

However, such complicated chromosomal systems did not explain inhibition of segregation of heterozygous alleles after self-pollination in an offspring. As mentioned above, this finding did not correlate with Mendelian laws. It has been discovered that factors responsible for this irregularity are gametophytic and zygotic lethal factors (Fig. 3) (Renner 1914; Cleland 1972; Harte 1994).

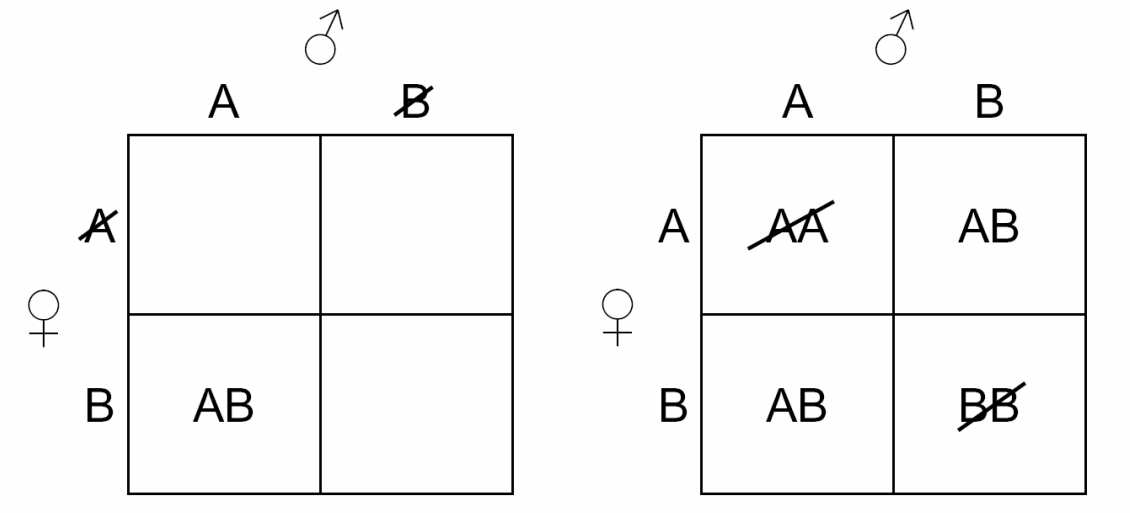

Fig. 3 Diagram to show the effect of balanced lethals. At the left, the B complex is inactivated by a male gametophytic lethal, the A complex is inactive by a female lethal allele. At the right, zygotes that received the same zygotic lethal from both parents fail to develop. Both gametophytic and zygotic lethal factors lead to stable complex-heterozygosity (Cleland 1972).

As was shown before, one reciprocal translocation is necessary to connect four chromosomes in one ring. To have all 14 chromosomes in one circle at least six reciprocal translocations are required. The maximal number of chromosome configurations with 14 chromosomes is 15 and all combinations are presented in Table 2 (Cleland 1972).

The combinatory results of interspecific crosses made it possible to determine chromosomal arrangements and then predict the chromosome configuration in certain hybrid combinations. The diversity in chromosome arrangement that now exists in

populations could be enormous. Cleland (1972) reported 162 different chromosome arrangements in North American races. Additional arrangements have been found in European material derived from North America. The total number of arrangements must be in the thousands. Another indication of diversity that exists is seen when one realizes that each from 91 possible associations of 14 arms have already been found. The frequency of 91 possible chromosomes (Fig. 4) shows a non-uniform distribution of chromosomal segment combinations in the North American material.

Table 2 Possible chromosome configuration in a plant with 2n = 14 (Cleland 1972)

Chromosome configuration
⊙*14
⊙10, ⊙4
⊙8, ⊙6
⊙6, ⊙4, ⊙4
⊙12, 1 pair
⊙8, ⊙4, 1 pair
⊙6, ⊙6, 1 pair
⊙4, ⊙4, ⊙4, 1 pair
⊙10, 2 pairs
⊙6, ⊙4, 2 pairs
⊙8, 3 pairs
⊙4, ⊙4, 3 pairs
⊙6, 4 pairs
⊙4, 5 pairs
7 pairs

*⊙ = chromosomal circle

Modern molecular approaches can be used to uncover the genetic mechanisms in *Oenothera*. The comparison of genetic maps which contain segment-specific, co-dominant markers (SNP, CAPS, microsatellites) allows the investigation of the dynamics and positions of reciprocal translocations. Information provided from this work may lead to the discovery of new molecular mechanisms in plant development and speciation processes.

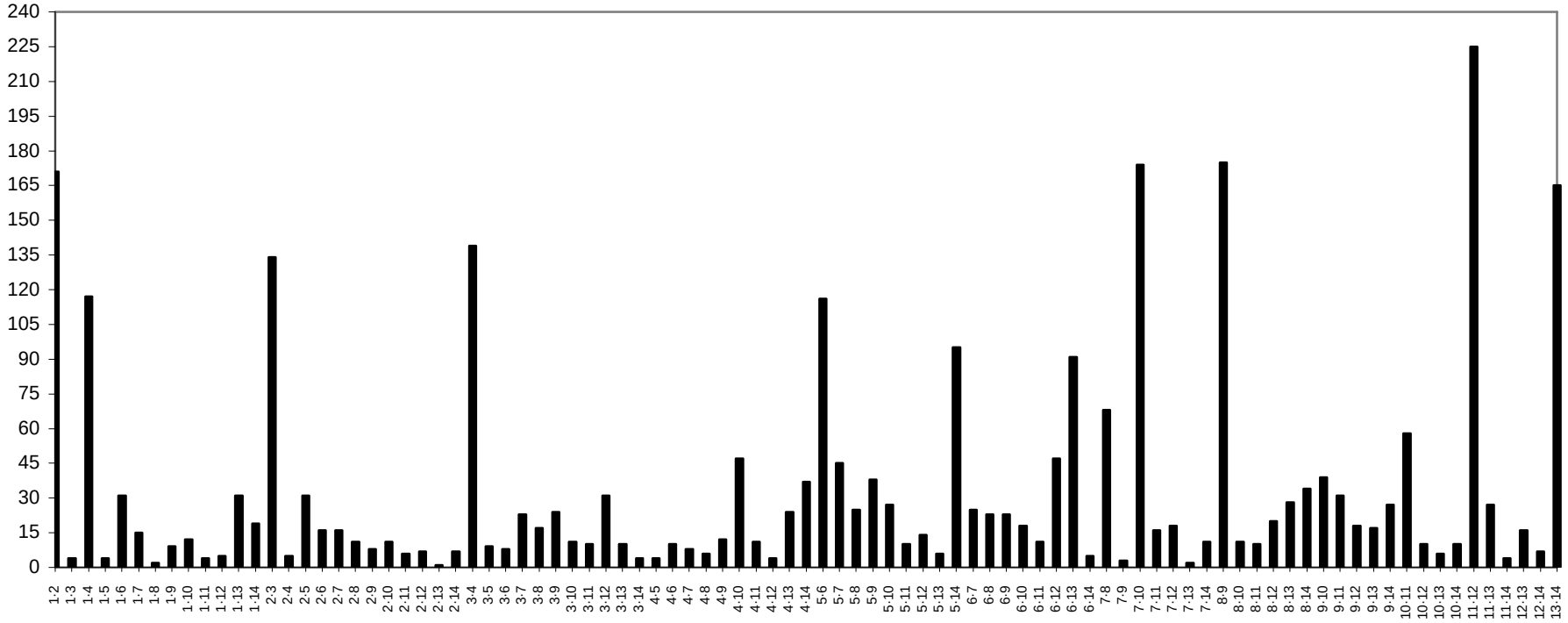

Fig 4 Diagram shows the frequency of 91 possible chromosomes from the standpoint of association of end segments which has been found among the North American materials. Axis X represents different kinds of chromosomes (combinations of chromosome arms), axis Y represents their frequency as they have been found among the North American materials. (Cleland 1972)

1.1.2 Genome-plastome incompatibility in *Oenothera*

The genetic study of Stubbe (1959) on *Oenothera* led him to categorize plastids of the subsection *Oenothera* into five basic, genetically distinguishable plastomes (I, II, III, IV and V) according to their compatibilities with three basic nuclear genomes (A, B and C), which occur in homozygous (AA, BB, CC) as well as in stable (complex) heterozygous (AB, BC, AC) combinations (Fig. 5). The degree of compatibility between the plastome and the genome in *Oenothera* has been defined by the ability of a plastid to become fully pigmented in a given nuclear background. The incompatible genome-plastome combinations result in hybrid bleaching as well as in quantitative differences in chlorophyll and carotenoid content (Schötz and Bathelt 1964). In extreme cases of incompatibility, no viable offspring is produced or the leaves are completely white. Often seedlings can only survive if they are grown on a medium supplemented with sucrose (Kutzelnigg and Stubbe 1974; Tilney-Bassett 1978; Stubbe and Herrmann 1982). Inhibition of plastid reproduction, cell division and gametophytic development are other examples of defects resulting from extremely incompatible genome-plastome combinations (Stubbe 1989). Different combinations of genomes and plastomes have been found in North America or Europe (Table 3).

1.1.3 Incompatible genome-plastome combination AA-III

The AA-III genome-plastome configuration has been described as a periodically pale combination (Stubbe 1959). The incompatible virescent phenotype is observed in plant development between 3 to 11 weeks at the base of leaves. Plastids in green areas on the leaf remained normal in size and internal structure. In contrast, the plastids of virescent regions of the leaf had enlarged, were swollen and had disorganized thylakoids membranes. The internal degeneration of the thylakoids proceeded, resulting in giant amoeboplasts that contain only fragments of thylakoid membranes, membrane vesicles and osmiophilic granules. The chlorophyll content and the chl_a/chl_b ratio is affected in both the green and bleached areas. The PSII activity of chloroplasts from both the green and bleached areas of leaves was significantly lower. After several weeks the wild-type phenotype is completely

restored, the leaves regreened, and the chloroplast ultrastructure becames normal (Glick and Sears 1994). The remaining incompatibilities have not been intensively analysed at the molecular or physiological levels.

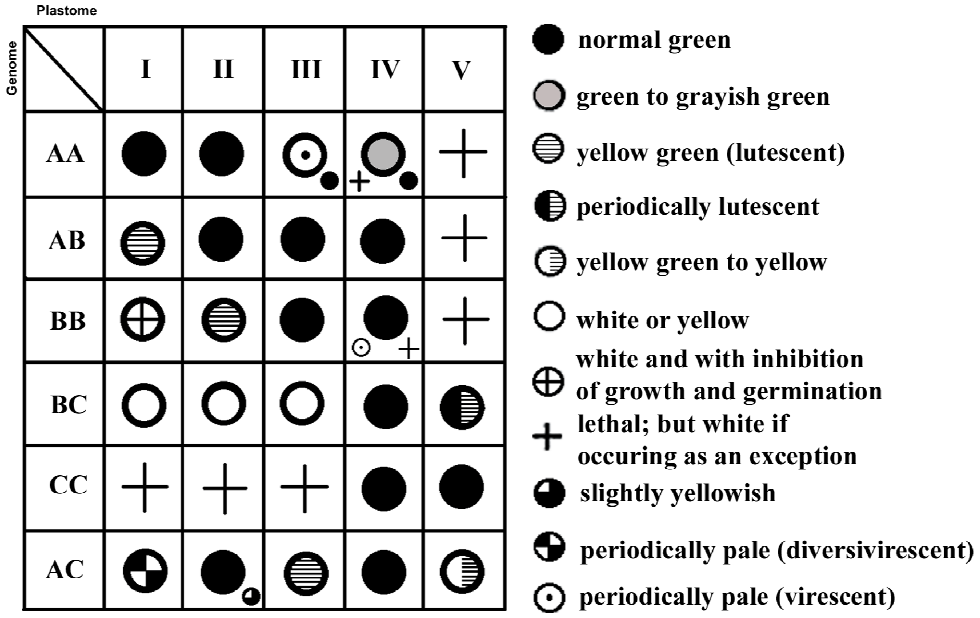

Fig. 5 Compatibility relations between different diploid genotypes and plastids types. More than one symbol in some squares indicates certain differences among the A- and B-complexes (Stubbe 1959)

Table 3 Classes of plastome and genome found in the various groupings of North American members of subgenus *Oenothera*, and in material found in Europe, according to Stubbe (1960, 1963, 1989)

Race or group of races	Class of plastome	Classes of genome	Race or group of races	Class of plastome	Classes of genome
EUROPEAN MATERIAL			NORTH AMERICAN MATERIAL		
lamarckiana	III	AB	*hookeri*	I	A_2A_2
biennis of de Vries	II	AB	*elata*	I	AA
suaveolens	II	AB	*strigosa*	I	A_1A_1
grandiflora of de Vries	II	AB	*biennis I*	III	BA
purpurata	II	AA	*biennis II*	II	AB
ammophila	IV	AC	*biennis III*	III	BB
atrovirens	IV	BC	*grandiflora (true)*	III?	BB
muricata	IV	AC	*parviflora I*	IV	BC
parviflora	IV	BC	*parviflora II*	IV	AC
silesiaca	IV	BC	*argillicola*	V	CC
bauri	I	AA			

1.2 *Passiflora* – a new model plant for genome-plastome incompatibility?

The genus *Passiflora* L. is one of 12 to 18 genera of the family *Passifloraceae* (Killip 1938; Holm-Nielsen et al. 1988) and is numerically and economically the most important genus of this family. It is distributed throughout the tropics and subtropics, the vast majority is endemic to the New World, but occurs also in Southeast Asia, Australia and in Southern Pacific Islands (de Wilde 1972; Killip 1938). The principal industrial economic interest is the fruit production. A majority of the species are also of interest as ornamental plants given the spectacular exotic forms and colours of their flowers. Some others are of pharmaceutical interest due to their sedative, antispasmodic, antibacterial and insecticidal secondary metabolism (Echeverry et al. 1991; Perry et al. 1991).

In molecular biology, *Passiflora* plays a role in several fields. Investigations of plastome variability have shown maternal, paternal and bi-parental transmission of chloroplasts (Do et al. 1992; reported in this study). The self-incompatibility, identification of carotenoids, regulation of ethylene biosynthesis and secondary metabolism of glycosides are other subjects investigated in *Passifloraceae* (Mercadante et al. 1998; Mita et al. 1998; Rêgo et al. 1999; Christensen and Jaroszewski 2001; Suassuna et al. 2003).

1.3 Nuclear genome analyses

1.3.1 Expressed Sequence Tag (EST)

Genome information provides a powerful tool for understanding biological mechanisms and functions and represents an important strategy in molecular biology, medical science, and agriculture (Mita et al. 2003). The analysis of gene expression is entering a new period of rapid advancement enabled by the development of efficient high-throughput technologies. Automated sequencing of cDNAs to generate expressed sequence tags (ESTs) allows the identification of genes and the characterization of expression patterns simultaneously (Adams et al. 1991; Matsubara and Okubo 1993; Fields 1994). ESTs are essential for rapid gene discovery (Adams et

al. 1992; Adams et al. 1993; Medzhitov et al. 1997; Nakamura et al. 1997; Sterky et al. 1998, Liang et al. 2000; Ohlrogge and Benning 2000; Sorek and Safer 2003) and have been used to build genetic and physical maps of the human genome (Hudson et al. 1995; Schuler et al. 1996; Deloukas et al. 1998), to annotate genomic sequences (McCombie et al. 1992; Waterston et al. 1992), to locate exons (Brody et al. 1995, Kan et al. 2001), to compare and contrast genomes of different organisms (Tugendreich et al. 1994), to find new members of gene families (Papadopoulos et al. 1994), to study gene expression on a large scale (Adams et al.1995; Allikmets et al. 1995; Braren et al. 1997) and to reconstruct metabolic pathways (Nelson et al. 2000). Analysis of large EST datasets led to the identification of putative single nucleotide polymorphisms (Buetow et al. 1999; Garg et al. 1999) and to the recognition of the prevalence of alternative splicing in the human genome (Mironov et al. 1999; Brett et al. 2000). The largest public collection of ESTs is dbEST (Boguski et al. 1993), a division of GenBank that currently contains more than 11 million sequences.

1.3.2 DNA Fingerprinting and Amplified Fragment Length Polymorphism (AFLP)

DNA fingerprinting involves the display of a set of DNA fragments from a specific DNA sample. A variety of DNA fingerprinting techniques is presently available (Jeffreys et al. 1985; Nakamura et al. 1987; Tautz 1989; Weber and May 1989; Welsh and McClelland 1990; Williams at al. 1990; Caetano-Anolles at al. 1991; Edwards et al. 1991, Jeffreys et al. 1991; Welsh and McClelland 1990; Beyermann et al. 1992), most of which use PCR for detection of fragments. The choice which of the fingerprinting technique to use is dependent on the application, e.g. DNA typing, DNA marker mapping and the organism under investigation, e.g. prokaryotes, plants, animals, and humans. Ideally, a fingerprinting technique should require no prior investments in terms of sequence analysis, primer synthesis or characterization of DNA probes. A number of fingerprinting methods which meet these requirements have been developed over the past few years, including random amplified polymorphic DNA (RAPD; Williams et al. 1990), DNA amplification fingerprinting (DAF; Caetano-Anolles at al. 1991), arbitrarily primed PCR (AP-PCR; Welsh and McClelland 1990, 1991), single nucleotide polymorphisms (SNPs; Halldorsson et al.

2004) and amplified fragment length polymorphism (AFLP; Vos et al. 1995). These methods are all based on the amplification of random genomic DNA fragments by arbitrarily selected PCR primers. DNA fragment patterns may be generated on any DNA without prior sequence knowledge. A comparison of genotyping methods is provided in Table 4.

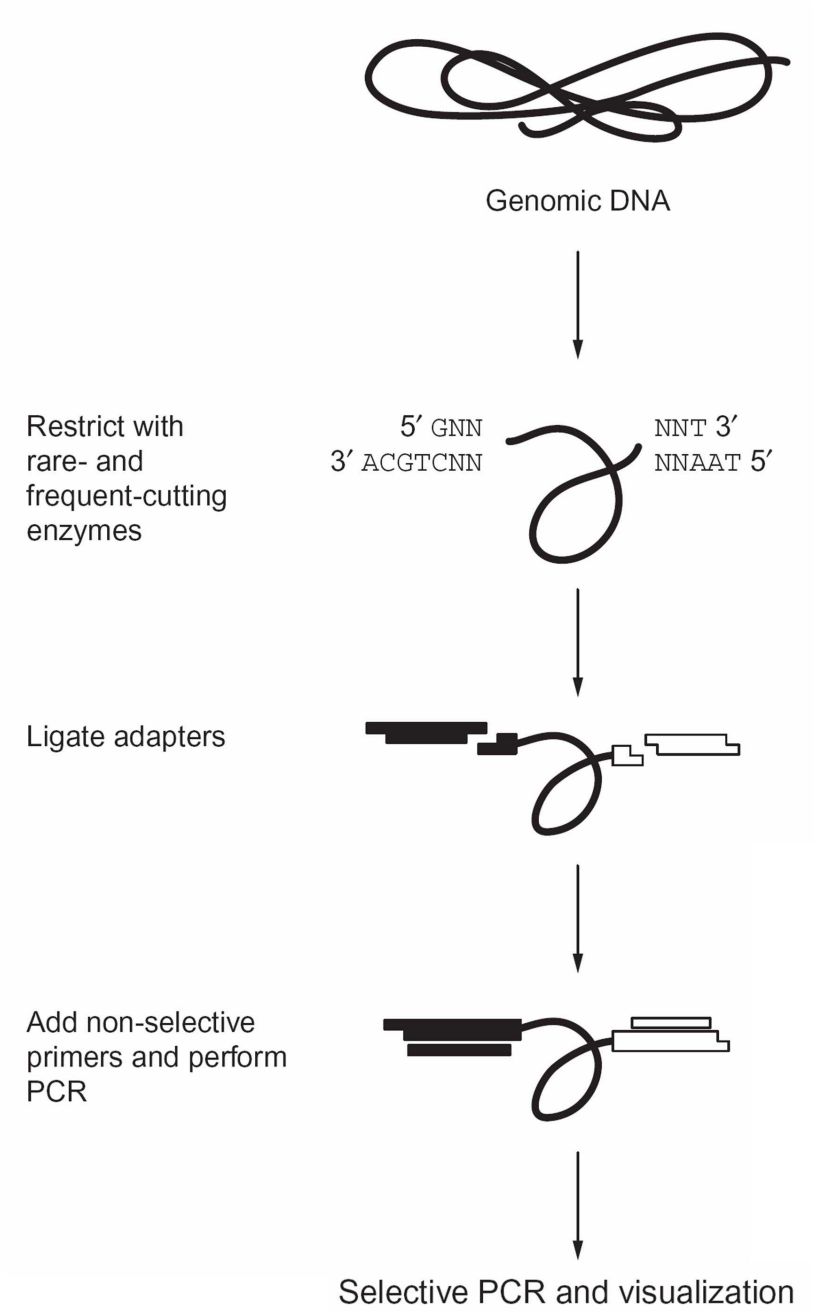

Fig. 6 Overview of AFLP technique (Ridout and Donini 1999)

Table 4 Comparison of various genotyping methods (Ridout and Donini 1999)

Feature	Marker system			
	AFLP	**RFLP**	**RAPD**	**SSR**
DNA required (mg)	0.5–1.0	10	0.02	0.05–0.10
PCR-based	Yes	No	Yes	Yes[a]
Level of polymorphism (Hav[b])	0.11	0.28	N/A[c]	N/A
	0.29–0.64	N/A	0.33–0.34	0.47–0.76
	0.32	0.41	0.31	0.6
	Medium	High	Medium	Very high
Effective multiplex ratio[d]	50–100	N/A	20–50	1.0
	19.2	0.25	1.56	1.0
	24.7	3.0	N/A	N/A
Marker index (MI[e])	6.14	0.1	0.48	0.6
Reproducibility	Very high	Very high	Fair[f]	Very high
Cost	N/A	N/A	2xAFLP	N/A
	$105[g]	$178	N/A	N/A
	Medium	N/A	Low	High[h]
Ease of use	Difficult initially[i]	Labour intensive	Easy	Easy[j]

[a] Sequence information required.
[b] Hav, average heterozygosity. An average for the probability that two alleles taken at random can be distinguished.
[c] N/A, data not available in the reference cited.
[d] Effective multiplex ratio is the number of polymorphic loci analysed per experiment in the germplasm tested.
[e] Marker index is the product of the average expected heterozygosity and the effective multiplex ratio.
[f] Between laboratories, influenced by *Taq* polymerase and thermocycler.
[g] Also savings of time compared to RFLP.
[h] Cost of initial sequencing high.
[i] Became easier with practise.
[j] Some technical problems associated with silver staining.

Amplified fragment length polymorphism (AFLP) is a PCR-based technique that involves restriction of genomic DNA, followed by ligation of adapters to the fragments generated and selective PCR amplification of a subset of these fragments (Vos et al. 1995). The amplified fragments are separated on polyacrylamide gels and visualized, usually by autoradiography or fluorescent sequencing equipments (Fig. 6). Several different restriction enzymes and primers are available, which gives a high degree of flexibility, enabling the complex final fingerprint to be manipulated for particular applications and efficient scanning of the genome for polymorphisms. The choice of enzymes and primer length is crucial for optimizing results in different

applications and the rationale for choosing restriction enzymes, adapters and primers is described both here and in original publications (e. g. Ridout and Donini 1999). The AFLP technique is robust and reliable because stringent reaction conditions are used for primer annealing: the reliability of the RFLP technique (Botstein et al. 1980; Tanksley et al. 1989) is combined with the power of the PCR technique (Mullis and Faloona 1987; Saiki et al. 1988). AFLP markers, which are produced by different combinations of restriction enzymes, are randomly distributed throughout the genome (Mackill et al. 1996; Waugh et al. 1997; Zhu et al. 1998), although clustering of markers in centromeric regions has also been reported (Qi et al. 1998). However, there is evidence that AFLP markers lie outside regions that are heavily populated with RFLPs (Becker et al. 1995; Waugh et al. 1997).

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Plant material

2.1.1.1 *Oenothera* EST library

The EST library was prepared from the youngest two leaves of 9-week-old plants of *Oenothera elata ssp. hookeri*, line *hookeri*.

2.1.1.2 *Oenothera* genotyping and linkage analysis

DNA was isolated from young leaves of the F2 generation from a single F1 progeny of a cross between cybrid *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III and *Oenothera grandiflora* ssp. *tuscaloosa* BB-III.

2.1.1.3 *Passiflora*

Passiflora menispermifolia, *Passiflora oerstedii*, and their F1 hybrids were used for analysis. The plant material was kindly provided by Ms. Irmi König.

2.1.2 Chemicals

All chemicals used in this study were of p. a. quality. They were purchased from the following companies: Roche (Basel, Switzerland), Roth GmbH & Co. (Karlsruhe) and Sigma Chemical Company (Munich).

2.1.3 Reagents and kits

ATP (Roche, Mannheim, Germany)
Bio-Rad AG 501-X8 (Bio-Rad, Hercules, California, USA)
DNeasy Plant Mini Kit (Qiagen, Oslo, Norway)

Dynabeads® mRNA DIRECT™ kit (Dynal Biotech, Oslo, Norway)
GENESCAN-500 ROX (Applied Biosystems, Foster City, California, USA)
Long Ranger gel solution – 50% stock solution (Cambrex, Rockland, ME USA)
Nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Germany)
SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning (Invitrogen, Carlsbad, California, USA)
TEMED (Invitrogen, Karlsruhe, Germany)
The "Random primed DNA labeling kit" (Roche Diagnostics, Switzerland)
"Thermo Sequenase™ DYEnamic Direct sequencing kits" (Amersham Biosciences, Uppsala, Sweden)
Whatman paper (Whatman plc, Brentford, Middlesex, UK)

2.1.4 Devices

EM 912 or EM 109 electron microscopes (Zeiss, LEO, Oberkochen)
Mixer Mill MM 300 (Qiagen, Oslo, Norway)
ABI PRISM 377 DNA Sequencer (Applied Biosystems, California, USA)
PCR Express (Thermo Hybaid GmbH, Heidelberg)

2.1.5 General buffers

TE Buffer

Tris-HCl, pH 8.0	10 mM
EDTA	1 mM

50x TAE Buffer (per litre)

Tris base	242 g
acetic acid	57.1 ml
0.5 M EDTA, pH 8.0	100 ml

TBE Buffer, pH 8.3

Tris	89 mM
Boric acid	89 mM
EDTA	2 mM

20x SSC (per litre)

NaCl	175.3 g
Sodium citrate, dihydrate	100 g

pH 7.0 adjusted with HCl or NaOH

10X MOPS Buffer

MOPS	41.85 g
Na-acetate	6.8 g
EDTA	3.72 g

pH 7.0 adjusted with NaOH

2.1.6 Software

Adobe Photoshop (San Jose, California, USA)
Bioedit (Ibis Therapeutics, Carlsbad, California, USA)
Fast PCR (University of Helsinki, Finland)
GeneScan (Applied Biosystems, Foster City, California, USA)
Genotyper (Applied Biosystems, Foster City, California, USA)
Hashed Position Tree2 (HPT2) algorithm (Biomax Informatics AG, Martinsried, Munich)
JoinMap 3.0 (Kyazma B.V., Wageningen, Netherlands)
Map Manager (Jane Meer, Robert H. Cudmore, Jr. and Kenneth F. Manly)
Microsoft Access Database (Microsoft, Redmond, WA, USA)
Microsoft Excel (Microsoft, Redmond, WA, USA)
Microsoft Word (Microsoft, Redmond, WA, USA)
RepeatBeater software (Biomax informatics AG, Martinsried, Munich)
Sequencher (Gene Codes Corporation, Ann Arbor, USA)
Sputnik (http://sputnik.btk.fi/)

2.1.7 Bacterial strains

MAX Efficiency® DH5α™ Competent Cells (Invitrogen, Carlsbad, California, USA)

2.1.8 Vectors

pSPORT1 Invitrogen (Carlsbad, California, USA)

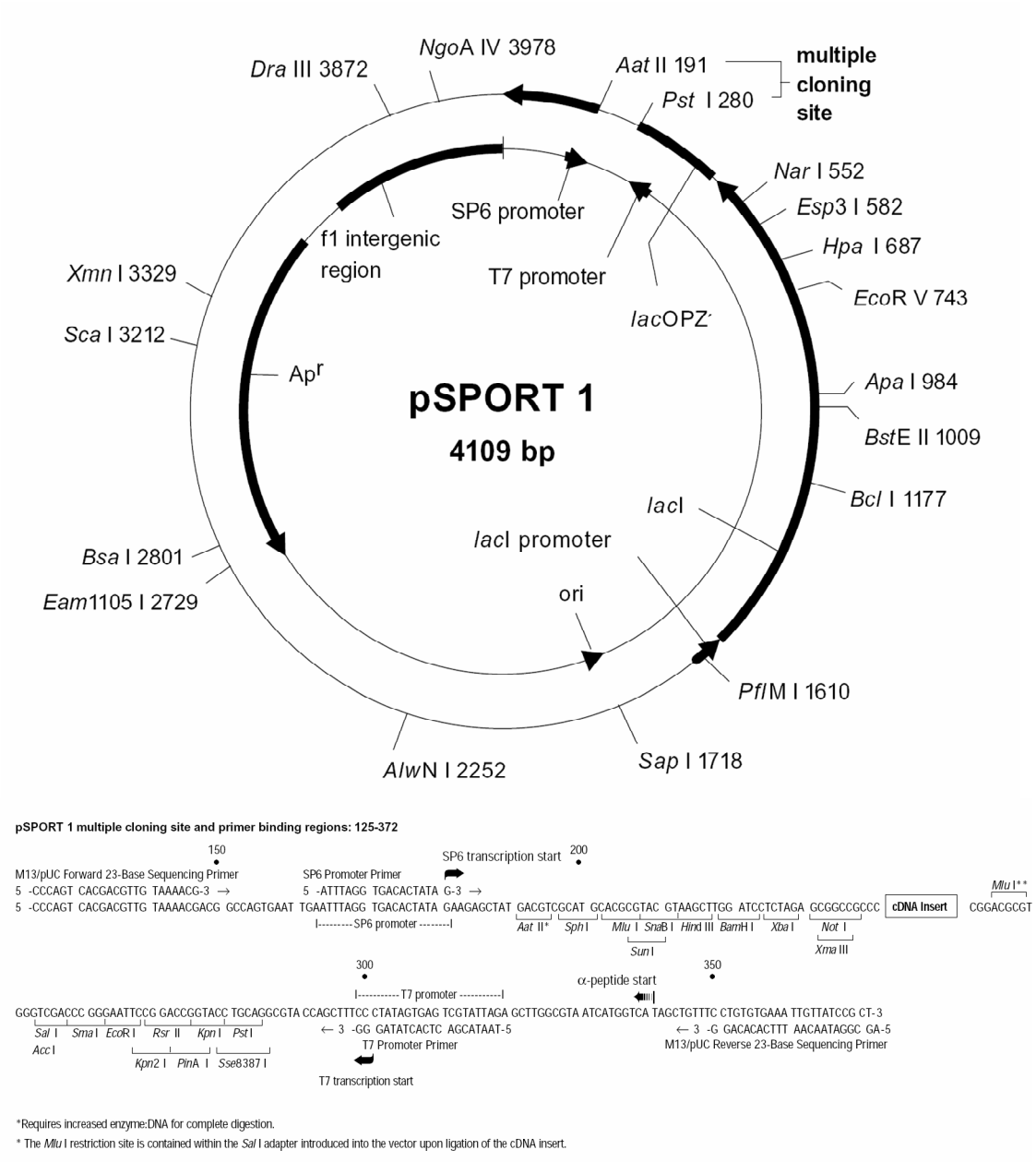

Fig. 7 The vector map and multiple cloning sites of plasmid pSPORT 1. This figure also includes the position of cDNA insertions (Handbook, Invitrogen, Carlsbad, California, USA).

2.1.9 Oligonucleotides

All nucleotides listed below were obtained from MWG-BIOTECH (Ebersberg, Germany), Termo Electron Corporation (Dreieich, Germany) or Applied Biosystems (Foster City, California, USA).

2.1.9.1 Unmodified oligonucleotides

ArbcL	5'-GGACTTACCAGCCTTGATCG-3'
M13 forward	5'-CCCAGTCACGACGTTGTAAAACG-3'
M13 reverse	5'-AGCGGATAACAATTTCACACAGG -3'
Not I primer-adapter	5'-GACTAGTTCTAGATCGCGAGCGGCCGCCC(T)15-3'
rbcL.P2	5'-GATCATTTCTTCGCATGTACC-3'
rpoB4forP	5'- GACTCATGTGACAAGCCAAGGCTCTG -3'
rpoB5Pfor	5'- ATGAGGGAATATCTACAATACC -3'
rpoBp1rev	5'- ATTTTGCCTAGRCAAGATATGCC -3'
rpoC1p	5'- GCGATGTGTGATTTGATCGAA -3'
SalI adapter I	5'-TCGACCCACGCGTCCG-3'
SalI adapter II	5'-CGGACGCGTGGG-3'
T7 promoter	5'-GCTCTAATACGACTCACTATAGGG-3'

2.1.9.2 Unmodified oligonucleotides used in the development of co-dominant markers

Oen_ATP_1343_for	5'- ATGAAAGCACAAGGAGTCCTC -3'
Oen_ATP_1343_rev	5'- CGAGAATGAAGCTGCCTAAGA -3'
Oen_psbS_1098_for	5'- AGCAGCAGCAGATCTCCTCAG -3'
Oen_psbS_1098_rev	5'- TGCTTCACCCAATATAGACGCTG -3'
Oen_PSI_1342_for	5'- ACTACTACTACAACTTCACTGGTG -3'
Oen_PSI_1342_rev	5'- AGTCATGAAGTTCTTGGTGATGTG -3'
Oen_PSII_1313_for	5'- AAGCCGAGATCATCCTGCAATGG -3'
Oen_PSII_1313_rev	5'- AGGCAAAATAAAACGGGGATACAGC -3'
Oen_reff_1188_for	5'- ACCAGCTCAGCTCTCTACAATGG -3'

Oen_reff_1188_rev	5'- TCAATAACAAGGTCGCTCTGCGG -3'
oenM10for	5'- TGGGTCCAAGCCAAGCAGGTCC -3'
oenM10rev	5'- CACATCCTCCAAAGTCCAGTGG -3'
oenM1for	5'- AGGGTCTGTTCTTGTTGCAGAG -3'
oenM1rev	5'- TGGCACTTCTCTTTGAAGCCTC -3'
oenM2for	5'- TGGCCATGGCGACACAAGCCTC -3'
oenM2rev	5'- CCTCAACCTGAGCCTTACGGAG -3'
oenM3for	5'- ATATCACCTGGTACTGCTAGCT -3'
oenM3rev	5'- AACTCCCTCCAATCTGAAGGGT -3'
oenM4for	5'- GCGGCTGCTGCAACAGCATCTC -3'
oenM4rev	5'- CTAACCGCGGTTCGGTATGCAG -3'
oenM5for	5'- CCTTAACCGGAGCTCCGAGCAG -3'
oenM5rev	5'- CCTCCGCAAGCGCAGGCAATGG -3'
oenM6for	5'- GCTATGGTCAAACCGACGATCC -3'
oenM6rev	5'- ACTTAGCCTCGACCCATGCAGG -3'
oenM7for	5'- ACCATACCCATATACCCAGTGC -3'
oenM7rev	5'- TCAAGCGGCTTCGGTGCATCTC -3'
oenM8for	5'- CTCAGCCAGGAGGACCTCAAGC -3'
oenM8rev	5'- GAGGTGGGTATCGACCTCGTCG -3'
oenM9for	5'- AGCGTTCCTCAGAACTCCAAGC -3'
oenM9rev	5'- AGGCTTTCCAGCGAGCTGCTGG -3'

2.1.9.3 AFLP unmodified primers and adapters

AFLP primers consist of three parts: a core sequence, an enzyme specific sequence (ENZ) and a selective extension (EXT) (Zabeau & Vos 1993).

	CORE	**ENZ**	**EXT**
SacI	5-GACTGCGTACA	AGCTC	NNN-3
MseI	5-GATGAGTCCTGAG	TAA	NNN-3

MseI adapter I	5'-GACGATGAGTCCTGAG-3'
MseI adapter II	5'-TACTCAGGACTCAT-3'

SacI adapter I	5'-CTCGTAGACTGCGTACAAGCT-3'
SacI adapter II	5'-TGTACGCAGTCTAC-3'
MseIC	5'-GATGAGTCCTGAGTAAC-3'
SacIG	5'-GACTGCGTACAAGCTCG-3'
MseICAC	5'-GATGAGTCCTGAGTAACAC-3'
MseICGC	5'-GATGAGTCCTGAGTAACGC-3'
MseICCG	5'-GATGAGTCCTGAGTAACCG-3'
MseICTC	5'-GATGAGTCCTGAGTAACTC-3'
MseICGG	5'-GATGAGTCCTGAGTAACGG-3'
MseICGA	5'-GATGAGTCCTGAGTAACGA-3'
MseICGT	5'-GATGAGTCCTGAGTAACGT-3'
MseICTA	5'-GATGAGTCCTGAGTAACTA-3'
MseICTT	5'-GATGAGTCCTGAGTAACTT-3'
MseICTC	5'-GATGAGTCCTGAGTAACTC-3'
MseICTG	5'-GATGAGTCCTGAGTAACTG-3'

2.1.9.4 AFLP modified primers

Name	Sequence	5' end modification
SacIGG-FAM	5'-GACTGCGTACAAGCTCGG-3'	6 FAM
SacIGA-FAM	5'-GACTGCGTACAAGCTCGA-3'	6 FAM
SacIGC-Joe	5'-GACTGCGTACAAGCTCGC-3'	Joe
SacIGT-Joe	5'-GACTGCGTACAAGCTCGT-3'	Joe

2.1.10 Enzymes

MseI (NEB, Beverly, USA)

NotI (Invitrogen, Carlsbad, California, USA)

Qiagen *Taq* polymerase (QIAGEN, Oslo, Norway)

SacI (NEB, Beverly, USA)

SalI (NEB, Beverly, USA)

T4 DNA ligase (NEB, Beverly, USA)

VspI (NEB, Beverly, USA)

2.1.11 Antisera

All antisera used were from the collection of Prof. Herrmann's laboratory.

2.2 Methods

2.2.1 Nucleic acid analysis

2.2.1.1 DNA analysis

2.2.1.1.1 Isolation of total DNA

2.2.1.1.1.1 Isolation of total DNA from *Oenothera*

DNA isolations were performed with DNeasy Plant Mini Kit (Qiagen, Oslo, Norway) using standard protocols with following adaptations: Disruption of 100 mg fresh leaf materials were performed by a Mixer Mill MM 300 (Qiagen, Oslo, Norway) (2 times 1.5 min at 30 Hz) in 2 ml reactions tubes with 400 µl of buffer AP1, 4 µl RNase A (100 mg/ml), 4 µl 10% PVP (polyvinyl pyrrolidone), and 0.4 µl 1M sodium 1-ascorbic acid. Then samples were incubated for 10 min at 65°C with 2 – 3 times mixing during incubation. To the lysate, 130 µl of buffer AP2 was added. The solutions were mixed and the mixture was incubated for 5 min on ice. The lysate was then centrifuged for 5 min at 20,000 x *g*, applied to the QIAshredder Mini Spin Column (Quigen, Oslo, Norway) and centrifuged for 2 min at 20,000 x *g*. The flow-through fraction was mixed with 1.5 volume of buffer AP3/E in new tube by pipetting. Then all mixtures were applied to the DNeasy Mini Spin Column (Qiagen, Oslo, Norway) and centrifuged for 1 min at 6,000 x g. The DNeasy Mini Spin Column was washed 2 times with 500 µl buffer AW. DNA was eluted with 50 µl of preheated (65°C) buffer AE. Buffers AP1, AP2, AP3/E, AW and AE were components from DNeasy Plant Mini Kit (Qiagen, Oslo, Norway).

2.2.1.1.1.2 Isolation of total DNA from *Passiflora*

DNA Extraction Buffer

sorbitol	0.35 M
Tris base	0.1 M
EDTA	5 mM
pH 7.5	

"Nuclei Lysis Buffer"

Tris base	0.2 M
EDTA	0.05 M
NaCl	2 M
CTAB	2%

Sarkosyl 5%

sodium lauroyl sarcosinate	5% (w/v)

Microprep Buffer was prepared immediately before use

DNA extraction buffer	2.5 parts
"nuclei lysis buffer"	2.5 parts
sarkosyl 5%	1.0 part
DTT	10 mM
sodium bisulfite	0.3 to 0.5 g /100 ml

DNA isolation was performed according to Fulton at al. (1995). The exposure of grinded plant material to air was crucial for successful isolation, in order to, the homogenisation were done under level of Microprep buffer. 50 to 100 mg of fresh leaf tissue was homogenized in 750 µl of Microprep buffer and incubated in 65°C for 30 to 120 min. 700 µl of chloroform : isoamyl (24:1) was added. The sample was mixed and centrifuged at 6,000 x g for 5 min. The aqueous phase was pipetted off, then mixed with 2/3 volume of cold isopropanol and centrifuged at 6,000 x g for 5 min. The pellet was washed in 70% ethanol, then dried and resuspend in 50 µl of TE buffer.

2.2.1.1.2 PCR product purification

PCR products were purified by 0.6 M ammonium acetate and precipitation in 75% ethanol.

2.2.1.1.3 Plasmid transformation

SOC medium

Bactotryptone	2 g
Yeast extract	0.55 g
1 M NaCl	1 ml
1 M KCl	1 ml
H_2O	97 ml

pH 7.0 adjusted with HCl or NaOH

Dissolve and autoclave

1 M $MgCl_2$; 1 M $MgSO_4$	1 ml
2 M glucose	1 ml

The medium was finally filtered through a 0.2 μm filter unit.

100 μl of MAX Efficiency® DH5α™ Competent Cells (Invitrogen, Carlsbad, California, USA) were gently mixed and placed into a 17 x 100 mm polypropylene tube on ice. 5 μl DNA from the ligation reaction was added and gently mixed by moving the pipette through the cells. Cells were incubated on ice for 30 minutes and then exposed to a heat shock (45 seconds in 42°C water bath). After heat shock they were incubated for 2 minutes on ice. Then 0.9 ml of SOC medium were added and the suspension was shaken at 225 rpm at 37°C for 1 h. Cells diluted with SOC medium were plated on LB medium plates with ampicilin and incubated for 14 h at 37°C.

2.2.1.1.4 Plasmid isolation

E1 Buffer

Tris-HCl, pH 8.0	50 mM
EDTA, pH 8.0	10 mM
RNase	100 µg/ml

E2 Buffer

NaOH	200 mM
SDS	1% (w/v)

E3 Buffer

K-Acetat	3.2 M

pH 5.5 adjusted with acetic acid

Plasmids were isolated according to Birnboim and Doly (1979). The cells were harvested by centrifugation for 5 min at 10,000 x g from 2 ml over-night grown culture. The pellet was resuspend in 200 µl of E1 buffer. To the sample 200 µl of E2 buffer were added, then mixed and incubated for 5 min at room temperature. Then 200 µl of E3 buffer were added, mixed, incubated for 15 min in ice, and centrifuged for 10 min at 10,000 x g. To the supernatant 0.7 volume of isopropanol were added, mixed, incubated 10 min on ice, and centrifuged for 30 min at 10,000 x g. The pellet was washed with 70% ethanol, dried, and resuspend in H_2O.

2.2.1.1.5 Southern analysis

Denaturation Solution (per litre)

NaCl	87.66 g
5 M NaOH	100 ml

Neutralization Solution (per litre)

NaCl	87.66 g
Tris	121.14 g

pH 8.0 adjusted with HCl

For Southern analyses, 5 μg of total DNA from leaf material was digested by appropriate restriction endonucleases and the fragments were electrophoretically separated in 1% TAE agarose gels. The gel was then incubated in denaturation solution for 1 hour and then another hour in neutralization solution. DNA transfer from the gel onto Nylon membranes (Hybond-N+, Amersham Biosciences, Uppsala, Sweden) was performed by capillary blotting as described by Sambrook et al. (1989). DNA was fixed to the membrane by 80°C incubation for 30 min.

2.2.1.2 RNA analysis

2.2.1.2.1 Isolation of total RNA

Homogenization Buffer

sorbitol	0.33 M
Tris-HCl, pH 9.0	0.2 M
NaCl	0.3 M
EDTA	10 mM
EGTA	10 mM
SDS	2% (w/v)

Tris-Borat Buffer

Tris-Borat, pH 8.0	80mM
EDTA	10mM

Total RNA was prepared using phenol extraction and the LiCl precipitation protocol (Lizardi 1983). 5 g of fresh leaf tissue was homogenized in liquid nitrogen and powder was added to 40°C preheated buffer (12 ml homogenisation buffer; 4 ml phenol). With an additional 4 ml of chloroform, the sample was incubated for 15 to 20

min at RT. Then the sample was centrifuged for 10 min at 7,500 x g. To the supernatant, 4 ml buffer saturated phenol and 4 ml chloroform were added, mixed and the emulsion was centrifuged for 10 min at 7,500 x g. To the supernatant, again 8 ml chloroform were added and centrifuged for 10 min at 7,500 x g. To the supernatant, 1/5 volume of Na-acetat, pH 6.0, and 1 volume of ice-cold isopropanol were added and incubated over night at -20°C. The sample was then centrifuged for 10 min at 12,000 x g. The pellet was resuspended in Tris-Borat buffer. To the sample, 1/3 volume of 8 M LiCl was added and the solution was incubated at +4°C over night. Then the sample was centrifuged for 10 min at 12,000 x g. The pellet was washed twice with 70% ethanol and resuspended in H_2O.

2.2.1.2.2 Isolation of poly(A)+ mRNA

Poly(A)+ mRNA was prepared from total RNA using Dynabeads® mRNA DIRECT™ kit (Dynal Biotech, Oslo, Norway), according to the manufacturer's protocol.

2.2.1.2.3 Northern analysis

10X Loading Buffer

glycerol	50%
$NaPO_4$, pH 7.0	10 mM
Bromophenol Blue	0.25%
Xylene Cyanol FF	0.25%

Preperation of 4.5 M (30%) Glyoxal

Glyoxal was deionized with Bio-Rad AG 501-X8 (or X8D) resin (Bio-Rad,Hercules, CCalifornia, USA) until the pH was 5.0 - 5.5. This was done immediately before use. Alternatively, after dionization it was stored in single-use aliquots in small tightly capped tubes at -70°C.

Probe Preparing

RNA (10 µg total RNA)	4 µl
deionized glyoxal	4 µl
DMSO (or H_2O)	9.5 µl
10x MOPS buffer	2.5 µl

The probe was incubated for 45 min at 50°C and then mixed with 5 µl 10x Loading Buffer. The RNA was electrophoretically separated in 1% agarose-MOPS gels. RNA transfer from the gel onto Nylon membranes (Hybond-N, Amersham Biosciences, Uppsala, Sweden) was performed by capillary blotting as described by Sambrook et al. (1989). DNA was fixed to the membrane by 80°C incubation for 30 min.

2.2.1.3 Radioactive labeling of PCR products

PCR products (10 - 20 ng DNA) were radiolabelled (^{32}P) by the random priming method with Klenow enzyme (Sambrook et al. 1989). The “Random primed DNA labeling kit” (Roche Diagnostics, Switzerland) was used according to the manufacturer’s protocol.

2.2.1.4 Hybridisation with radioactively labelled probe

Hybridisation Buffer

Na_2HPO_4	250 mM, pH 7.2
SDS	7% (w/v)
Na_2EDTA	2.5 mM

Washing Solution 1

SSC	1×
SDS	1% (w/v)

Washing Solution 2

SSC	0.5×
SDS	1% (w/v)

Washing Solution 3

SSC	0.2×
SDS	1% (w/v)

Washing Solution 4

SSC	0.1×
SDS	0.5% (w/v)

The membrane was pre-hybridized for at least 2 h at 65°C in hybridization buffer. Hybridization was performed by incubation of the membrane with denatured radiolabeled probe in 10 ml hybridization buffer for 12 – 16 hour at 65°C. Then the membrane was washed in multiple steps with washing solution 1 to 4 until the background signal was reduced to a minimum measurable intensity.

2.2.1.5 Automated sequencing on the ABI PRISM 377 DNA Sequencer

10x TBE, pH 8.3 (per litre)

Tris base	108 g
Boric acid	55 g
EDTA	7.4 g

Loading Buffer

formamide	80%
Dextran Blue	10 mg/ml
EDTA	5 mM

1x TBE Electrophoresis Buffer, pH 8.3

Tris	89 mM
boric acid	89 mM
EDTA	2 mM

Table 5 Pipeting scheme of sequencing gels

H_2O	22.8 ml
urea	18 g
10X TBE	6 ml
Accugel 29:1 40% (Geneflow Ltd, USA)	5.33 ml
APS (15%)	300 µl
Temed	20 µl

The dideoxy sequencing method (Sanger et al. 1977) was applied for automated sequencing using the ABI PRISM 377 DNA sequencer (Applied Biosystems, California, USA). The DNA was labelled with "Thermo Sequenase™ DYEnamic Direct sequencing kits" (Amersham Biosciences, Uppsala, Sweden) using the following reaction conditions: 1 cycle of 95°C denaturation for 2 min; 99 cycles at 95°C (10 s), 49°C (5 s), and 1 cycle 60°C (4 min). The reactions were then cleaned up to remove unincorporated fluorescent nucleotides by ethanol precipitation (65% ethanol final concentration). The pellet was resuspended in 4 µl loading buffer and the sample was denatured for 2 min at 80°C. The sequencing gel was prepared as is described in Table 5. The sequence run was performed using 48 cm glass plates, 0.2 mm spacers and a 36 well comb. Electrophoresis conditions were as following: 2.5 kV; 45°C; 11 h using 1x TBE electrophoresis buffer.

2.2.2 Protein analysis

2.2.2.1 Protein isolation

Sample Buffer

Na_2CO_3	100 mM
sucrose	10% (w/v)
DTT	50 mM

Homogenisation Buffer

Tris-HCl, pH 8.0	50 mM
EDTA	10 mM
EGTA	2 mM
DTT	10 mM

Frozen leaf material of mature plants was ground to a fine powder in liquid nitrogen with a mortar and pestle. Two volumes of ice-cold homogenisation buffer were added. The filtrate of the homogenate mixture obtained by two layers of Miracloth was centrifuged for 10 min at 10.000 x g. The supernatant containing soluble proteins was separated. The pellet containing the membrane proteins was washed with isolation buffer and resuspended into one volume of sample buffer.

2.2.2.2 Protein gel electrophoresis

Pipeting Scheme of PAA Gels

	15% resolving PAA gel	8% stacking PAA gel
30% acrylamide / 0.8% bisacrylamide	30ml	2.7 ml
3M Tris-HCl, pH 8.8	7.5 ml	-
1M Tris-HCl, pH 6.8	-	1.25 ml
10% (w/v) LDS	0.6 ml	0.1 ml
Temed	60 µl	17 µl
10% (w/v) APS	180 µl	50 µl
H_2O	Ad 60 ml	Ad 10 ml

10x Laemmli Buffer

Tris-HCl, pH 8.5	0.25 M
glycine	1.92 M
SDS	1% (w/v)

Electrophoreses buffer

Tris-HCl, pH 8.5	0.05 M
glycine	0.384 M
SDS	0.01% (w/v)

SDS-denaturing polyacrylamide (PAA) gels were prepared according to Laemmli (1970). Preparations involved casting of two different layers of acrylamide between glass plates. The lower layer (separating or resolving, gel) was responsible for separating polypeptides accurating to their sizes. The upper layer (stacking gel) including the sample wells was of a composition that causes the samples to become compressed (stacked) in order to get thin bands and correspondingly high resolution.

2.2.2.3 Staining of PAA gels

2.2.2.3.1 Coomassie Brilliant Blue staining

Staining Solution

methanol	30%
acetic acid	12%
Coomassie Brilliant Blue R 250 (Serva, Heidelberg)	0.1% (w/v)

Destaining Solution

acetic acid	12%

The gels were incubated in staining solution for 1 – 2 hour under constant shaking at 50°C and destained until protein bands appeared well.

2.2.2.3.2 Staining with silver nitrate

Fixation Solution

ethanol	50%
acetic acid	12%
37% formaldehyde	0.05%

Thiosulfate Solution

$Na_2S_2O_3$	0.02% (w/v)

Silver Nitrate Solution

$AgNO_3$	0.2% (w/v)
37% formaldehyde	200 µl/100 ml

Developing Solution

Na_2CO_3	6% (w/v)
37% formaldehyde	135 µl/100 ml
$Na_2S_2O_3$	4 mg/ml

Stopping Solution

methanol	50%
acetic acid	12%

The 1.5 mm gel was incubated for at least 1 h in fixation solution, washed three times for 30 min in 50% ethanol and soaked for 1.5 min in thiosulfate solution. Then, the gel was washed three times for 30 sec with water and incubated in silver nitrate solution for 30 min in darkness with constant agitation. The gel was then washed twice with water and incubated in developing solution until the bands reached the desired intensity. The reaction was ended in stopping solution.

2.2.2.4 Immunological detection of proteins on membranes

2.2.2.4.1 Transfer of proteins onto nitrocellulose and PVDF membranes

Anode Buffer I

Tris base	0.025 M

Anode Buffer II

Tris base	0.3 M

Cathode Buffer

ε-aminocapronic acid	40 mM
SDS	0.01% (w/v)

Transfer membranes and PAA gels were incubated for 10 min in anode buffer II prior to transfer. PVDF membranes were pre-wetted in 100% methanol. The proteins were transferred onto membranes using a semi-dry blotting system. Three sheets of Whatman paper (Whatman plc, Brentford, Middlesex, UK) were soaked in cathode buffer and placed onto the cathode. The gel was placed on the cathode buffer-soaked Whatman paper and covered with the transfer membrane. The gel and transfer membrane assembly was covered by two layers of Whatman paper soaked in anode buffer II and three layers of Whatman paper soaked in anode buffer I. The transfer was performed for 1.5 - 2.5 h with constant current according to the formula: mA = gel size in cm^2 x 0.8.

2.2.2.4.2 Staining of blots with Ponceau S

Ponceau S Solution

Ponceau S	0.2% (w/v)
acetic acid	1.0%

After blotting the membrane was incubated in Ponceau S (3-hydroxy-4-[2-sulfo-4-(sulfophenylazo) phenylazo]-2,7-naphtalene disulfonic acid) solution for 10 min at room temperature with agitation. The membrane was rinsed with water and the positions of the molecular marker bands were marked. The membrane was finally destained in water.

2.2.2.4.3 Western analysis

Blocking Buffer

PBS	1x
dry milk powder	5% (w/v)
Tween 20	1% (v/v)

Washing Buffer	
PBS	1x
Tween 20	1% (v/v)

Solution I Stock	
luminol (in DMSO)	0.25 M
P-coumaric acid (in DMSO)	0.09 M

Solution I	
luminol	2.5 mM
p-coumaric acid	0.4 mM
Tris-HCl, pH 8.5	0.1 M

Solution 2	
H_20_2	5.4 mM
Tris-HCl, pH 8.5	0.1 M

After electrophoretic transfer of proteins from a PAA gel, the membrane was incubated in blocking buffer for 1 h at room temperature. The antisera diluted to the desired concentration in blocking buffer were incubated with the membrane for 2 h at room temperature or overnight at 4°C. The first antibodies were removed by washing the membrane four times for 10 min in blocking buffer. Anti-rabbit antibodies were diluted in blocking buffer and then incubated with the membrane for 1 h at room temperature. The membranes were then washed four times for 10 min in washing buffer and developed in a mixture of solution 1 and 2 (1:1) by incubation for 1.5 min. They were exposed to X-ray films (Hyperfilm; Amersham Life Science, England) for 1 - 20 min.

2.2.3 Expressed sequence tags (ESTs)

On the *Oenothera* EST project Uwe Rauwolf, Martha Braun, Lada Mlčochová, Martina V. Silber, Pavan Umate, and Stephen Rudd participated.

2.2.3.1 Construction of a cDNA library

LB medium (per litre)

Bacto – Trypton	10 g
Yeast extract	5 g
NaCl	10 g

pH 7.0

The cDNA library was constructed by using the "SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning" (Invitrogen, Carlsbad, California, USA). The cDNA was synthesised from poly(A)+ mRNA using SuperScript II reverse transcriptase with NotI primer-adapter for first strand synthesis. *E. coli* DNA polymerase I was used for second strand synthesis. The cDNAs were ligated with SalI adapters and digested with NotI (Fig. 8). A column chromatographic step, used to size-fractionate the cDNAs, was included to make more effective the cloning of larger inserts. This procedure also ensures that residual adapters and NotI fragments released by restriction digestion of the primer-adapter do not contaminate the library. Size-fractioned cDNA was unidirectionally inserted into pSPORT1 vector exploiting SalI and NotI restriction sites, respectively. Plasmid transformations were performed with MAX Efficiency® DH5α™ Competent Cells (Invitrogen, Carlsbad, California, USA) and transformants were selected on LB medium containing 1.5% agar and ampicilin (100 mg/l). Clones were stored in 96-well microtiter plates in storage buffer (LB medium with 20% glycerol) at -70°C (Fig. 9).

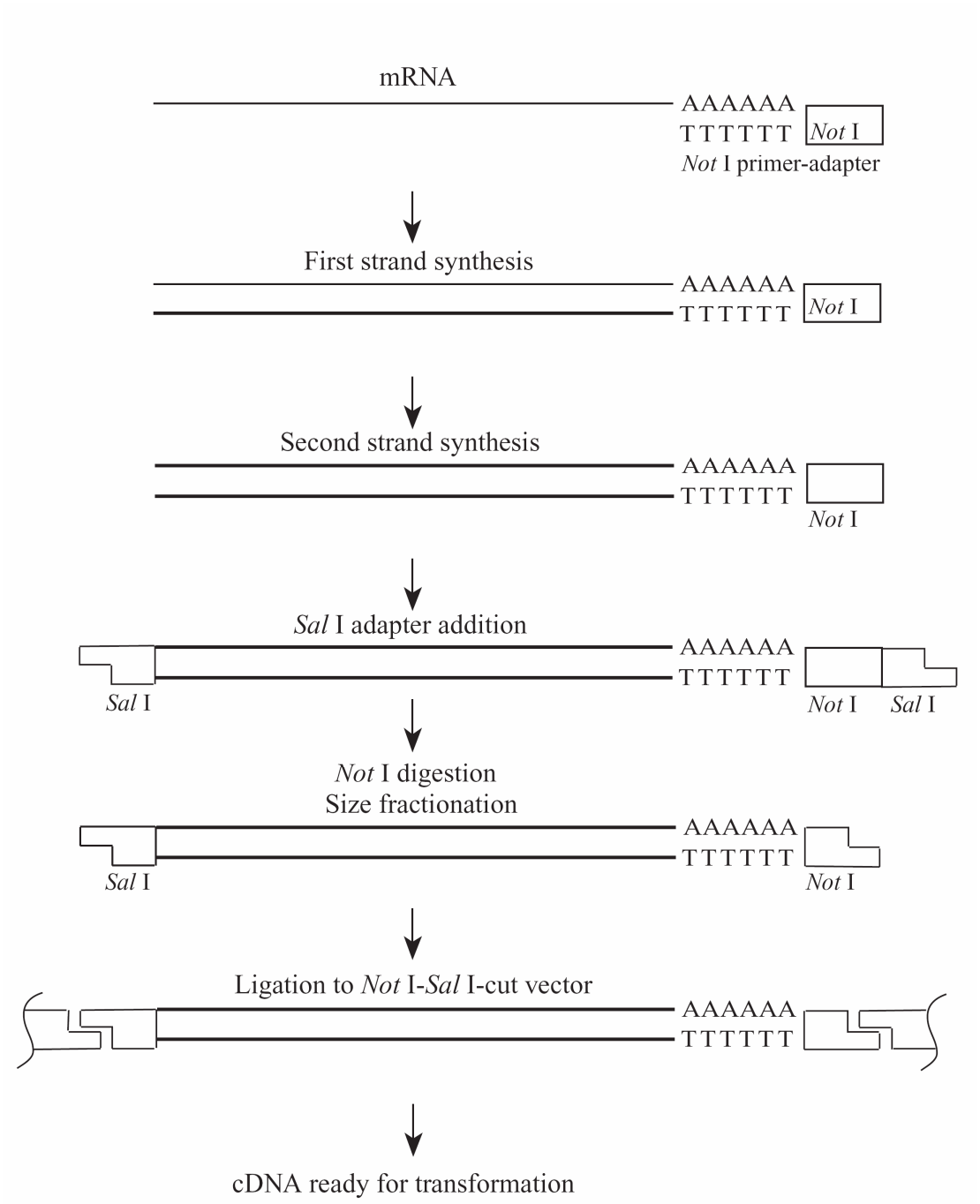

Fig. 8 Summary of the SUPERSCRIPT Plasmid System procedure to synthesise cDNAs (Handbook, Invitrogen, Carlsbad, California, USA)

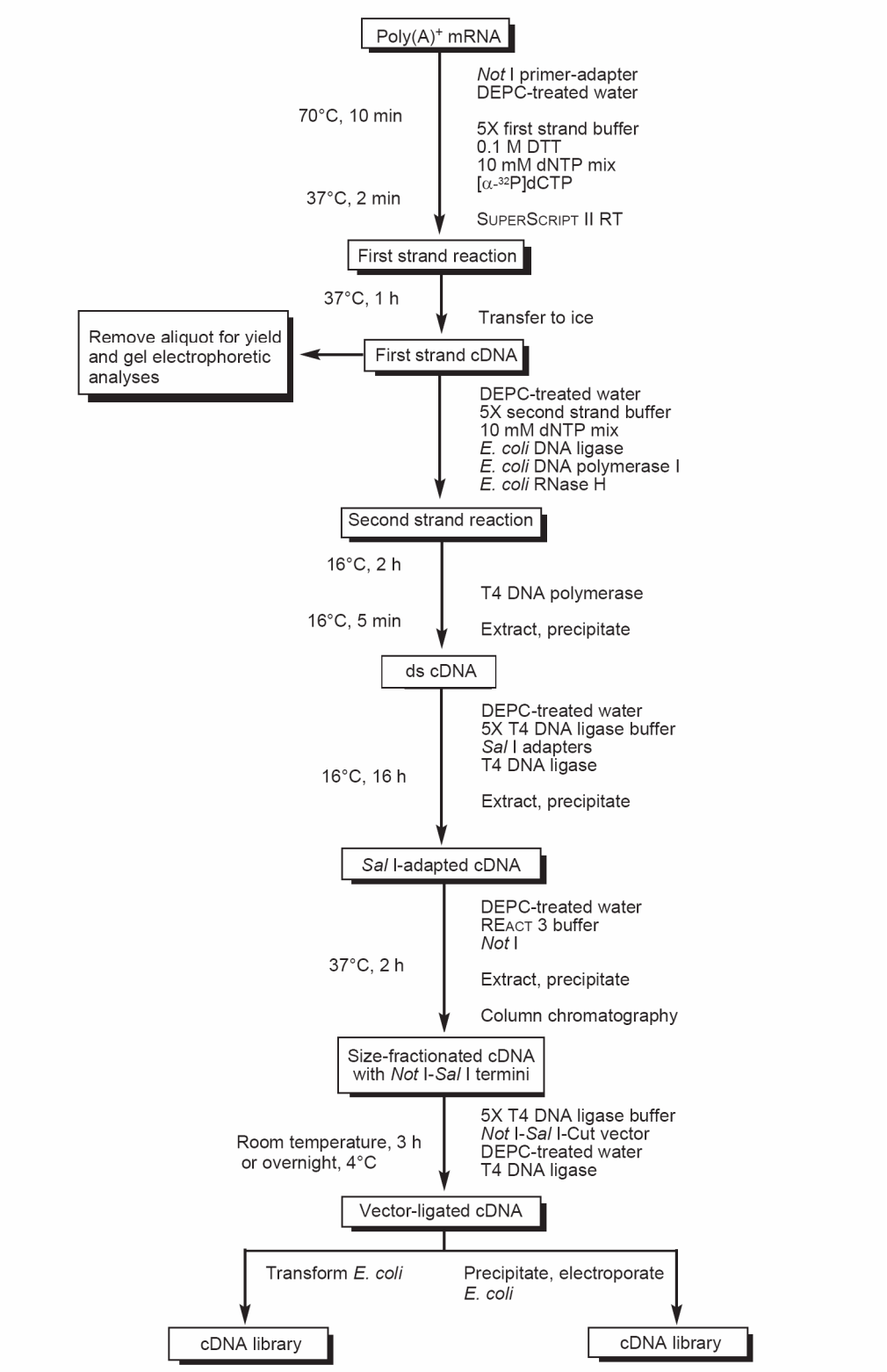

Fig. 9 Flow diagram used for construction of an *Oenothera* cDNA library (Handbook, Invitrogen, Carlsbad, California, USA)

2.2.3.2 Computer analyses of EST sequences

Processed sequence data were entered into the Microsoft Access database. Computer analyses of EST sequences were performed by Dr. S. Rudd. EST clustering and assembly, peptide predictions and sequence annotations were performed within the openSputnik sequence analysis pipeline (Rudd et al. 2003). EST sequences were clipped off any vector or polylinker sequence using the Crossmatch application (Phil Green, unpublished data) and the NCBI UniVec database was adapted to include the vector and polylinker sequences used during cDNA preparation. Simple repeats and regions of low complexity were masked using the RepeatBeater software and the ESTs were clustered using the Hashed Position Tree2 (HPT2) algorithm. HPT2 was optimised for over-clustering by imposing a similarity threshold of 0.7 and 300 iterations for cluster classification. The derived cluster sequences were assembled using the repeat-unmasked EST sequences with the CAP3 algorithm (Huang and Madan 1999) using the default settings. BLASTX (Altschul et al.1990) was performed against a non-redundant protein sequence database and filtered at 1e-10 to identify stretches of *Oenothera* sequences that correspond to probable CDSs. The aggregated CDS sequences were used to train the ESTScan application (Iseli et al. 1999) for the nuance of *Oenothera* codon usage. The subsequent *Oenothera* ESTScan model was used to derive peptide sequences from the unigene set. Unigene sequences were annotated for function using the MIPS funcat (Mewes et al. 2002) and Gene Ontology assignments (Harris et al. 2004). Homology (BLAST) methods were used to place the sequences within the context of both the rice and *Arabidopsis* genome scaffold and to place the sequences within the context of other plant EST collections. Peptide sequences were annotated for Interpro domains, subcellular and organellar localisation and transmembrane domain content. Sequences are available in a relational database and are presented through the WWW at http://sputnik.btk.fi.

2.2.4 Amplified Fragment Length Polymorphism (AFLP)

On the *Oenothera* AFLP project Uwe Rauwolf and Martha Braun participated.

The AFLP method was initially applied for mapping the *Arabidopsis thaliana* mutant hcf145 locus to obtain experience before it was applied to *Oenothera*. The data were used in *Oenothera* comparison analyses.

2.2.4.1 AFLP reactions

$TE_{0.1}$

Tris-HCl, pH 8.0	20 mM
EDTA, pH 8.0	0.1 mM

First AFLP reactions contained 50 to 500 ng DNA, 20 nmol ATP, 2.5 U MseI, 5 U SacI with 1x NEB-1 buffer, 15 μg BSA, 1.2 Weiss U T4 DNA ligase, 50 pmol per MseI adaptor, 5 pmol per SacI adaptor, in total volume 20 μl. The reaction was incubated in 37°C for 3 h and after was 10-fold diluted with $TE_{0.1}$ buffer. In first restriction and ligation took place simultaneously. In the second AFLP reactions, Qiagen Taq polymerase, 4 μl diluted AFLP reaction I and SacI and MseI primers with one selective base (SacI+1, MseI+1) were used with standard PCR conditions. Second AFLP reactions were as following: 2 min at 72°C cycle was followed by 20 sec 94°C; 30 sec 56°C; 2 min 72°C cycle. The second cycle was repeated 20 times, followed by a 30 min 60°C cycle. The second reaction was diluted 10-fold with $TE_{0.1}$ buffer. AFLP reactions III were used to label AFLP fragments with fluorescent dye. In our case, SacI+2 labeled primers with 6-FAM or Joe were employed. In third AFLP reactions, 4 μl diluted AFLP reaction II, 0.1 μM SacI+2 and 0.25 μM MseI+3 primers (listed in Table 6) were used with same PCR conditions as the AFLP II reaction. Third AFLP reactions were as following: 2 min at 94°C cycle was followed by 20 sec 94°C; 30 sec 66°C – 1°C each cycle; 2 min 72°C cycle. The second cycle was repeated 10 times. The next step was 20 sec 94°C; 30 sec 56°C; 2 min 72°C cycles repeated 20 times. The last cycle was performed for 30 min at 60°C.

Table 6 Primer combinations used for AFLP analyses

	MseICAC	MseICGC	MseICCG	MseICGG	MseICGA
SacIGG-FAM	sm263	sm260	sm261	sm290	sm291
SacIGA-FAM	sm267	sm264	sm265	sm297	sm298
SacIGC-Joe	sm271	sm268	sm269	sm283	sm284
SacIGT-Joe	sm275	sm272	sm273	sm276	sm277

	MseICGT	MseICTA	MseICTT	MseICTC	MseICTG
SacIGG-FAM	sm292	sm293	sm294	sm295	sm296
SacIGA-FAM	sm299	sm300	sm301	sm302	sm303
SacIGC-Joe	sm285	sm286	sm287	sm288	sm289
SacIGT-Joe	sm278	sm279	sm280	sm281	sm282

2.2.4.2 DNA fragment detection

Formamide Dye

formamide	80%
dextran blue	10 mg/ml
EDTA	5 mM

1x TBE Electrophoresis Buffer, pH 8.3

Tris	89 mM
boric acid	89 mM
EDTA	2 mM

The third AFLP reaction product was mixed with an equal volume (1 µl) formamide dye and with 0.15 µl of GENESCAN-500 ROX internal lane standard. Mixtures were heated at 90°C for 2 min and immediately cooled down to 4°C. Fragments were separated on a 5% denaturing (sequencing) polyacrylamid gel using the ABI Prism 377 DNA sequencer (Applied Biosystems, California, USA). The gel was prepared by using Long Ranger gel solution 50% stock solution. The gel contained 5% acrylamide, 6.0 M urea, 89 mM Tris, 89 mM boric acid and 2 mM

EDTA. To 30 ml of gel solution 150 µl of 10 % ammonium persulphate (APS) and 21 µl of TEMED were added. Electrophoresis conditions were as the following: 2.5 kV; 51°C; 4 h using 1 x TBE as buffer. The MATRIX file was created with dye 6 FAM, ROX, NED and Joe using virtual filter F. The gel images were captured by GeneScan software (Applied Biosystems, California, USA) (Fig. 10).

2.2.4.3 Computer analysis of genotyping data

GeneScan analysis software and Genotyper software were used to capture genotyping data. In genotyper, polymorphic bands were identified with 0.5 base tolerances (Fig. 11). Data were stored in Microsoft Access database. The AFLP markers were labelled using the following key (sm268_136.8). The first two letters represent restriction enzymes which were used (sm means SacI and MseI restriction endonucleases); next three numbers represent the unique ID for primer combinations (268) and last numbers represent marker sizes in bp (136.8 bp). Genotyping data were analysed in program Joinmap 3.0.

2.2.5 Electron microscopy

Electron microscopy was performed by Prof G. Wanner (Botanisches Institut der LMU, München). Small leaf pieces were fixed for 2 h in 2.5% glutardialdehyde (in 75 mM sodium cacodylate, 2 mM $MgC1_2$, pH 7.0) at room temperature. Subsequently, the material was rinsed several times in fixative buffer and post-fixed for 1 h with 1% osmium tetroxide in fixative buffer at room temperature. After two washing steps in distilled water, the tissue pieces were stained with 1% uranyl acetate in 20% acetone for 1h. Dehydration was performed with a graded acetone series. Tissue samples were then infiltrated and embedded in Spurr's low-viscosity resin (Spurr 1969). After polymerization, ultra-thin sections with thicknesses between 50 and 70 nm were cut with a diamond knife and mounted onto collodion-coated copper grids. The sections were post-stained with aqueous lead citrate (100 mM, pH 13.0). All micrographs were taken with the EM 912 or EM 109 electron microscopes.

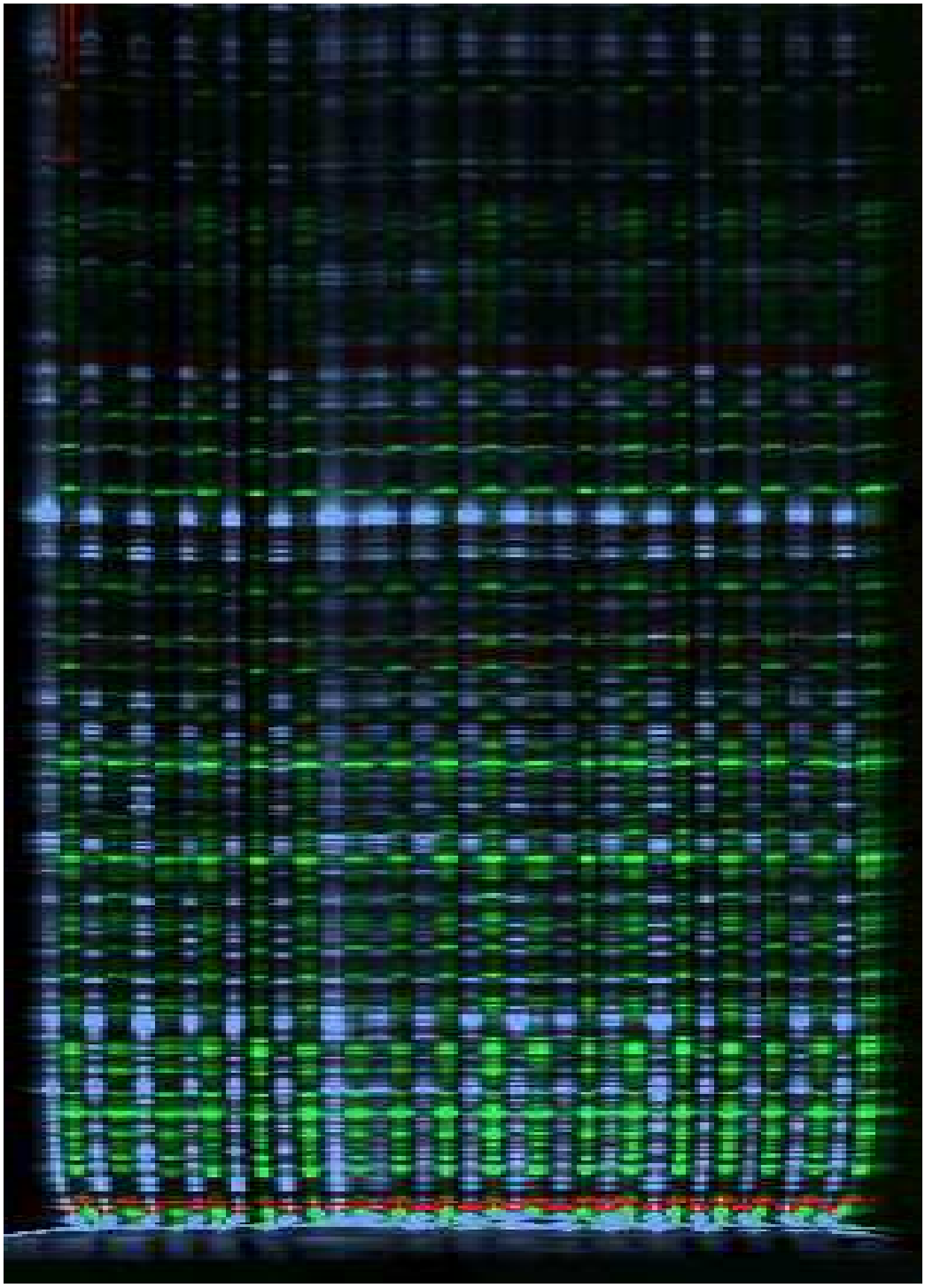

Fig. 10 Example of a gel image with three different Dyes: ROX (red-size standard), 6-FAM (blue), Joe (green)

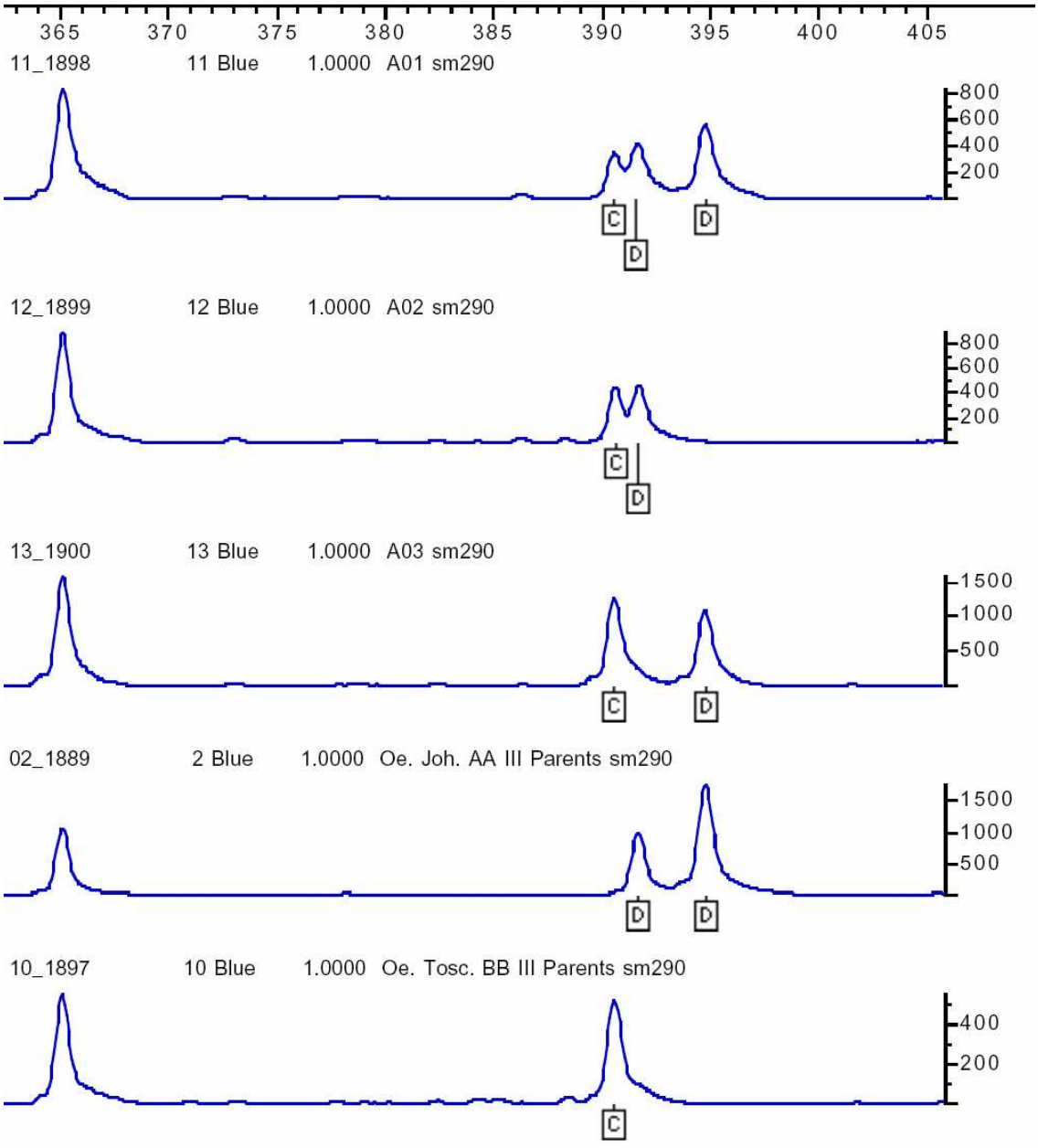

Fig. 11 Example of a genotyping analysis in the Genotyper program. The first three chromatographs represent plants from a F2 generation and the last two belong to parent's individuals. The SM290 primer combination was used in this example.

3 RESULTS

3.1 ESTs FROM *OENOTHERA*

3.1.1 Preparing of an EST library

An EST library was prepared from RNA of the youngest two leaves of 9-week-old plants of *Oenothera elata ssp. hookeri*, line *hookeri*. 127 ng cDNA was synthesised from 4 µg of poly(A)+ mRNA, and separated into twenty fractions by column chromatography. Only four of them were suitable for cloning but three were used for further analyses. The mean sizes of cDNA fragments obtained were 1.41 kb in fraction 1, 1.36 kb fraction 2 and 0.90 kb in fraction 3. The detailed information of the mean cDNA sizes is listed in Table 7 and Fig. 12.

The efficiency of cDNA transformation was determined as 12,000 colonies/ng cDNA, corresponding to 380,000 colonies/µg of poly(A)+ mRNA. 14,000 colonies were randomly selected and stored at -70°C (7,000 colonies of fraction 1; 3,500 colonies of fraction 2; and 3,500 colonies of fraction 3).

Table 7 The mean size of cDNA fragments in EST library

Size of cDNA inserts	Fraction 1	Fraction 2	Fraction 3
<1 kbp	9.7%	10.8%	36.8%
1 – 2 kbp	50.5%	58.9%	54.0%
2 – 3 kbp	28%	25.9%	8.1%
>3 kbp	11.8%	4.3%	1.1%

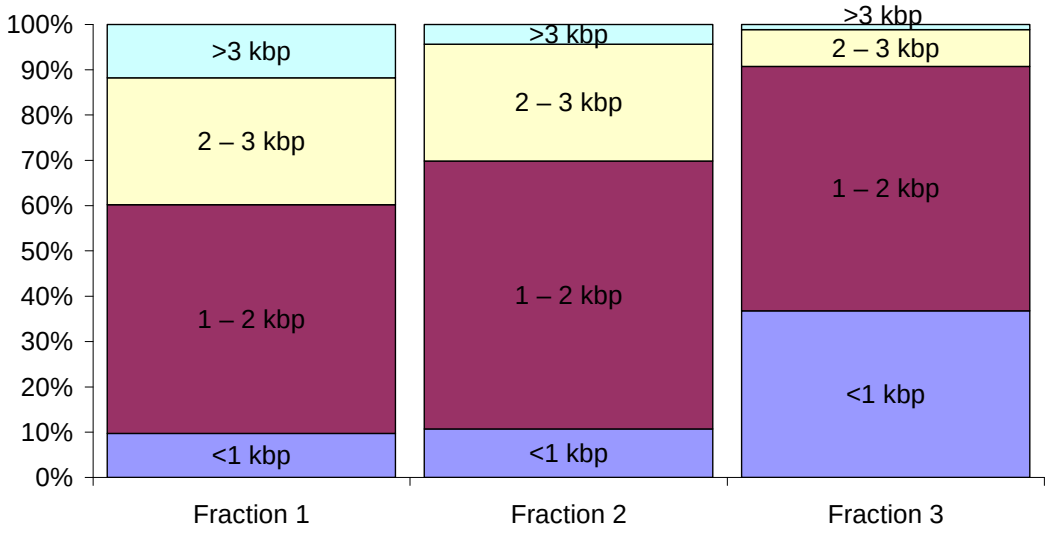

Fig. 12 Mean sizes of cDNA fragments in the EST library

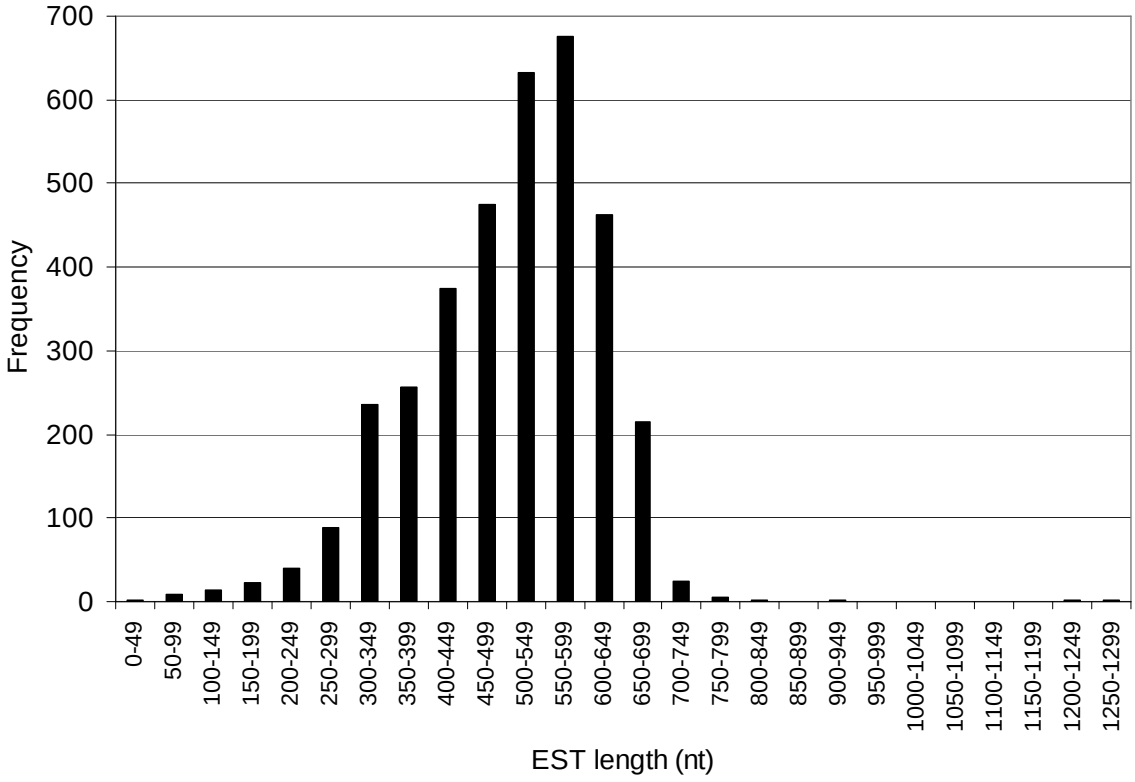

Fig. 13 Distribution of EST sequence lengths

3.1.2 Sequencing of ESTs

A total of 3,532 cDNA clones including 1,648 clones from fraction 1; 1,577 clones from fraction 2; and 307 clones from fraction 3 have been sequenced from their 5' ends. From their sequence information of a total of 1,774,576 nucleotides was received. The average sequence length was 502 nucleotides (Fig. 13).

3.1.3 Clustering of the EST sequences

A total of 1621 clusters (non-redundant ESTs) were formed including 1,133 singletons and 488 multi-member unigenes. A singleton is an IMAGE clone whose ESTs do not cluster with any other clone, and contain a minimium of 50 consecutive base pairs of non-repetitive sequences. Multi-member unigene is a candidate gene cluster containing sequences from more than one clone. A total of 875,940 non-redundant nucleotides were found after clustering. The average cluster length was 540 nucleotides (Fig. 14). Most of 488 multi-member unigenes contain less than 5 ESTs. Only 3.0% clusters contain more than 10 ESTs, 1.0% clusters have more than 20 redundant EST sequences (Table 8).

3.1.4 Distribution of GC content in the EST sequences

Sequences have been assembled into groups on the basis of their GC content (Fig. 15). Most of the ESTs contained a GC content between 46 and 47%.

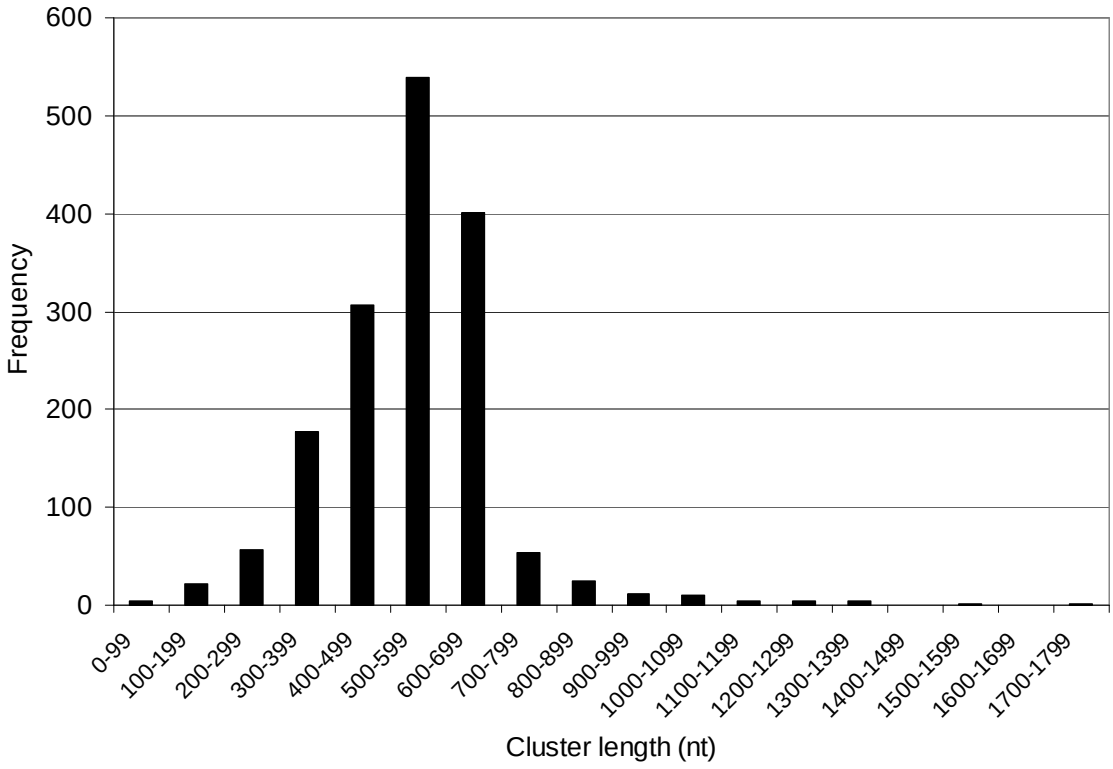

Fig. 14 Distribution of cluster sequence lengths

Table 8 Number of redundant EST sequences per cluster

No. of redundant EST sequences per cluster	Frequency	Frequency in %
1-4	1518	93,6%
5-9	55	3,4%
10-14	22	1,4%
15-19	8	0,5%
20-24	8	0,5%
25-29	4	0,2%
30-34	3	0,2%
45-49	1	0,1%
75-79	1	0,1%
205-209	1	0,1%

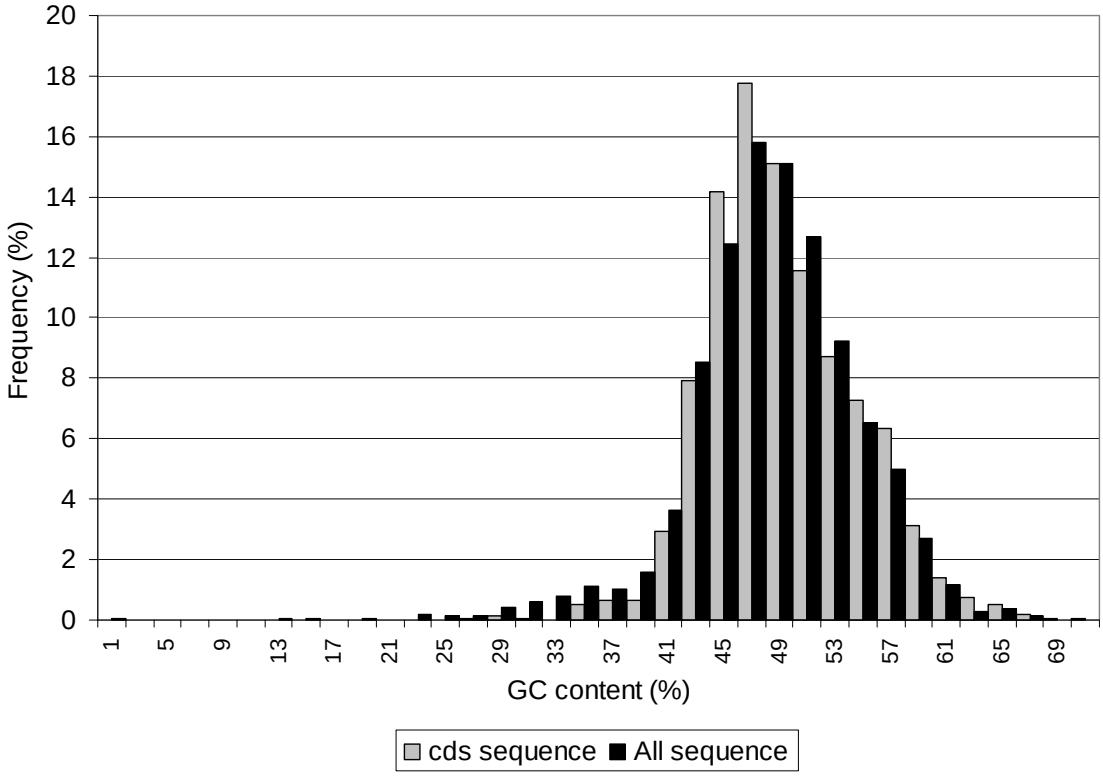

Fig. 15 Distribution of GC content for all sequences and for coding sequence (cds)

3.1.5 Annotation of EST sequences

ESTs have been annotated with respect to their putative function on the basis of the MIPS Functional Catalogue. An E-value of 1e-10 was used to define a significant database match for all analyses. As a result, 848 (52%) unigenes from 1621 non-redundant ESTs showed sequence similarity to genes registered in the public databases (Table 9). Then ESTs were grouped into functional categories as outlined in Fig. 16. 29.9% of the non-redundant ESTs by sequences homology correspond to the functional category UNCLASSIFIED PROTEINS (including the category CLASSIFICATION NOT YET CLEAR-CUT). From genes with known function, most of the redundant EST sequences could be assigned to the function category METABOLISM (13.6%) followed by CELLULAR ORGANIZATION (11.2%) and PROTEIN SYNTHESIS (7.5%). Unigenes that contained the most redundant EST sequences are listed in Table 10.

Table 9 The result of EST similarity search against the public databases

Similarity	Number of unigenes	Number of EST sequences
Genes of known function	632	1823
Unclassified proteins	387	641
No similarity	773	1133
Total	1621	3532

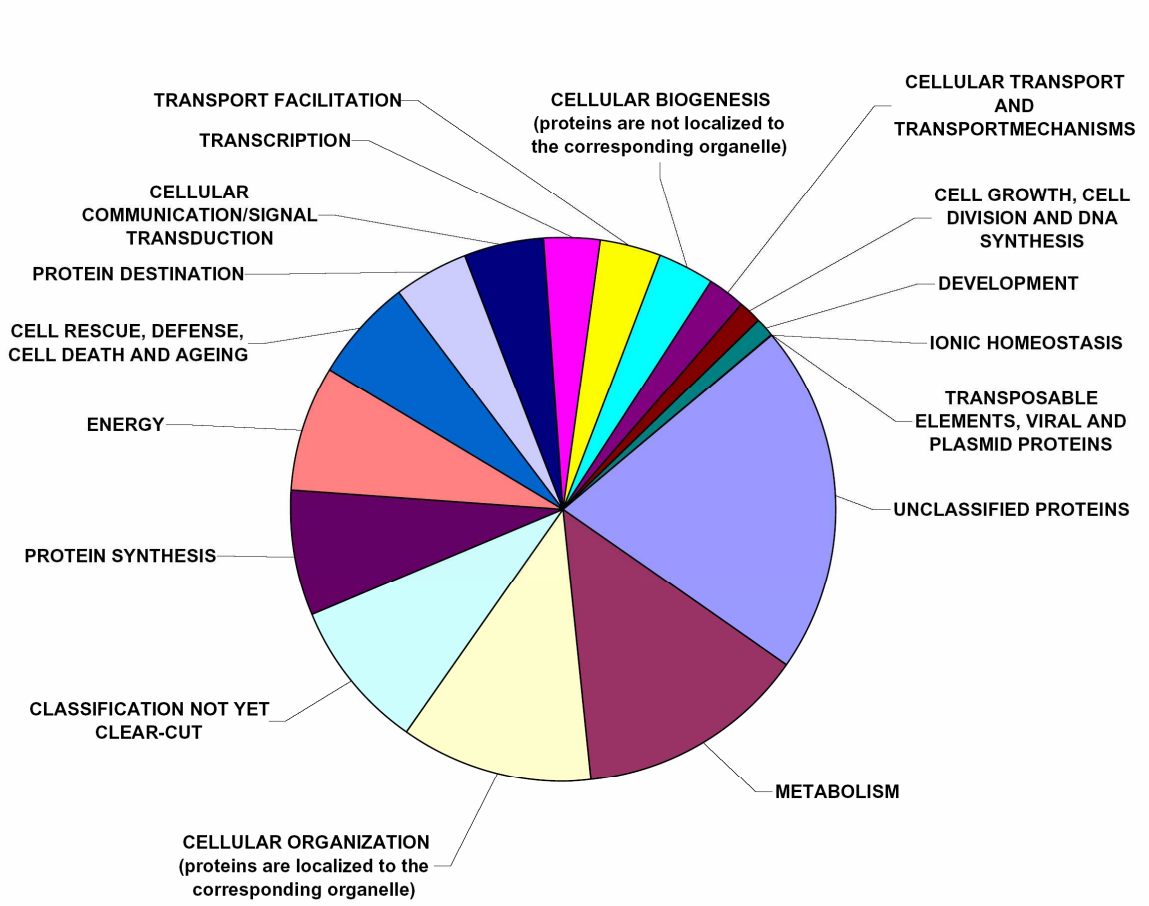

Fig. 16 Functional distribution of ESTs

Table 10 Clusters containing the most redundant EST sequences.

Cluster accession	number of ESTs	AtgCode	Gene description	Expect
C_2346-23-D11	207	At1g67090	ribulose bisphosphate carboxylase-like protein, small subunit	3,00E-64
C_46-4-A11	77	At2g34420	photosystem II type I chlorophyll *a/b* binding protein	1,00E-123
C_2253-22-D06	47	At4g38970	fructose-bisphosphate aldolase-like protein	0
C_2501-25-D11	32	At1g29920	chlorophyll *a/b* binding protein	1,00E-138
C_766-7-G12	31	At2g39730	Rubisco activase	1,00E-164
C_1201-10-G10	30	At4g10340	CP26, *lhcb*5	9,00E-78
C_495-5-F06	29	At2g39730	Rubisco activase	1,00E-79
C_2492-25-D02	29	*psb*C	chloroplast genome - PSII 43 KDa protein (*psbC*)	0
C_3449-84-A03	28	At4g05180	PSII-Q protein (*psbQ2*, OE16)	9,00E-50
C_1462-13-A09	28	At1g79040	unknown protein	1,00E-45
C_391-111-F03	24	At1g06680	PSII-P protein (*psbP1*, OE23)	1,00E-104
C_1303-11-H04	23	At2g34430	putative photosystem II type I chlorophyll *a/b* binding protein.	1,00E-99
C_5189-113-A11	23	At1g31330	PSI-F protein, PSI subunit III (*psaF*)	6,00E-77
C_1749-16-A07	23	At3g61470	Lhca2 protein	1,00E-100
C_3591-86-D03	22	At3g50820	PSII-O protein (*psbO2*, OE33)	1,00E-146
C_3898-88-B01	21	At5g20630	germin-like protein (GLP3b)	2,00E-67
C_5307-94-E09	21	At1g60950	ferrodoxin precursor	1,00E-34
C_1278-11-F03	20	At1g12900	glyceraldehyde 3-phosphate dehydrogenase A, chloroplast precursor, putative	1,00E-167
C_1821-16-G08	19	At1g15820	unknown protein	1,00E-111
C_604-6-G02	18	*psb*D	chloroplast genome - PSII D2 protein (*psbD*)	1,00E-151
C_1535-13-G10	17	At1g42970	putative glyceraldehyde-3-phosphate dehydrogenase	3,00E-82
C_2284-22-G03	17	-	-	
C_660-6-F06	16	At2g21330	fructose bisphosphate aldolase-like protein	4,00E-74
C_3639-85-B09	16	At1g13440	putative protein	1,00E-164
C_815-8-A07	15	At1g56600	galactinol synthase-like protein	1,00E-104
C_4044-89-F11	15	At1g03130	PSI-D protein, PSI subunit II (*psaD2*)	3,00E-82
C_3705-85-H03	14	At5g54270	Lhcb3 chlorophyll *a/b* binding protein (gb|AAD28773.1)	1,00E-102
C_311-111-B07	14	At2g30570	PSII-W protein (*psbW*, 6.1 kDa PSII protein)	3,00E-34

Table 10 continued

Cluster accession	number of ESTs	AtgCode	Description	Expect
C_5142-112-E06	14	At5g54770	thiazole biosynthetic enzyme precursor (ARA6) (sp|Q38814)	2,00E-79
C_3439-83-H05	14	At3g01500	chloroplast carbonic anhydrase, precursor	1,00E-121
C_1797-16-E08	13	At4g37930	glycine hydroxymethyltransferase-like protein	1,00E-114
C_1708-15-F02	13	-	-	
C_4678-97-C02	12	At4g04640	ATP synthase subunit gamma (*atpC1*)	4,00E-98
C_5199-113-B09	12	At3g47470	light-harvesting chlorophyll *a/b* binding protein (*cab*4)	1,00E-113
C_1202-10-G11	12	At2g06520	putative PSII-X protein (PSII subunit, *psbX*)	2,00E-14
C_943-8-C09	12	At1g12900	chloroplast glyceraldehyde 3-phosphate dehydrogenase A, precursor, putative	8,00E-83
C_1573-14-B11	12	At2g36830	putative aquaporin (tonoplast intrinsic protein gamma)	5,00E-75
C_1290-11-G03	12	At5g37600	glutamate-ammonia ligase	1,00E-85
C_749-7-F07	11	At5g01530	Lhcb4 protein (CP29, Lhcb4.1)	1,00E-129
C_179-81-E03	11	At1g61520	PSI type III chlorophyll *a/b* binding protein	1,00E-123
C_2287-22-G06	11	At5g64040	photosystem I reaction centre subunit *psa*N, precursor (PSI-N) (sp|P49107)	9,00E-56
C_1098-9-G03	11	At1g44575	photosystem II chlorophyll-binding protein *psbS*	2,00E-71
C_4674-97-B09	11	At3g09390	metallothionein-like protein (AtMT-K)	5,00E-12
C_5290-94-D04	11	At4g12800	PSI-L protein (*psaL*, PSI subunit XI)	1,00E-77
C_3809-87-C12	11	-	-	
C_5350-115-B05	10	-	-	
C_3443-83-H09	10	At3g16140	PSI-H protein (*psaH1*, PSI subunit VI)	3,00E-45
C_4896-99-G04	10	At4g28750	photosystem I subunit PSI-E (*psaE1*)	4,00E-26
C_4612-96-E05	9	-	-	
C_469-111-E06	9	At1g30380	photosystem I subunit K (*psaK*)	1,00E-46
C_2290-22-G09	9	At4g01150	unknown	1,00E-36
C_2222-21-G10	9	At3g53420	plasma membrane intrinsic protein 2a	4,00E-94
C_1366-12-E03	9	-	-	
C_4797-98-F09	9	At1g51400	unknown protein	1,00E-12
C_1649-15-A03	8	At1g29930	unknown protein	1,00E-131
C_1721-15-G03	8	At4g35100	plasma membrane intrinsic protein PIP3	1,00E-69

Table 10 continued

Cluster accession	number of ESTs	AtgCode	Description	Expect
C_3913-88-C06	8	At4g09650	ATP synthase delta subunit (*atpD*)	1,00E-62
C_3805-87-C08	8	At3g12780	phosphoglycerate kinase-like protein	4,00E-73
C_3349-83-B05	8	-	-	
C_3535-84-G10	8	At1g08380	PSI-O protein (PSI subunit, *psaO*)	1,00E-55
C_4625-96-F06	7	At1g07920	elongation factor 1-alpha	1,00E-171
C_2590-26-F11	7	At1g32060	phosphoribulokinase (EC 2.7.1.19) precursor-like protein	4,00E-76
C_489-5-E12	7	At4g38770	extensin-like protein	2,00E-27
C_2191-21-D05	7	At1g20340	plastocyanin (*petE2*)	2,00E-44
C_1234-11-B07	7	At2g45290	putative transketolase precursor	8,00E-50
C_2388-24-B03	7	At4g18360	glycolate oxidase-like protein	1,00E-111
C_2343-23-D08	7	At1g23310	putative alanine aminotransferase	1,00E-98
C_1795-16-E06	7	At3g56940	putative dicarboxylate diiron protein (CHL27)	1,00E-159
C_4393-93-C02	7	At5g01410	pyridoxine biosynthesis protein-like	2,00E-98
C_1932-18-B04	7	At2g36830	putative aquaporin (tonoplast intrinsic protein gamma)	3,00E-82
C_5220-113-D06	7	At1g55670	photosystem I subunit V, precursor (*psaG*)	5,00E-50
C_5178-112-H12	7	At1g67740	PSII-Y protein (PSII subunit, *psbY*)	8,00E-38
C_86-4-D12	6	At5g35630	chloroplast glutamate-ammonia ligase (EC 6.3.1.2) precursor (clone lambdaAtgsl1) (pir||S18600)	2,00E-64
C_966-9-C04	6	At4g32260	ATP synthase subunit b' (*atpG*)	3,00E-32
C_866-8-E11	6	At5g15950	S-adenosylmethionine decarboxylase (adoMetDC2)	1,00E-17
C_4154-90-G03	6	At5g64840	ABC transporter protein 1-like	4,00E-19
C_3991-89-B06	6	*atp*H	chloroplast genome-ATP synthase subunit III (*atpH*)	1,00E-37
C_3695-85-G05	6	At1g24020	pollen allergen-like protein	2,00E-09
C_1608-14-E10	6	At1g09340	putative RNA-binding protein	6,00E-93
C_1953-18-D02	6	At4g36130	putative ribosomal protein L8	1,00E-117

Comparison was made with *Arabidopsis thaliana* annotation from MIPS.

3.2 GENOTYPING AND LINKAGE ANALYSIS

3.2.1 Genotyping analysis

Three different *Oenothera* species were genotyped using AFLPs, namely cybrid *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III, *Oenothera grandiflora* ssp. *tuscaloosa* BB-III, and *Oenothera grandiflora* ssp. *bellamy* BB-III. The genotyping analysis has shown that single primer combinations can result in up to 35 polymorphic bands that can correspond up to 30% of all bands.

3.2.2 Development of AFLP markers and construction of linkage maps

Genetic linkage maps of *Oenothera* were generated based on 202 AFLP markers and 246 genotyped individuals of an F2 generation obtained from a single F1 progeny of a cross between cybrid *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III, and *Oenothera grandiflora* ssp. *tuscaloosa* BB-III (Fig. 17b, d, e, f, and g). The incompatible *Oenothera elata* ssp. *hookeri*, line *johansen* AA with plastome III exhibits a virescent phenotype (Stubbe 1989). In contrast, the genomic combination AA-I is compatible as is *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III (Fig. 17a-d). The F1 generation did not show an incompatible phenotype and was uniform. The F2 generation segregated incompatible phenotypes, in at least two different types. The first one corresponded to a virescent phenotype which is typical for the AA-III combination (Fig. 17e-middle, Fig. 17f). The incompatibility of the second one was even stronger than the first one. Growth was persistently retarded in plants with the strong incompatible phenotype, and many of these plants were not able to survive under normal greenhouse conditions (Fig. 17e-left and f).

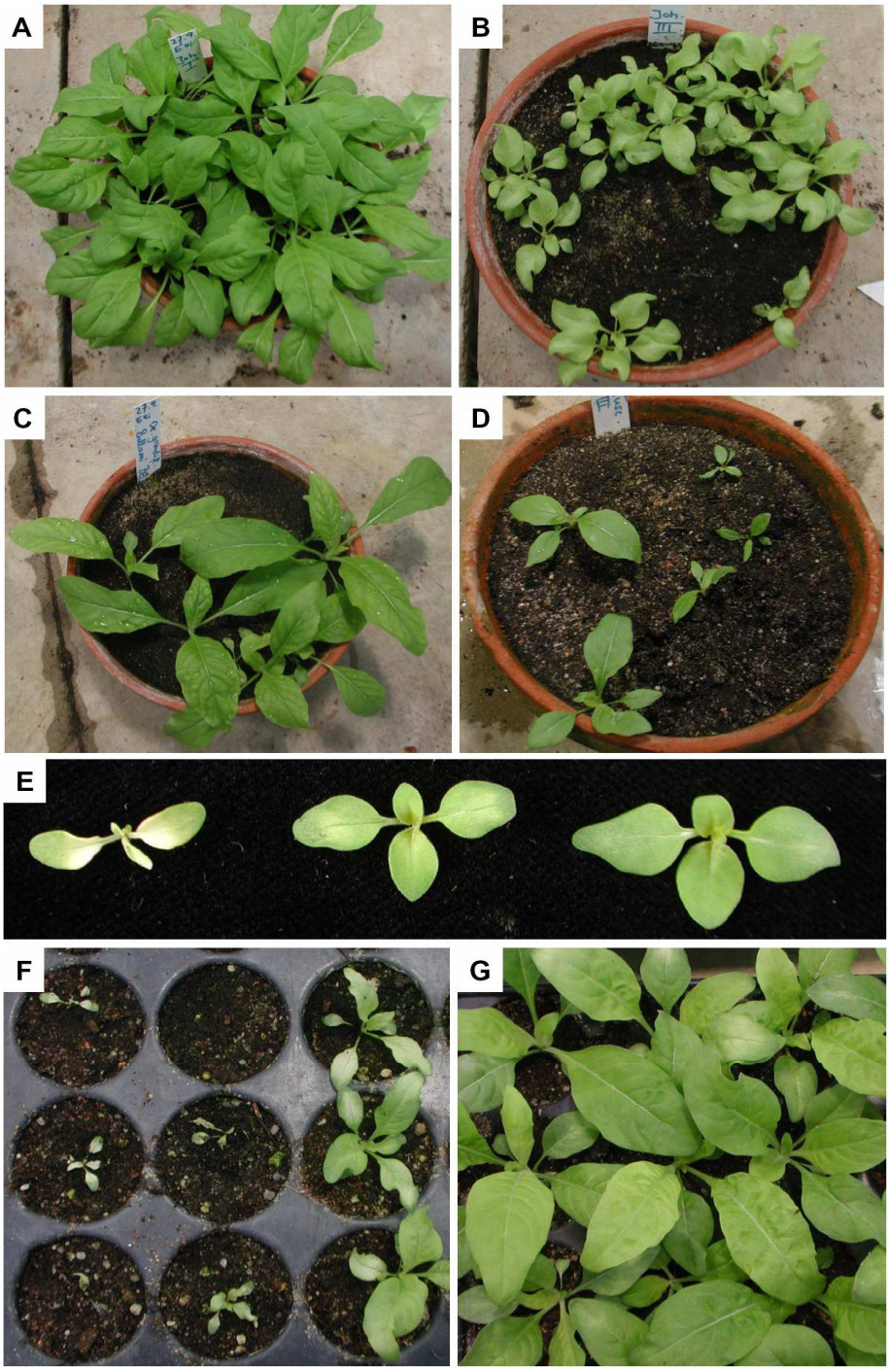

Fig. 17 *Oenothera* plants used in genotyping analyses. **A** *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-I; **B** *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III; **C** *Oenothera grandiflora* ssp. *bellamy*, BB-III; **D** *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III; **E - G** F2 hybrid *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III x *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III

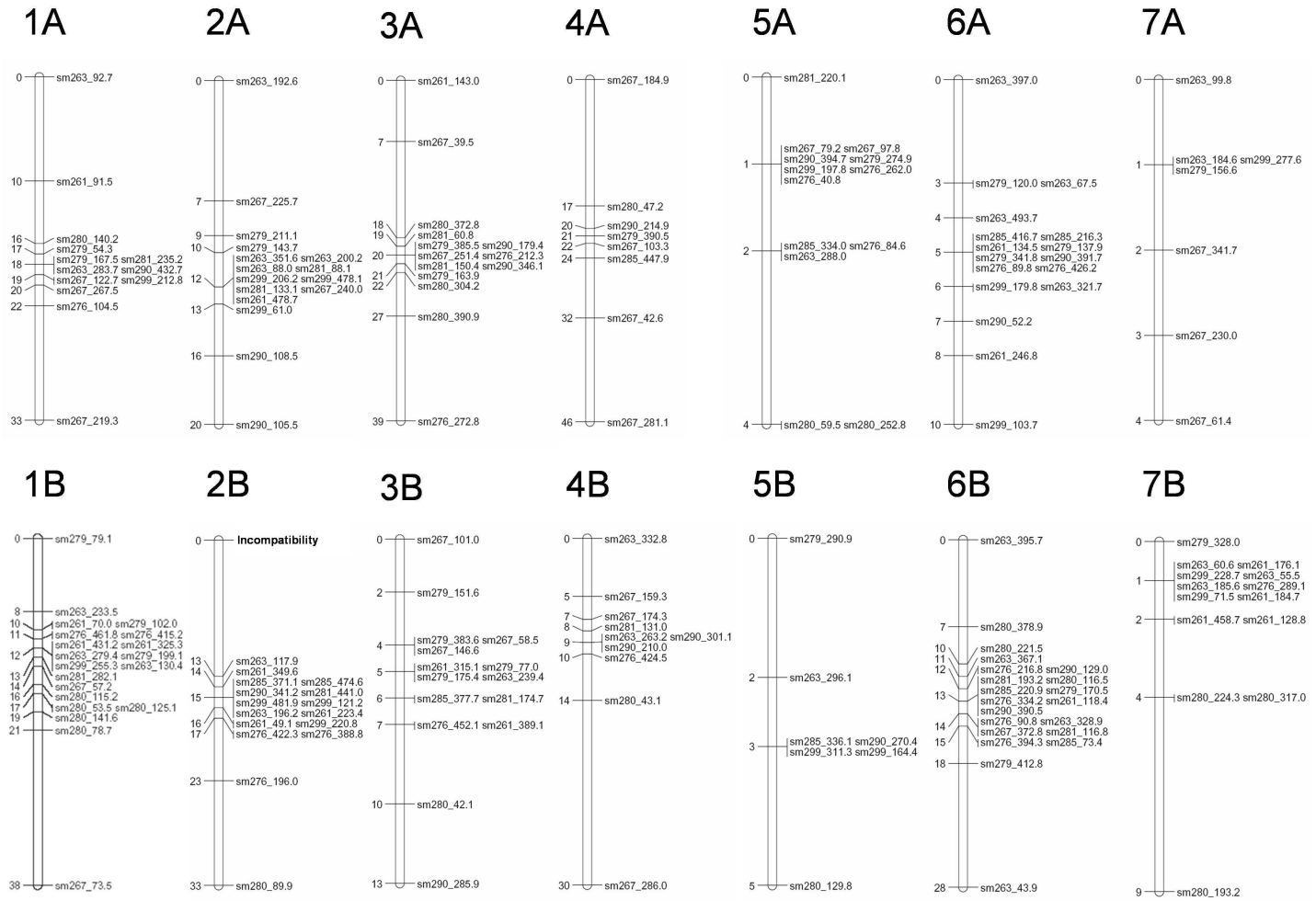

Fig. 18 The first genetic map from *Oenothera*. The first seven coupling groups labeled with A contain dominant markers for the *johansen* genotype. The groups labeled with B represent dominant markers for the *tuscaloosa* genotype.

Table 11 Detail information about markers assigned to map A and B

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
1A	0	sm263_92.7	0	77	0	169	0	5.2	**	
1A	10.192	sm261_91.5	0	74	0	172	0	3.4	*	31.81
1A	15.656	sm280_140.2	0	72	0	172	2	2.6	-	30.56
1A	17.168	sm279_54.3	0	76	0	170	0	4.6	**	48.66
1A	17.690	sm279_167.5	0	73	0	173	0	2.9	*	55.50
1A	17.999	sm281_235.2	0	77	0	169	0	5.2	**	58.14
1A	18.279	sm263_283.7	0	75	0	171	0	4	**	61.67
1A	18.279	sm290_432.7	0	75	0	171	0	4	**	65.70
1A	18.867	sm267_122.7	0	75	0	171	0	4	**	60.73
1A	19.104	sm299_212.8	0	72	0	174	0	2.4	-	59.12
1A	20.369	sm267_267.5	0	71	0	175	0	2	-	50.91
1A	22.230	sm276_104.5	0	78	0	168	0	5.9	**	42.21
1A	32.504	sm267_219.3	0	101	0	145	0	33.8	*******	21.80

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
2A	0	sm263_192.6	0	54	0	192	0	1.2	-	
2A	7.446	sm267_225.7	0	54	0	192	0	1.2	-	16.67
2A	9.217	sm279_211.1	0	64	0	182	0	0.1	-	35.56
2A	9.712	sm279_143.7	0	61	0	185	0	0	-	54.58
2A	11.577	sm263_351.6	0	60	0	186	0	0.1	-	46.28
2A	11.720	sm263_200.2	0	62	0	184	0	0	-	55.51
2A	11.841	sm263_88.0	0	62	0	184	0	0	-	55.39
2A	11.994	sm281_88.1	0	61	0	185	0	0	-	57.61
2A	11.994	sm299_206.2	0	61	0	185	0	0	-	59.84
2A	11.994	sm299_478.1	0	61	0	185	0	0	-	59.84
2A	11.994	sm281_133.1	0	61	0	185	0	0	-	59.84
2A	12.226	sm267_240.0	0	62	0	184	0	0	-	57.61
2A	12.348	sm261_478.7	0	62	0	184	0	0	-	55.39
2A	13.151	sm299_61.0	0	64	0	182	0	0.1	-	52.36
2A	15.556	sm290_108.5	0	55	0	191	0	0.9	-	42.03
2A	20.038	sm290_105.5	0	46	0	200	0	5.2	**	27.87

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
3A	0	sm261_143.0	0	15	0	231	0	46.9	*******	
3A	7.042	sm267_39.5	0	24	0	222	0	30.5	*******	4.40
3A	18.385	sm280_372.8	0	48	0	198	0	4	**	19.71
3A	19.482	sm281_60.8	0	48	0	198	0	4	**	41.11
3A	19.810	sm279_385.5	0	49	0	193	4	2.9	*	50.23
3A	19.813	sm290_179.4	0	49	0	197	0	3.4	*	52.95
3A	19.813	sm267_251.4	0	49	0	197	0	3.4	*	53.34
3A	19.813	sm276_212.3	0	49	0	197	0	3.4	*	53.34
3A	19.996	sm281_150.4	0	48	0	198	0	4	**	50.61
3A	20.443	sm290_346.1	0	51	0	195	0	2.4	-	47.78
3A	21.136	sm279_163.9	0	46	0	200	0	5.2	**	44.37
3A	22.226	sm280_304.2	0	49	0	197	0	3.4	*	41.33
3A	27.037	sm280_390.9	0	39	0	206	1	10.8	****	32.58

Table 11 Continued

3A	39.426	sm276_272.8	0	16	0	230	0	44.9	*******	9.73

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
4A	0	sm267_184.9	0	53	0	193	0	1.6	-	
4A	16.504	sm280_47.2	0	55	0	191	0	0.9	-	7.79
4A	19.988	sm290_214.9	0	55	0	191	0	0.9	-	36.96
4A	20.938	sm279_390.5	0	54	0	192	0	1.2	-	49.78
4A	21.757	sm267_103.3	0	53	0	193	0	1.6	-	49.24
4A	24.330	sm285_447.9	0	62	0	184	0	0	-	41.08
4A	32.120	sm267_42.6	0	36	0	210	0	14.1	******	23.34
4A	45.630	sm267_281.1	0	31	0	215	0	20.2	*******	2.82

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
5A	0	sm281_220.1	0	59	0	187	0	0.1	-	
5A	0.664	sm267_79.2	0	62	0	183	1	0	-	49.60
5A	1.145	sm267_97.8	0	59	0	187	0	0.1	-	53.52
5A	1.440	sm290_394.7	0	60	0	186	0	0.1	-	56.65
5A	1.440	sm279_274.9	0	60	0	186	0	0.1	-	59.35
5A	1.440	sm299_197.8	0	60	0	186	0	0.1	-	59.35
5A	1.440	sm276_262.0	0	60	0	186	0	0.1	-	59.35
5A	1.440	sm276_40.8	0	60	0	186	0	0.1	-	59.35
5A	1.627	sm285_334.0	0	61	0	185	0	0	-	57.14
5A	1.863	sm276_84.6	0	59	0	187	0	0.1	-	55.03
5A	2.197	sm263_288.0	0	60	0	186	0	0.1	-	52.34
5A	3.726	sm280_59.5	0	57	0	189	0	0.4	-	45.56
5A	3.726	sm280_252.8	0	57	0	189	0	0.4	-	57.83

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
6A	0	sm263_397.0	0	105	0	141	0	41	*******	
6A	2.661	sm279_120.0	0	88	0	158	0	15.2	*******	46.19
6A	3.321	sm263_67.5	0	92	0	154	0	20.2	*******	49.81
6A	4.258	sm263_493.7	0	93	0	152	1	21.9	*******	53.93
6A	4.748	sm285_416.7	0	94	0	152	0	22.9	*******	60.25
6A	4.842	sm285_216.3	0	94	0	152	0	22.9	*******	66.04
6A	5.066	sm261_134.5	0	91	0	155	0	18.9	*******	64.63
6A	5.188	sm279_137.9	0	92	0	154	0	20.2	*******	68.00
6A	5.188	sm279_341.8	0	92	0	154	0	20.2	*******	70.62
6A	5.188	sm290_391.7	0	92	0	154	0	20.2	*******	70.62
6A	5.188	sm276_89.8	0	92	0	154	0	20.2	*******	70.62
6A	5.189	sm276_426.2	0	92	0	151	3	21.4	*******	70.01
6A	5.584	sm299_179.8	0	94	0	152	0	22.9	*******	65.80
6A	5.752	sm263_321.7	0	90	0	156	0	17.6	*******	62.92
6A	7.125	sm290_52.2	0	88	0	158	0	15.2	*******	54.84
6A	7.954	sm261_246.8	0	90	0	156	0	17.6	*******	46.90
6A	9.578	sm299_103.7	0	87	0	159	0	14.1	******	41.19

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
7A	0	sm263_99.8	0	100	0	146	0	32.1	*******	

Table 11 Continued

7A	0.879	sm263_184.6	0	96	0	150	0	25.8	*******	64.16
7A	1.094	sm299_277.6	0	98	0	148	0	28.9	*******	67.22
7A	1.233	sm279_156.6	0	97	0	149	0	27.3	*******	69.23
7A	2.073	sm267_341.7	0	96	0	150	0	25.8	*******	64.62
7A	2.866	sm267_230.0	0	94	0	152	0	22.9	*******	62.65
7A	3.616	sm267_61.4	0	97	0	149	0	27.3	*******	61.21

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
1B	0	sm279_79.1	31	0	215	0	0	20.2	*******	
1B	8.146	sm263_233.5	54	0	192	0	0	1.2	-	19.44
1B	9.893	sm261_70.0	48	0	198	0	0	4	**	38.06
1B	10.263	sm279_102.0	54	0	192	0	0	1.2	-	44.55
1B	10.894	sm276_461.8	53	0	193	0	0	1.6	-	42.77
1B	11.183	sm276_415.2	52	0	194	0	0	2	-	55.95
1B	11.622	sm261_431.2	51	0	195	0	0	2.4	-	48.12
1B	11.816	sm261_325.3	52	0	194	0	0	2	-	52.38
1B	11.816	sm263_279.4	52	0	194	0	0	2	-	55.10
1B	11.909	sm279_199.1	52	0	194	0	0	2	-	50.24
1B	12.109	sm299_255.3	52	0	194	0	0	2	-	50.24
1B	12.418	sm263_130.4	54	0	192	0	0	1.2	-	47.36
1B	13.068	sm281_282.1	56	0	190	0	0	0.7	-	45.15
1B	13.560	sm267_57.2	50	0	196	0	0	2.9	*	42.06
1B	15.791	sm280_115.2	47	0	199	0	0	4.6	**	32.45
1B	16.650	sm280_53.5	48	0	198	0	0	4	**	36.65
1B	17.432	sm280_125.1	52	0	194	0	0	2	-	39.82
1B	18.549	sm280_141.6	44	0	202	0	0	6.6	***	31.80
1B	20.551	sm280_78.7	55	0	191	0	0	0.9	-	27.75
1B	37.645	sm267_73.5	52	0	194	0	0	2	-	10.01

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
2B	0	Incompatibility	55	0	191	0	0	0.9	-	
2B	13.020	sm263_117.9	58	0	188	0	0	0.3	-	15.76
2B	14.013	sm261_349.6	56	0	190	0	0	0.7	-	46.18
2B	14.651	sm285_371.1	55	0	191	0	0	0.9	-	46.87
2B	14.785	sm285_474.6	57	0	189	0	0	0.4	-	53.01
2B	15.215	sm290_341.2	56	0	190	0	0	0.7	-	50.84
2B	15.338	sm281_441.0	58	0	188	0	0	0.3	-	53.53
2B	15.482	sm299_481.9	57	0	189	0	0	0.4	-	53.01
2B	15.482	sm299_121.2	57	0	189	0	0	0.4	-	57.83
2B	15.482	sm263_196.2	57	0	189	0	0	0.4	-	57.83
2B	15.482	sm261_223.4	57	0	189	0	0	0.4	-	57.83
2B	15.774	sm261_49.1	56	0	190	0	0	0.7	-	55.12
2B	16.092	sm299_220.8	55	0	191	0	0	0.9	-	54.59
2B	16.910	sm276_422.3	56	0	190	0	0	0.7	-	46.87
2B	17.294	sm276_388.8	57	0	189	0	0	0.4	-	55.12
2B	22.557	sm276_196.0	53	0	193	0	0	1.6	-	49.26
2B	32.929	sm280_89.9	64	0	182	0	0	0.1	-	17.45

Table 11 Continued

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
3B	0	sm267_101.0	62	0	184	0	0	0	-	
3B	1.959	sm279_151.6	76	0	170	0	0	4.6	**	36.22
3B	3.659	sm279_383.6	70	0	176	0	0	1.6	-	45.25
3B	4.168	sm267_58.5	72	0	174	0	0	2.4	-	49.33
3B	4.475	sm267_146.6	73	0	173	0	0	2.9	*	62.29
3B	5.134	sm261_315.1	73	0	173	0	0	2.9	*	56.25
3B	5.334	sm279_77.0	72	0	174	0	0	2.4	-	62.29
3B	5.334	sm279_175.4	72	0	174	0	0	2.4	-	64.59
3B	5.439	sm263_239.4	73	0	173	0	0	2.9	*	62.29
3B	5.577	sm285_377.7	71	0	175	0	0	2	-	60.22
3B	6.168	sm281_174.7	68	0	178	0	0	0.9	-	57.59
3B	6.567	sm276_452.1	71	0	175	0	0	2	-	50.38
3B	7.400	sm261_389.1	72	0	172	0	2	2.6	-	52.07
3B	9.677	sm280_42.1	74	0	172	0	0	3.4	*	40.63
3B	13.346	sm290_285.9	63	0	183	0	0	0.1	-	28.42

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
4B	0	sm263_332.8	84	0	162	0	0	11	*****	
4B	4.593	sm267_159.3	64	0	182	0	0	0.1	-	31.27
4B	7.301	sm267_174.3	68	0	178	0	0	0.9	-	47.67
4B	8.083	sm281_131.0	65	0	181	0	0	0.3	-	52.40
4B	8.524	sm263_263.2	67	0	179	0	0	0.7	-	57.79
4B	8.525	sm290_301.1	67	0	178	0	1	0.7	-	62.42
4B	8.776	sm290_210.0	68	0	178	0	0	0.9	-	60.16
4B	9.933	sm276_424.5	67	0	179	0	0	0.7	-	52.46
4B	14.475	sm280_43.1	69	0	177	0	0	1.2	-	36.01
4B	29.724	sm267_286.0	67	0	179	0	0	0.7	-	12.20

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
5B	0	sm279_290.9	52	0	194	0	0	2	-	
5B	1.974	sm263_296.1	57	0	189	0	0	0.4	-	41.07
5B	2.977	sm285_336.1	54	0	192	0	0	1.2	-	51.12
5B	2.977	sm290_270.4	54	0	192	0	0	1.2	-	56.23
5B	3.302	sm299_311.3	53	0	193	0	0	1.6	-	53.51
5B	3.302	sm299_164.4	53	0	193	0	0	1.6	-	55.67
5B	4.960	sm280_129.8	54	0	192	0	0	1.2	-	45.81

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
6B	0	sm263_395.7	42	0	204	0	0	8.2	****	
6B	6.529	sm280_378.9	38	0	197	0	11	9.8	****	10.02
6B	9.734	sm280_221.5	34	0	212	0	0	16.4	*******	23.89
6B	11.343	sm263_367.1	31	0	215	0	0	20.2	*******	26.74
6B	12.163	sm276_216.8	29	0	216	0	1	22.6	*******	35.47
6B	12.410	sm290_129.0	27	0	219	0	0	25.8	*******	29.96
6B	12.699	sm281_193.2	28	0	218	0	0	24.3	*******	31.07
6B	12.889	sm280_116.5	28	0	218	0	0	24.3	*******	33.22
6B	13.186	sm285_220.9	27	0	219	0	0	25.8	*******	35.09

Table 11 Continued

Group	Position	Locus	a	b	c	d	-	X2	Signif.	LOD
6B	13.186	sm279_170.5	27	0	219	0	0	25.8	*******	36.97
6B	13.186	sm276_334.2	27	0	219	0	0	25.8	*******	30.18
6B	13.186	sm261_118.4	27	0	219	0	0	25.8	*******	36.97
6B	13.186	sm290_390.5	27	0	219	0	0	25.8	*******	36.97
6B	13.607	sm276_90.8	28	0	218	0	0	24.3	*******	35.09
6B	13.607	sm263_328.9	28	0	218	0	0	24.3	*******	37.87
6B	13.931	sm267_372.8	27	0	219	0	0	25.8	*******	31.07
6B	14.448	sm281_116.8	30	0	216	0	0	21.5	*******	29.08
6B	14.761	sm276_394.3	26	0	220	0	0	27.3	*******	30.93
6B	15.221	sm285_73.4	27	0	219	0	0	25.8	*******	34.19
6B	17.887	sm279_412.8	36	0	208	0	2	13.7	******	24.88
6B	27.868	sm263_43.9	54	0	192	0	0	1.2	-	13.69
7B	0	sm279_328.0	35	0	211	0	0	15.2	*******	
7B	0.634	sm263_60.6	36	0	210	0	0	14.1	******	37.59
7B	1.041	sm261_176.1	35	0	210	0	1	15	******	41.65
7B	1.043	sm299_228.7	35	0	211	0	0	15.2	*******	43.64
7B	1.043	sm263_55.5	35	0	211	0	0	15.2	*******	43.70
7B	1.043	sm263_185.6	35	0	211	0	0	15.2	*******	43.70
7B	1.043	sm276_289.1	35	0	211	0	0	15.2	*******	43.70
7B	1.194	sm299_71.5	36	0	210	0	0	14.1	******	41.72
7B	1.368	sm261_184.7	34	0	212	0	0	16.4	*******	39.56
7B	1.778	sm261_458.7	37	0	209	0	0	13	******	38.39
7B	2.074	sm261_128.8	33	0	213	0	0	17.6	*******	36.61
7B	4.364	sm280_224.3	35	0	211	0	0	15.2	*******	29.05
7B	4.364	sm280_317.0	35	0	211	0	0	15.2	*******	43.70
7B	8.924	sm280_193.2	25	0	221	0	0	28.9	*******	26.02

significance levels: *:0.1 **:0.05 ***:0.01 ****:0.005 *****:0.001 ******:0.0005 *******:0.0001

Table 12 Relative frequency of alleles in F2 generation.

Genotypes	**A (AA)**	**D (AA,AB)**	**C (AB,BB)**	**B (BB)**
Frequency	50	178	196	67

The allele frequency was normalized to eliminate different numbers of dominant markers for the A and B genomes. Allele A represents the *johansen* genome and B the *tuscaloosa* genome, respectively.

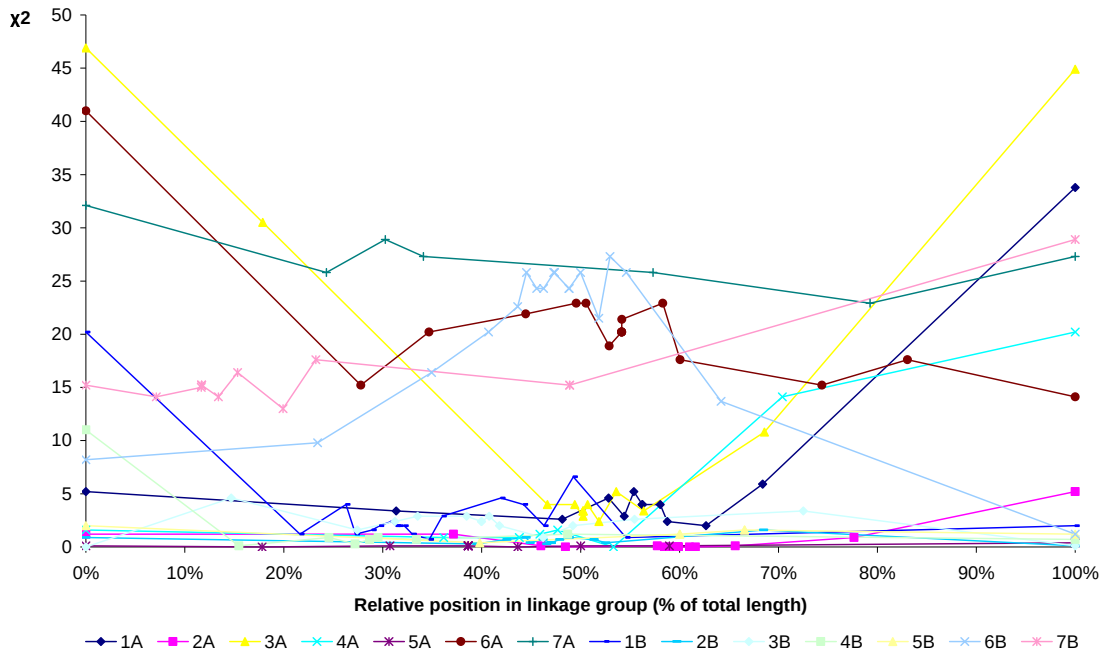

Fig. 19 Summary of allele disturbance in the F2 generation from Table 10. In this chart axis X represents the relative length of linkage group (% of total length) and axis Y the segregation of distortion tested by the chi square test.

The markers were assigned to two genetic maps (Fig. 18). The first linkage map contained 96 dominant markers for the *johansen* AA genome and mapped into seven linkage groups covering a total length of 154.4 cM using the Kosambi function (Kosambi 1944). Eight loci were removed by JoinMap analysis due to internal conflicts within the linkage groups. The second linkage map contained 107 dominant markers for the *tuscaloosa* BB genome present on seven linkage groups covering a total length of 155.3 cM using the Kosambi function. Three loci were removed by JoinMap analysis, again due to internal conflicts within the linkage groups. Detail information for individual marks including genotype frequencies for genotype a (aa), b (bb), c (ab, bb), d (aa, ab) and missing genotypes (-) were assembled into Table 11. The Table also contains data from segregation distortion analysis and linkage relationship analysis among adjacent loci using LOD score.

Segregation distortion was tested against the normal expectation ratio (3:1) using the chi square test. A total of 84 markers (44%) showed significant deviation ($\alpha = 0.05$) from the expected segregation and 68 markers (35%) showed deviations at the higher threshold of $\alpha = 0.005$. Four coupling groups, namely 6A, 6B, 7A and 7B, contain nearly exclusively markers with significant distortion of allele segregation (threshold $\alpha = 0.005$). Five coupling groups (namely 1A, 3A, 4A, 1B and 4B) include at least one marker with significant segregation distortion at the end of the coupling group (Fig. 19). The analyses of allele frequency (Table 12) deduce that calculation of A:B allele ratio in the F2 generation differed from the expected 1:1, instead a ratio of 1:1.15 was found.

3.2.3 Development of co-dominant markers derived from EST sequencing

EST sequences were used to develop co-dominant markers. The first primer has been develop close to 5' end of EST sequence and the second primer in about 300 bp downstream to prevent problems with introns. The PCR products from *Oenothera elata* ssp. *hookeri*, line *hookeri*, AA-I, *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-I, *hybrid Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III, *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III and *Oenothera grandiflora* ssp. *bellamy*, BB-III, were used for sequencing. Detail information about amplified fragments is shown in Table 13. From 15 primer combinations checked, ten were sequenced. The analysis discovered potential EST sequences to develop gene specific co-dominant markers with a reasonable yield of polymorphism. Only one primer combination (M6) did not provide any differences. From eight combinations it was possible to develop CAPS markers, namely A2; A3; M1; M2; M4; M7; M8; M10.

Table 13 Description of amplified fragments used for development co co-dominant markers. Cluster accession represent accession number of cluster in *Oenothera* EST database (Sputnik).

Primer comb.	**Cluster accession**	**Primers used for PCR**	**Gene description**
A1	C_495-5-F06	oen_PSI_1342_for, oen_PSI_1342_rev	rubisco activase
A2	S_1348-12-C09	oen_ATP_1343_for, oen_ATP_1343_rev	sodium-dicarboxylate cotransporter-like
A3	C_1202-10-G11	oen_PSII_1313_for, oen_PSII_1313_rev	putative PSII-X protein (*psbX*)
A4	C_1098-9-G03	oen_psbS_1098_for, oen_psbS_1098_rev	photosystem II chlorophyll-binding protein (*psbS*)
A5	C_5307-94-E09	oen_Reff_1188_for, oen_Reff_1188_rev	ferredoxin
M1	C_1975-18-F05	oenM1for, oenM1rev	delta subunit of mitochondrial F1-ATP synthase
M2	C_4044-89-F11	oenM2for, oenM2rev	PSI-D protein, synonym PSI subunit II (*psaD1*)
M3	C_4496-95-C09	oenM3for, oenM3rev	unknown protein
M4	C_5290-94-D04	oenM4for, oenM4rev	PSI-L protein (*psa*L, PSI subunit XI)
M5	C_966-9-C04	oenM5for, oenM5rev	ATP synthase subunit b' (*atpG*)
M6	S_1155-10-C12	oenM6for, oenM6rev	photosystem II protein W-like
M7	S_4170-90-H07	oenM7for, oenM7rev	fructose-bisphosphatase-like protein
M8	S_3501-84-D07	oenM8for, oenM8rev	putative ribose 5-phosphate isomerase
M9	S_2749-28-E05	oenM9for, oenM9rev	putative protein
M10	S_2579-26-E08	oenM10for, oenM10rev	chlorophyll synthetase-like protein

Two primer combinations (A2 and A3) gave length polymorphism. Two primer combinations (A3, M4) provided polymorphisms between *hookeri* I, *johansen* I or *johansen* III including one primer combination (M4) which showed polymorphism between *johansen* AA-I and *johansen* AA-III. A polymorphism between *bellamy* BB-III and *tuscaloosa* BB-III was found in two primer combinations (A3, M1). Detail information is shown in Table 14 and the data are summarized in Table 15.

Table 14 Detail information about polymorphic bases in individual primer combinations.

Primer comb.	**A2**					**A3**						
Position	69	188	221	278	290	64	89	104	117	142	149	155
***hookeri* AA-I**	+18	G	A	T	G	T	G	G	C	C	G	G
***johansen* AA-I**	+18	G	A	T	G	T	G	T	A	C	A	A
***johansen* AA-III**	+18	G	A	T	G	T	G	T	A	C	A	A
***bellamy* BB-III**	0	A	G	A	A	A	A	T	A	C	G	G
***tuscaloosa* BB-III**	0	A	G	A	A	A	A	T	A	A	G	G

Primer comb.	**A3**								**M1**			
Position	156	163	191	206	242	264	272	393	272	308	388	400
***hookeri* AA-I**	A	C	C	G	T	T	G	+11	C	C	G	A
***johansen* AA-I**	C	T	G	A	T	T	G	+11	N/A	N/A	N/A	N/A
***johansen* AA-III**	C	T	G	A	T	T	G	+11	C	C	G	A
***bellamy* BB-III**	A	T	C	G	G	C	C	0	T	G	G	A
***tuscaloosa* BB-III**	A	T	C	G	G	C	C	0	C	C	T	G

Primer comb.	**M1**									**M2**		**M3**
Position	404	405	406	407	423	428	435	438	451	111	148	48
***hookeri* AA-I**	A	G	T	C	T	C	T	G	C	T	T	G
***johansen* AA-I**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	T	T	G
***johansen* AA-III**	A	G	T	C	T	C	T	G	C	T	T	G
***bellamy* BB-III**	G	A	C	T	G	T	G	T	G	G	C	A
***tuscaloosa* BB-III**	G	A	C	T	G	T	G	T	G	G	C	A

Primer comb.	**M4**		**M7**		**M8**		**M10**		
Position	80	172	21	130	41	189	349	462	464
***hookeri* AA-I**	C	T	A	G	T	T	T	G	T
***johansen* AA-I**	C	T	A	G	T	T	T	G	T
***johansen* AA-III**	G	G	A	G	N/A	N/A	T	G	T
***bellamy* BB-III**	G	T	C	T	N/A	N/A	G	C	G
***tuscaloosa* BB-III**	N/A	N/A	C	T	C	C	N/A	N/A	N/A

Table 15 Summary of polymorphisms between *Oenothera* species. Two different kinds of polymorphism were detected: Single Nucleotide Polymorphism (SNP) and Length Polymorphism (LP)

	hookeri AA-I	*johansen* AA-I	*johansen* AA-III	*bellamy* BB-III
johansen AA-I	8 SNP	X		
johansen AA-III	10 SNP	2 SNP	X	
bellamy BB-III	32 SNP 2 LP	23 SNP 2 LP	34 SNP 2 LP	X
tuscaloosa BB-III	31 SNP 2 LP	22 SNP 2 LP	31 SNP 2 LP	5 SNP

3.3 DETECTION AND INVESTIGATION OF INTERSPECIFIC GENOME-PLASTOME INCOMPATIBILITY IN *PASSIFLORA*

Most *Passiflora* species are heterogamous and self-incompatible. Crossings of various *Passiflora* species (*Passiflora menispermifolia, P. oerstedii., P. chocoenesis, P. edulis, P. malimorfis, P. ´Minai´, P. cearuela,* and *P. actinia*) have shown that all hybrids whose female or male parent plant was *Passiflora menispermifolia,* displayd bleached leaf sectors, the colour varied from pale green through yellow to completely white. Hybrids between *Passiflora menispermifolia* ♀ and *Passiflora oestedii* ♂ show a strong incompatible phenotype during ontogenesis. Besides this, many individuals of the F1 generation contained both white/pale and green leaf sectors and very often even within the same branch or sometime on the same leaf (Fig. 20). The white tissue did not survive after several weeks. When the apical meristem was affected, plant growth was retarded and the plant later died. The hybrid between *P. menispermifolia* ♀ and *P. oestedii* ♂ was used for further investigation. Reciprocal crosses provided the same bleaching phenotype on seedlings (Fig. 20). Surprisingly, we found that the leaf shape in the progeny was expressed more the female parent than the male parent (Fig. 20).

3.3.1 Investigation of *Passiflora* cpDNA

The inheritance pattern of *Passiflora* plastids was investigated. Using three independent approaches, Southern analyses of cpDNA (RFLP), restriction analyses of PCR products from cpDNA (CAPS markers), and sequencing of amplified regions of cpDNA, we detected that the plastids of *Passiflora menispermifolia* (Pm) and *Passiflora oerstedii* (Po) are biparentally transmitted. The DNA of the female parent (Pm), male parent (Po) as well as that of green (G) and white leaf sectors (W) in the F1 generation were analysed separately.

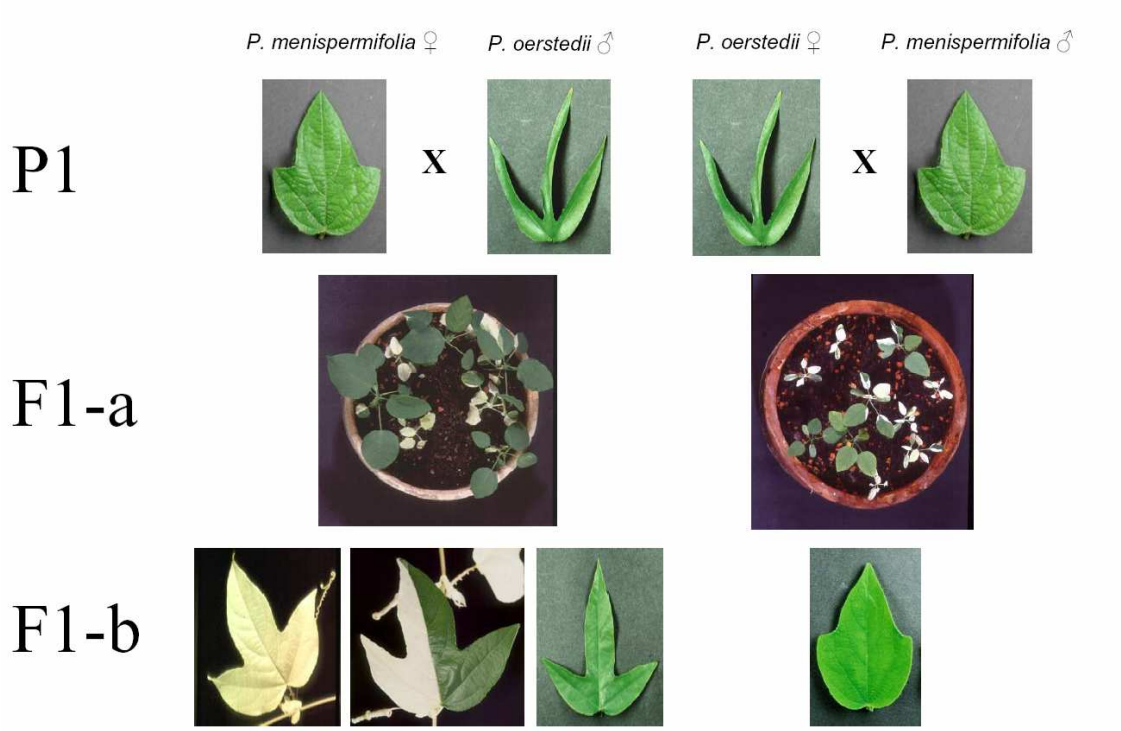

Fig. 20 Leaves of *Passiflora* parent plants (P1), seedlings of F1 hybrids (F1-a) and leaves from mature F1 hybrids (F1-b).

Southern analyses of cpDNA were performed with a PCR probe from the *rpo*B/C operon. The *rpo* operon encodes subunits of the organelle RNA polymerase. The female parent (Pm) and the white hybrid (W) showed the same pattern, the male parent (Po) the same as the green hybrid (G) (Fig. 21).

The one-base pair polymorphism in the *rbcL* (RuBisco) gene was analyzed by sequencing. The sequence provided evidence of a transmission of the cpDNA from the female parent (Pm) to the white hybrid (W) and from the male parent (Po) to the green hybrid (G) (Fig. 22).

The CAPS markers confirmed as well that chloroplasts in *Passiflora* are inherited biparentally. Fragment polymorphism was obtained after the digestion of the 2 kbp PCR product generated from the *rpo*B gene by restriction with VspI. The female parent (Pm) and the white hybrid (W) again show the same restriction pattern, with fragments sizes of 1.7 kbp and 0.2 kbp, respectively. The male parent (Po) and the green progeny (G) showed identical restriction patterns with fragment sizes of 1.0, 0.7, and 0.2 kbp (Fig. 23).

Taken together, these results confirm the occurrence of biparental transmission of plastids in *Passiflora*. The white and green leaf sectors within individual plants of F1 hybrids identified also genome-plastome incompatibility between the progeny genome and the *P. menispermifolia* plastome (Fig. 24).

Plastome similarities between *P. menispermifolia* and *P. oerstedii* were then studied by sequencing analyses. Because sequences of *Passiflora* plastid chromosomes are unknown, sequencing of randomly chosen DNA segments from *P. menispermifolia*, *P. oerstedii* were carried out, using primers designated originally for tobacco, *Arabidopsis*, or *Oenothera*. In addition, new primers were designed for conserved regions of cpDNA. Sequence information has shown that both *P. menispermifolia* and *P. oerstedii* carry expectedly very similar plastomes. 8.5 kb of sequence contains only 15 deleted and 17 single nucleotide polymorphisms between both species.

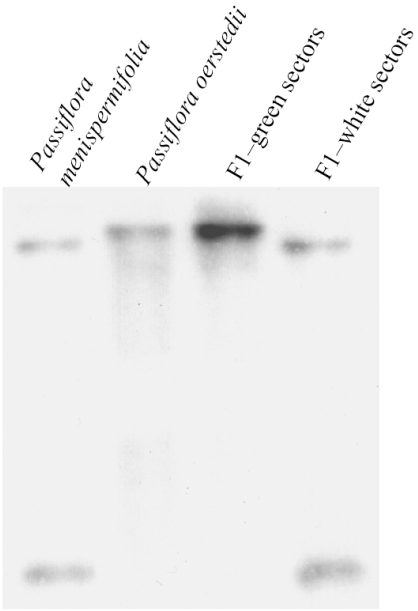

Fig. 21 Southern analysis of total DNA, digested with SalI and transferred onto the membrane. The PCR product from the *rpo*B/C operon obtained with the *rpo*C1p and *rpo*Bp1rev primers was used as a probe.

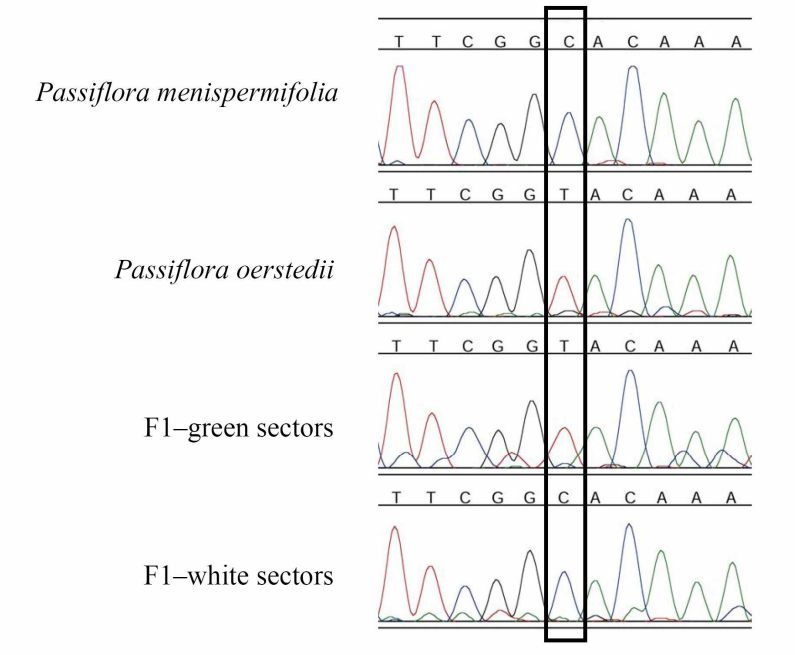

Fig. 22 Comparison of sequences from the *Passiflora rbc*L gene. The PCR product was obtained with the primers ArbcL and rbcL.P2.

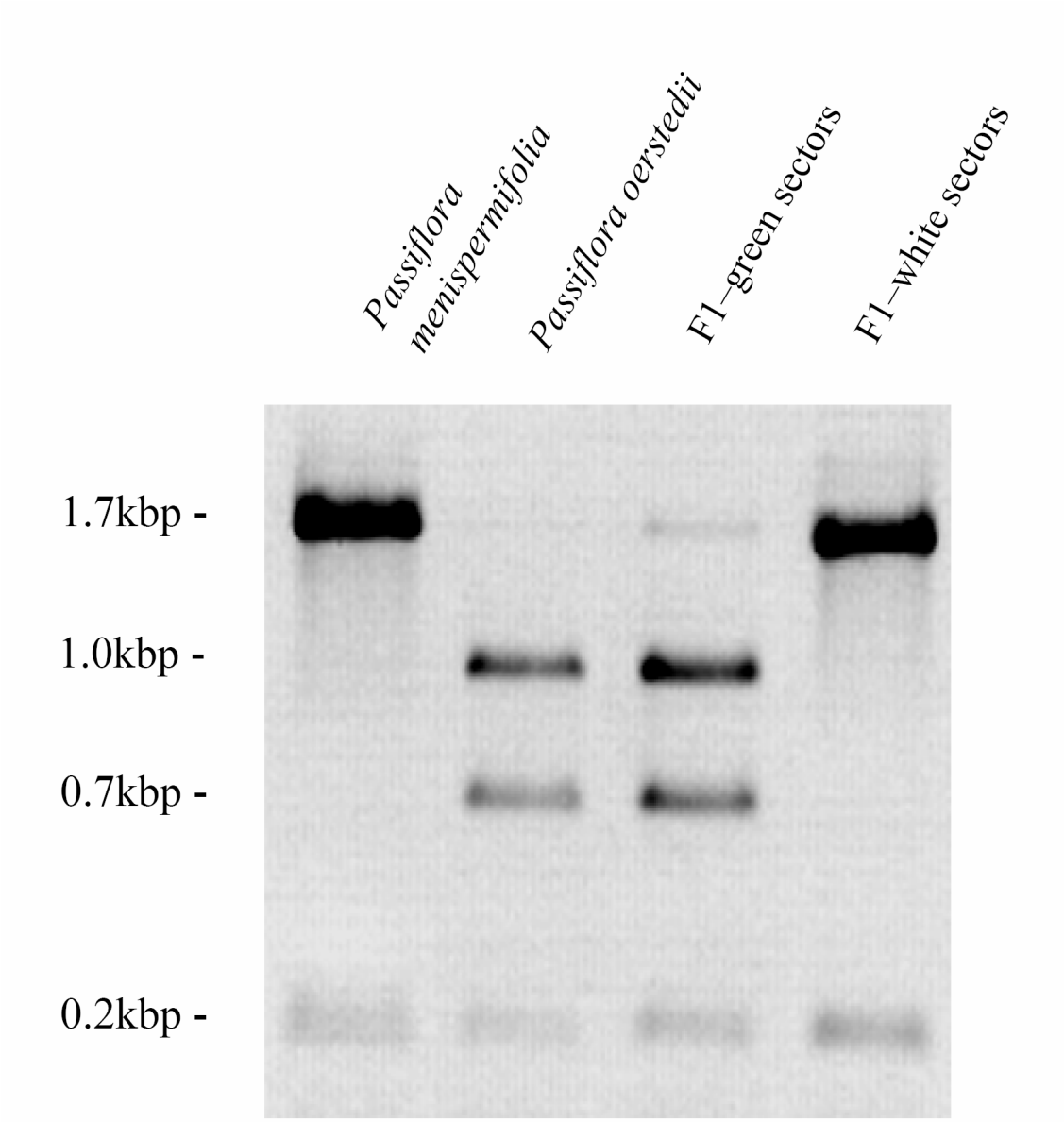

Fig. 23 Digested PCR product, generated from the *rpo*B gene (primers used: rpoB4forP and rpoB5Pfor) with VspI. *P. menispermifolia* plastid DNA cut twice and *P. oerstedii* possesses one additional restriction site.

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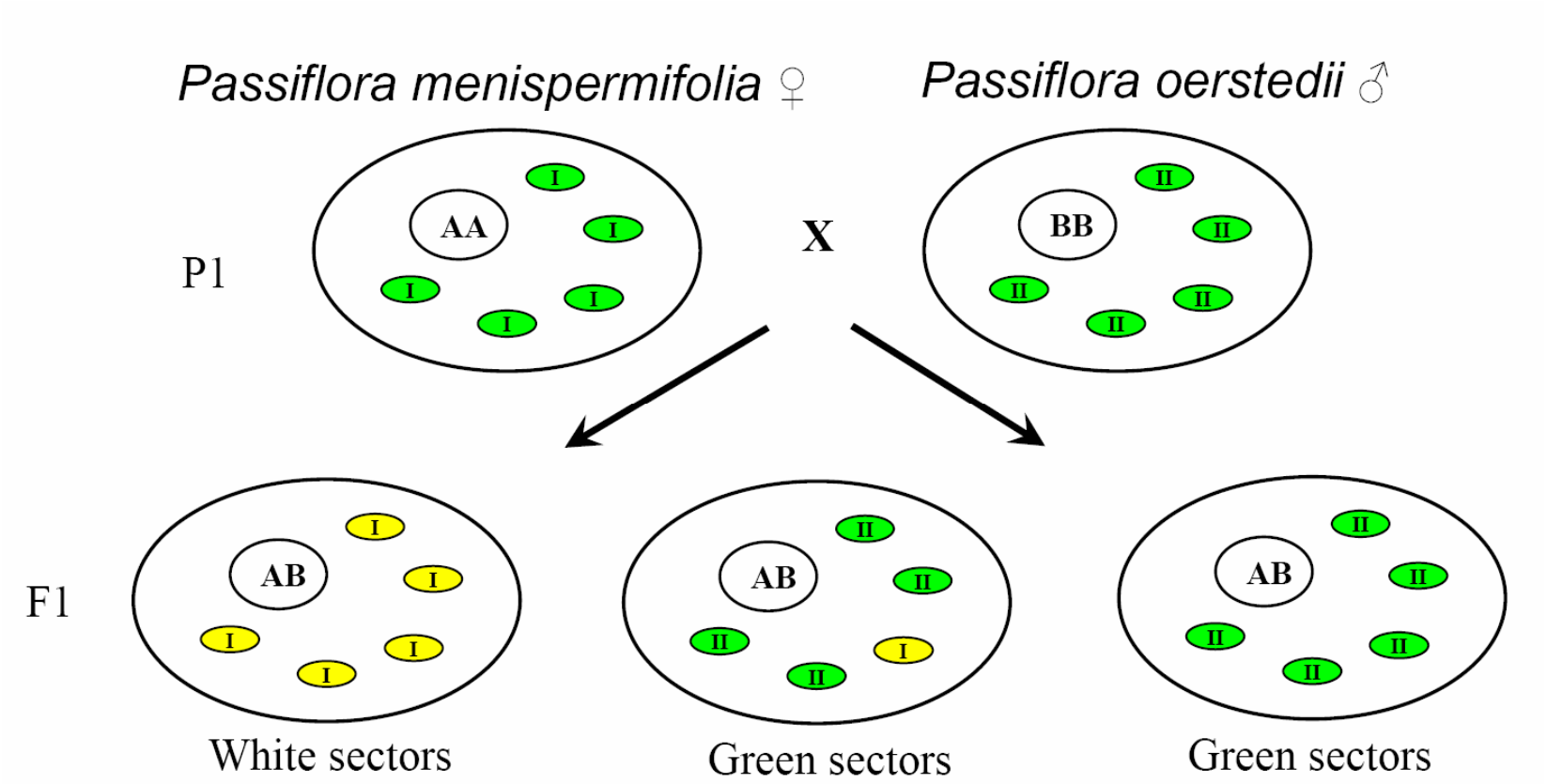

Fig.24 Scheme of genome-plastome incompatibility in the genus *Passiflora*. The genome AB is incompatible with the plastome of *P. menispermifolia* (I), but compatible with the plastid of *P. oerstedii* (II).

3.3.2 Ultrastructure of the *Passiflora* chloroplasts

Electron micrographs of plastids have shown typical lens-shaped fully developed chloroplasts with prominent stroma and grana thylakoids in *P. oerstedii* and in the green sectors of F1 hybrids (Figs. 25B and D). White sectors contain non-differentiated proplastids that accumulate vesicles and rudimentary thylakoid membrane structures (Fig. 25C). Electron micrographs from *Passiflora menispermifolia* (Fig. 25A) show unusual thylakoid formations, probably due to retarded vesicle fusions. Plastids of *P. menispermifolia* significantly accumulated such vesicles. Plastids from same leaf section were found in different developmental stages including proplastids (Figs. 26A and 26B), etioplasts (Fig. 26C) and fully mature plastids that contain grana and stroma thylakoids (Fig. 26D). A picture series depicts a possible dynamics of vesicle fusion heading in thylakoid grana stack formation (Fig. 27), because thylakoid biogenesis was probably retarded due to retarded vesicle fusion.

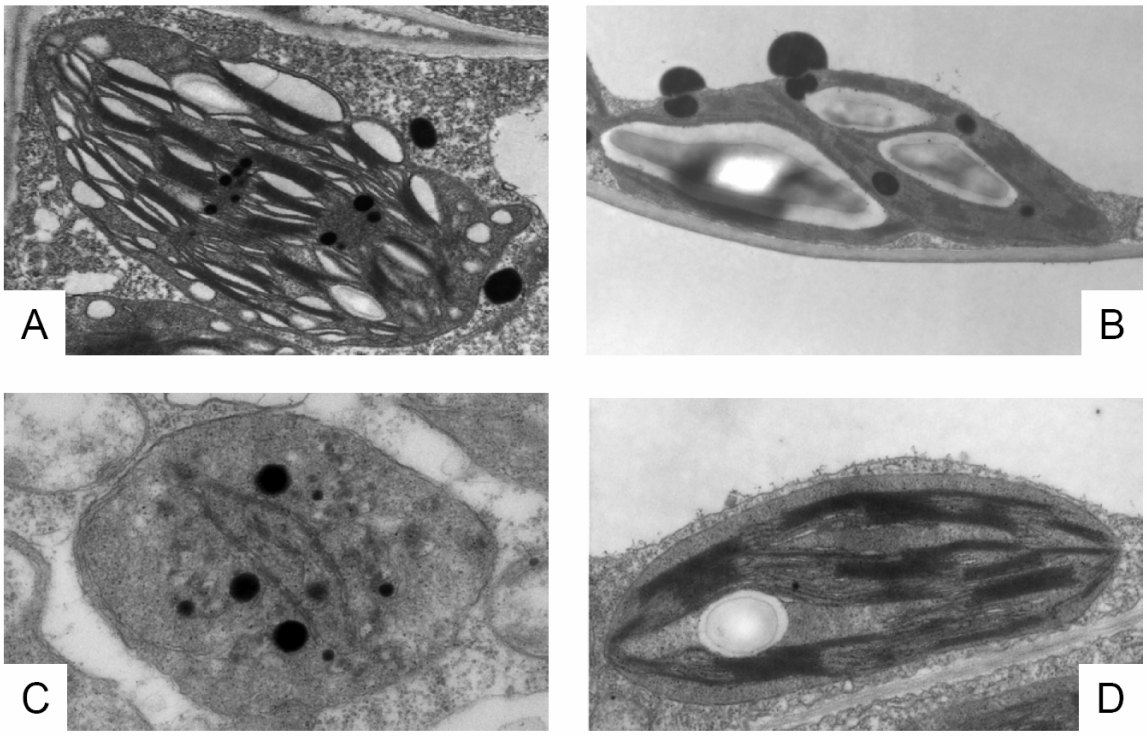

Fig. 25 Electron micrographs of plastids from (A) *Passiflora menispermifolia*, (B) *Passiflora orstedii*, (C) white regions of the F1 hybrid, and (D) green regions of the F1 hybrid.

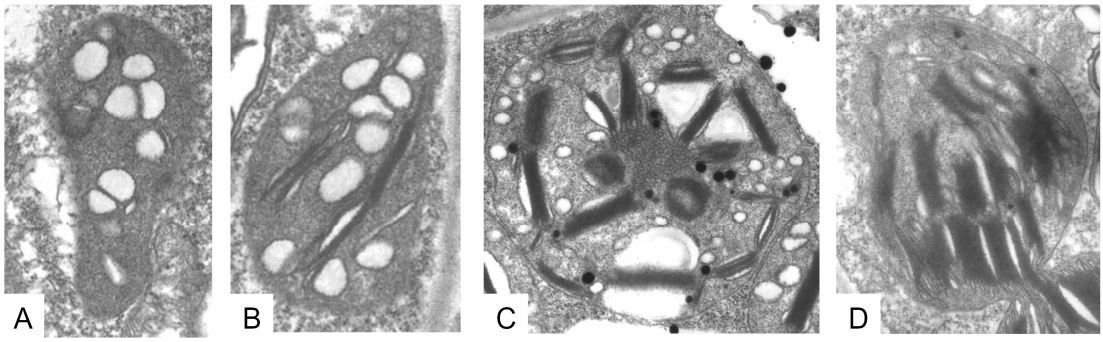

Fig. 26 Plastids from *Passiflora menispermifolia*. (A), (B) proplastids with basic membrane structure and vesicles, (C) etioplast with a characteristic prollamelar body and grana thylakoids, and (D) mature chloroplast with grana and stroma thylakoids and minimum disturbance

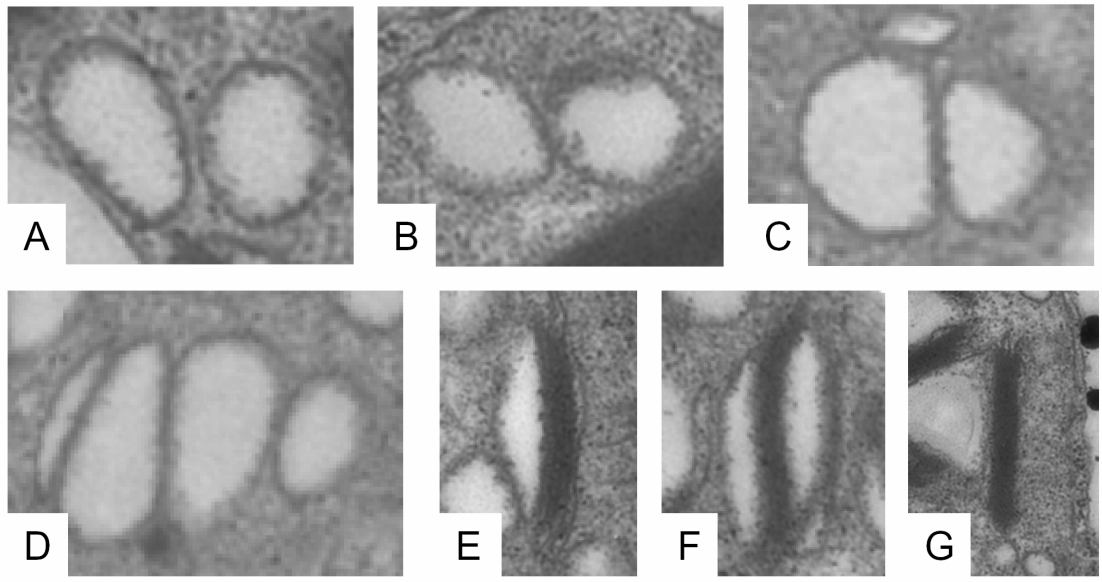

Fig. 27 Different steps of thylakoid membrane biogenesis, taken from plastids of *Passiflora menispermifolia*. Single vesicles (A) attaches another one (B). Then the vesicle shape is flattening (C and D) and this process continues from first occurrence of grana stacks (E and F) to fully formed grana stacks (G).

3.3.3 Northern analysis of *Passiflora* RNA

The following genes were tested by Northern analysis: *psaA* (encoding subunit Ia of PS I), *psbA* (encoding subunit D1 of PSII) and *petA* (encoding subunit cytochrome *f* of the cytochrome *b6/f* complex). Before hybridization blots were stained to check equal loading of RNA estimated on the amount of ribosomal RNA (Fig. 28). Northern analyses showed that the amounts of all RNAs tested were similar in *P. menispermifolia*, *P. oerstedii* and in green sectors of F1 hybrid. The white sectors of the F1 hybrid showed significant lower amounts of *psaA* and *psbA* RNAs. In contrast, *petA* RNA of white sectors accumulated in comparison with parents and green sectors of F1 hybrid (Fig. 29).

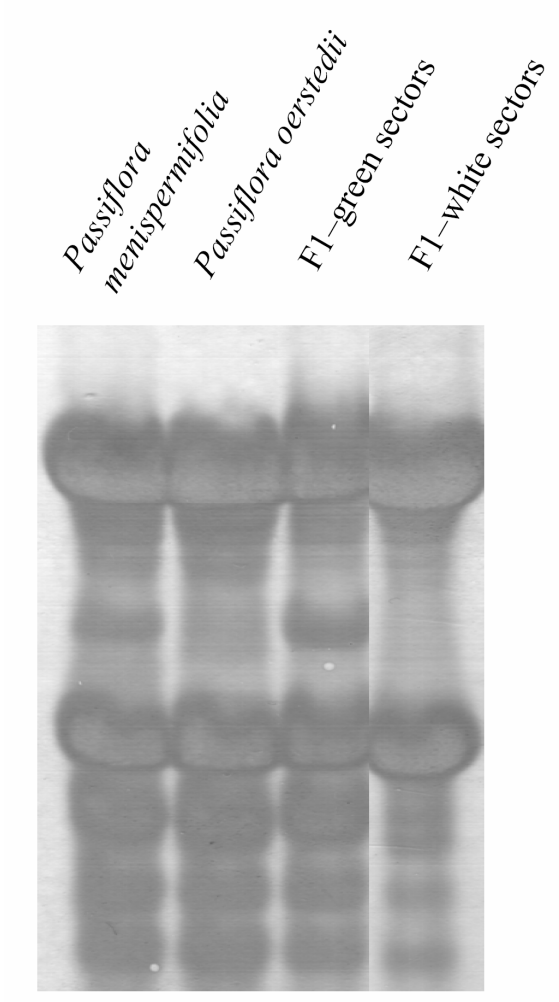

Fig. 28 Stained RNA blot from *Passiflora* to estimate equal concentrations of ribosomal RNA

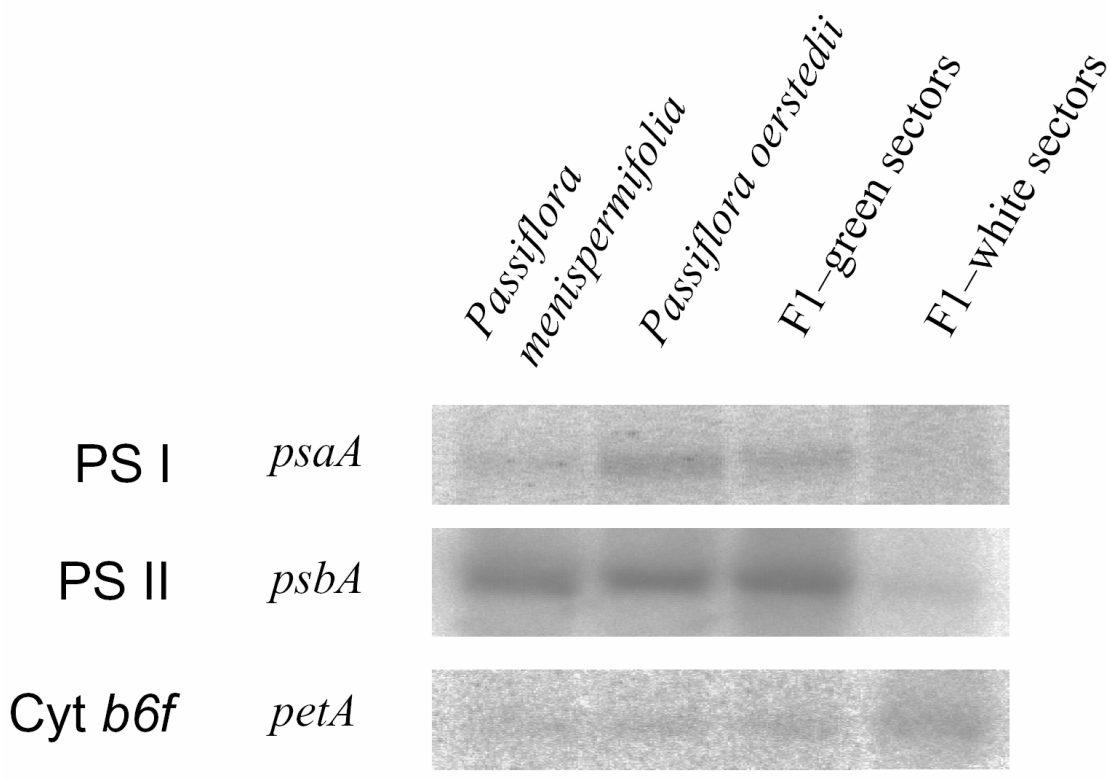

Fig. 29 Northern analysis of RNA from *Passiflora menispermifolia*, *P. oerstedii*, as well as from white and green regions of the F1 hybrid.

3.3.4 Protein analyses of *Passiflora*

3.3.4.1 SDS-PAGE protein gels

SDS-PAGE protein gels have shown various differences in the patterns of membrane and soluble proteins between parents. A soluble protein of 23 kDa, which is abundant in *P. oerstedii*, and green and white sectors of F1 hybrid, was completely missing in *P. menispermifolia*. Membrane proteins were significantly reduced in white sectors of the F1 hybrid in comparison with parents and green sectors (Fig. 30).

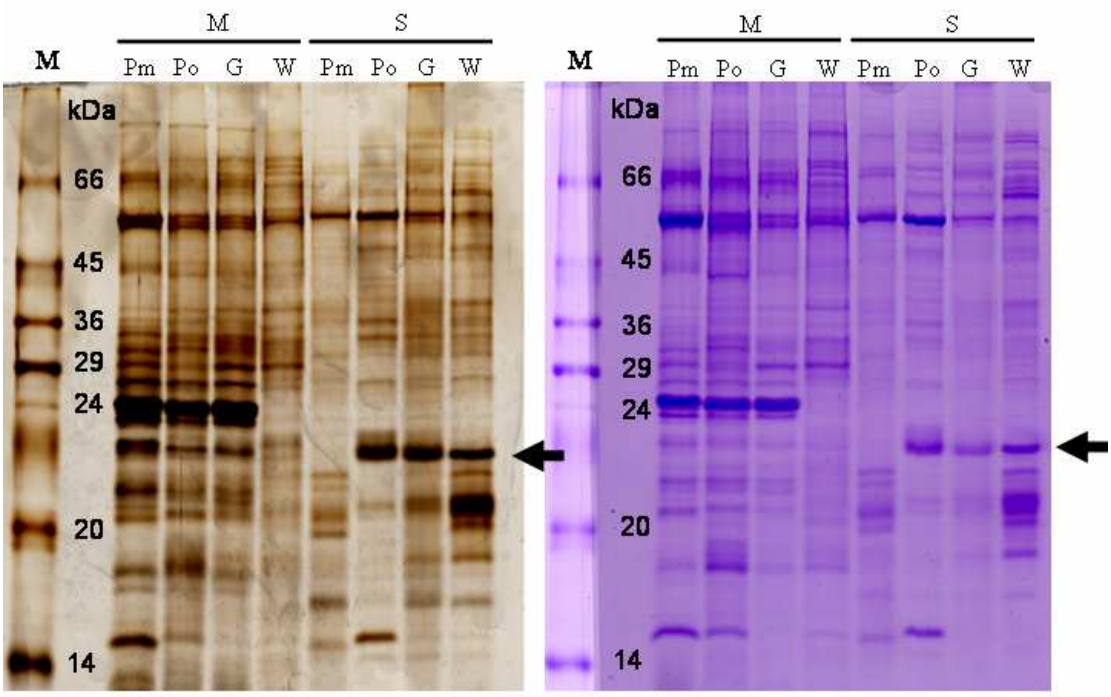

Fig. 30 SDS-PAGE protein gels with silver staining (left) and Coomassie Brilliant Blue staining (right). The gels contain membrane (M) and soluble (S) proteins from *P. menispermifolia* (Pm), *P. oerstedii* (Po), green (G) and white (W) sectors of their F1 hybrid. Arrows show an abundant band which is polymorphic between Pm and Po

3.3.4.2 Western analysis

Different genes encoding polypeptides of PSI, PSII, Cytochrome *b6/f* complex and ATP synthase were tested (Table 16). Western analyses uncovered comparable protein amounts for all proteins tested between *P. menispermifolia*, *P. oerstedii* and green sectors of their F1 hybrid. The plastid encoded proteins of *psaA*, *psaD*, *petB*, and *atpA* were undetectable in white sectors of the F1 hybrid, but the nuclear encoded protein *psbO*, which encodes the extrinsic 33 kDa polypeptide of the oxygen-evolving complex of PSII, was significantly reduced in comparison with parents and green sectors of their F1 hybrid (Fig. 31). The D1 protein of PSII showed only one band in the white sectors of the F1 hybrid in comparison with three bands for green material which could represent precursor or phosphorylated form of D1 which accumulate to a higher level in the white sectors. The plastome encoded protein of *pet*A, which encodes the cytochrome *f* of the cytochrome *b6f* complex, was significantly reduced and the molecular weight of protein was higher than the mature protein presented in

Pm, Po and green sectors of their F1 hybrid. This band probably represents unprocessed cytochrome *f* precursor.

Table 16 Genes used in western analysis of *Passiflora*

Complex	**Gene**	**Poduct**
PSI	*psaA*	P700 chl-a protein of the PSI core
	psaD	ferredoxin-binding protein of PSI
PSII	*psbA*	D1 protein of the PSII core
	psbO	33 kDa OEC protein
Cytochrome *b6f* complex	*petA*	cytochrome *f*
	petB	cytochrome b_6
ATP synthase	*atpA*	α subunit of ATP synthase

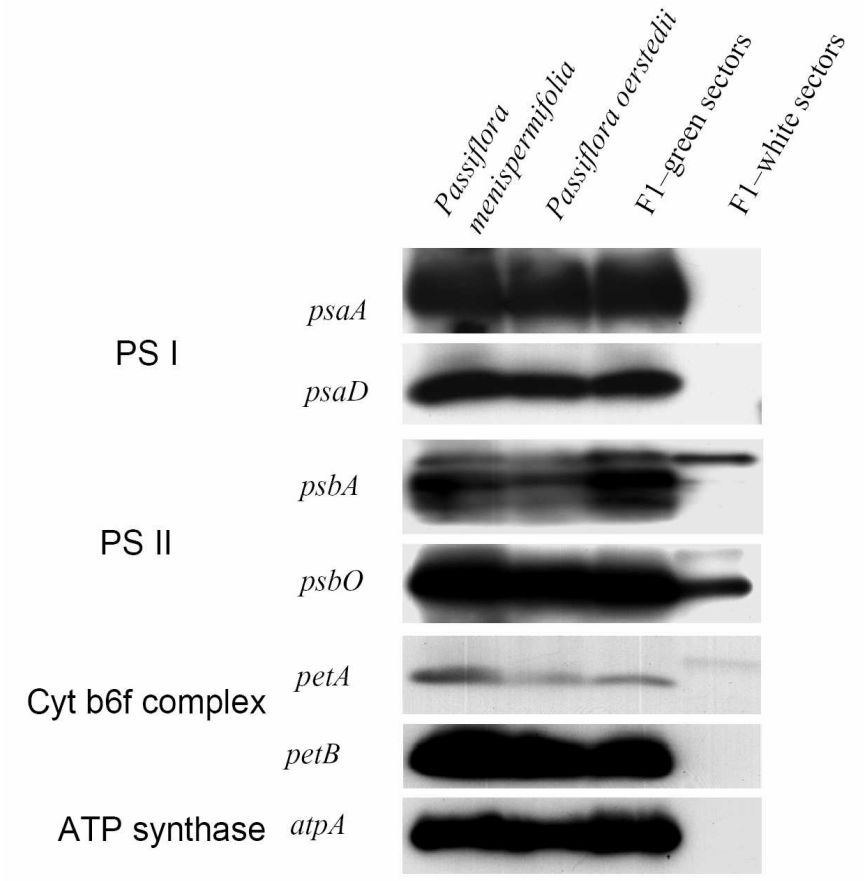

Fig. 31 Western analysis from membrane proteins of *Passiflora*

4 DISCUSSION

4.1 Expressed sequence tags (ESTs)

Oenothera as a model plant offers an advantageous potential in basic research, especially to mechanisms of genome-plastome incompatibility which have not been studied, and can hardly be studied, with other commonly favoured models. The first EST database from *Oenothera* presented in this thesis provides useful information of gene sequences and functions as in *Arabidopsis*, rice, wheat, barley and various other plant models (Delseny et al. 1997; La Rota et al. 2005).

The computational processing of EST sequences has an enormous impact on data redundancy and quality. The redundancy within an EST collection depends on computational methods for the clustering of sequences to reduce the complexity of the dataset while concomitantly improving the overall quality of the data (Rudd et al. 2003). Due to these elements, EST sequence analysis was performed by Sputnik algorithms (http://sputnik.btk.fi/), because this analysis system implements clustering and peptide management, annotation and a data display pipeline, and it is based on SQL databases.

In this study we present the first analyzed EST data set for *Oenothera*. The 3,532 cDNA sequences derived from RNA of 9-week-old *Oenothera* plantlets were assembled into 1,621 non-redundant clusters including 1,133 singletons and 488 multi member unigenes. A total of 875,940 nonredundand nucleotides provide a substantial amount of information. The EST library was generated with the aspect of a high fraction of full length clones. They represented about 75% of all clones in the library.

A significantly large percentage of EST sequences with no similarity or unknown functions illustrate the potential of the strategy to discover new genes. The cDNA library can be directly used for macroarray applications including gene expression studies. Furthermore, EST sequences allowed the development of gene specific PCR-based co-dominant markers (SNPs, CAPS, micro-satellites) as was previously described by La Rota et al. (2005). ESTs can also be used to assemble

BAC libraries and to enable physical mapping of genes (Sterky et al. 1998; Childs et al. 2001; Schibler et al. 2004). Sequence analyses of *Oenothera* ESTs compared to complementary sequences from other *Oenothera* species provide sufficient information to generate phylogenetic trees reflecting the dynamic formation and the diversity of *Oenothera* species.

4.2 Genotyping and linkage analyses in *Oenothera*

In addition to the EST project genotyping and linkage analyses have been performed for several species of the *Oenothera* genus. Genotyping analyses were applied to three *Oenothera* species with A and B genomes namely cybrid *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III, *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III, and *Oenothera grandiflora* ssp. *bellamy*, BB-III, using the AFLP strategy. Analyses detected between 50 and 150 bands per primer combination using two selective bases for the SacI primer and three bases for the MseI primer. Genotyping with 22 prime combinations has shown that the two subspecies with the B genome (*bellamy*, *tuscaloosa*) are more closely related than with the *johansen* A genome (Fig. 32). When *Oenothera* genotyping data were compared with those from *Arabidopsis thaliana*, it can be concluded that *bellamy* and *tuscaloosa* are genetically related like the *Arabidopsis* ecotypes *Columbia* and *Landsberg erecta*, but the genetic distances between the *johansen* genome and species with B genomes were about three times higher (Table 17).

Table 17 Genetic distances between *Oenothera* species and between *Arabidopsis thaliana* ecotypes

	Polymorphic bands *
Oenothera johansen - bellamy	11.3
Oenothera johansen - tuscaloosa	10.8
Oenothera bellamy - tuscaloosa	4.1
Arabidopsis thaliana Col/Ler**	6.3

* represent number of polymorphic bands per primer combination
** (Peters et al. 2001)

From the literature it is known that *Oenothera* species contain relatively small genomes of approximately 1.4 Gb (Bennett et al. 1982). The comparison analyses of

AFLPs between *Oenothera* and *Arabidopsis* obtained from mapping of the *hcf*145 mutant (data not shown) was used to astimate an approximate genome size. According to our genotyping data the *Oenothera* genomes are only six times larger than that of *Arabidopsis* corresponding to size of approximately 750 Mb (6 times 125 Mb).

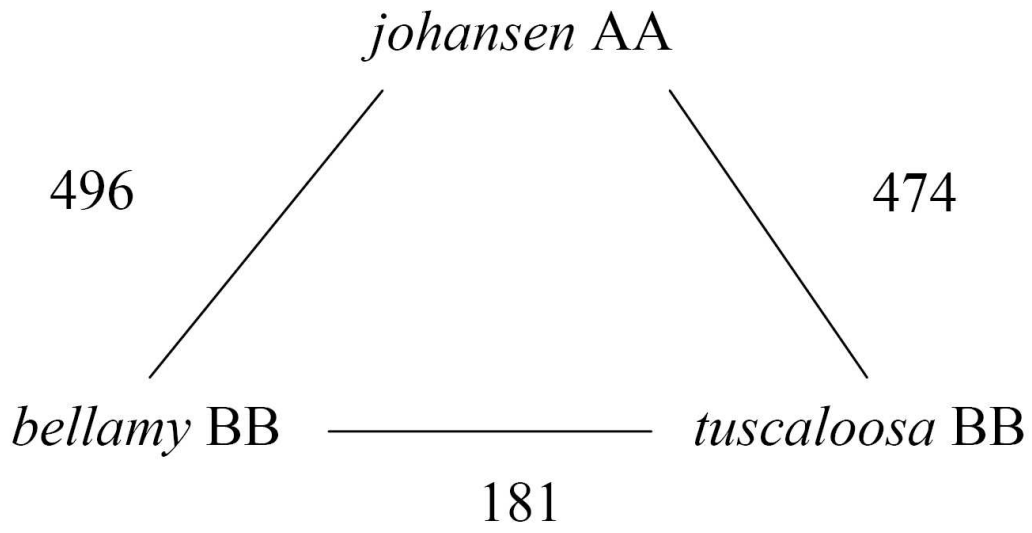

Fig. 32 Genetic distances between three *Oenothera* species. Distances represent value of polymorphic bands in 22 primer combinations.

The AFLP markers were also successfully applied to analyse F2 mapping populations of interspecific hybrids between the cybrid *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III x *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III. Markers were assembled by linkage analysis into 2x seven coupling groups, directly corresponding to the chromosome number in all *Oenothera* species. Therefore, it was concluded that each coupling group represents a single chromosome of *Oenothera*. The first two linkage maps for *Oenothera* contain 88 AFLP markers covering a total map length of 154.4 cM for dominant markers in the *johansen* AA-III genotype (Map A) and 104 AFLP markers and a total size of 155.3 cM for dominant markers in the g*randiflora* BB-III genotype (Map B). The average distance between markers is 1.75 cM for map A, and 1.49 cM for map B. Individual distances differ between single coupling groups from 0.29 cM (Group 5A) to 5.63 cM (Group 4A). Each *Oenothera* chromosome has centromers in almost symmetrical position and the sizes do not differ significantly

(Harte 1994). The uniform karyotype is the reason why individual chromosomes cannot be readily identified without hybridization with chromosome specific probes. This observation does not correlate with the present results of the genetic map because the smallest and largest sizes registered for coupling groups are 3.7 cM for Group 7A and the 45 cM for Group 4A. Both *Oenothera, johansen* AA-III and *tuscaloosa* BB-III, have the same chromosomal arrangements, therefore the F1 hybrid should assembly chromosomes during meiosis in seven bivalents and they should allow normal homologous recombination. The recombination frequencies vary significantly between species as well as along single chromosomes (Anderson and Stack 2002). The high frequency of recombination correlates with gene-rich regions rather than non-coding regions. This is probably related to higher levels of DNA sequence conservation of CDS that allow successful homology searching which is followed by the occurrence of crossing-over (Okagaki and Weil 1997). The inhibition of crossing-over in coupling groups 5A, 5B, 7A, and 7B may correspond to lower homology of A and B genomes and mainly variability of homology on chromosome sequences in singular bivalents of the F1 hybrid.

Analyses of allele distribution have shown significant disturbances in transmission ratios on several loci. Distorted transmission ratios are typical for interspecific hybrids in general (Zamir and Tadmor 1986; Fishman et al. 2001) and can result from selection or mortality (negative selection) acting at different stages of the life cycle (Schwarz-Sommer et al. 2003). The genome *Oenothera* provides a variety of causes that could explain these observations such as gametophytic and zygotic lethal factors, which contribute or ensure the stability of complex-heterozygous species. When selection acts solely on the haploid state of F1 gametes or gametophytes, it will affect the frequency of an allele in the F2 generation. When it acts after pollination due, for example, to self-incompatibility, it will affect one of the homozygote classes disproportionately (Schwarz-Sommer et al. 2003). Analysis of allele frequency showed that the A:B allele ratio in the F2 generation was not the expected 1:1 but 1:1.15 which demonstrates a predominance of *grandiflora* genotypes in the F2 hybrids. This finding corresponds with the prediction that incompatibility caused by the *johansen* AA genotype decreases viability in comparison with the heterozygotic AB and homozygotic BB genotypes. The reasons for segregation

distortion are varied and more complex than genome-plastome incompatibility alone would support. They could belong to the following factors: nonrandom population, selections, incompatibilities, or gametophytic and zygotic lethal factors. We can also conclude that selection acts already at the haplophase, because allele frequencies differed from expected ration in F2 generation but there were no effects on frequencies of homozygote genotypes (Table 12).

Analysis of *Oenothera* species with A and B genomes shows two main aspects: (i) subspecies are much more closely related than species with different genomes. The description of *Oenothera* genomes based on genome-plastome interactions (Stubbe 1959) corresponds directly with our genotyping analysis. (ii) Even between plants within same subspecies which differ only in the plastome, like *johansen* AA-I and *johansen* AA-III, it is possible to detect genetic polymorphisms. The naturally occurring *johansen* possesses genome AA with plastome I. The species with other plastomes were created artificially. The prediction that ring formation during meiosis results in restriction of crossing-over and consequential preservation of parental genomes were applied in hybridisation program (Stubbe 1989). Therefore, new artificial species should have identical nuclear genome to original species and they should only differ in the type of plastome. The sequence analyses of *johansen* AA-I and *johansen* AA-III provide evidence of recombination between parental genomes during development of artificial species in the hybridisation program.

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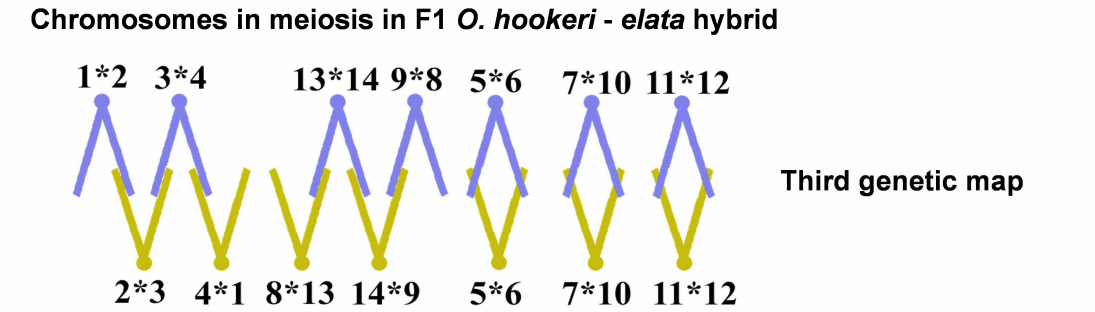
The segregation analyses made it possible to assign the genome-plastome incompatibility to the locus on the edge of coupling group 2B with 13 cM distance to the next AFLP marker. This represents a first step in the identification of primary causes for genome-plastome incompatibility. The physical mapping of the incompatibility (responsible gene(s) for incompatibility) is possible by the assembly of the AFLP map, co-dominant markers derived from ESTs, and EST clones with BAC libraries.

	Complex	Chromosomal arrangement
First genetic map	*O. hookeri* ^{h}*Johansen*	1•2 3•4 5•6 7•10 9•8 11•12 13•14
	O. grandiflora ^{h}*Tuscaloosa*	1•2 3•4 5•6 7•10 9•8 11•12 13•14
Second genetic map	*O. elata* ^{h}*Toluca*	1•4 3•2 5•6 7•10 9•14 11•12 13•8
	O. elata ^{h}*Chapultepec*	1•4 3•2 5•6 7•10 9•14 11•12 13•8
Third genetic map	*O. hookeri* ^{h}*Johansen*	1•2 3•4 5•6 7•10 9•8 11•12 13•14
	O. elata ^{h}*Toluca*	1•4 3•2 5•6 7•10 9•14 11•12 13•8



The chromosomal configuration of hybrid is 2 circles with 4 chromosomes each and 3 bivalents. The F2 generation will therefore show 5 linkage groups instead of 7.

Fig. 33 *Oenothera* genotyping program for localization of reciprocal translocations followed by the study of affects of ring conformation on recombination.

Oenothera, as a model plant, could provide a new stage in research. The EST project followed by genotyping analysis enlarges knowledge not only in discovering primary causes of genome-plastome incompatibility. *Oenothera* chromosomes with arm translocations and the occurrence of a variety of chromosomal arrangements provide a tool to study speciation processes and species evolution in a continental dimension. Genetic maps complemented by gene specific markers derived from ESTs represent a first basic stone for the localization of the positions of chromosome translocations and the study of ring conformations. The program, summarized in Fig. 33, supposes to analyse differences between first and second genetic maps. The analyses should provide information of positions of arm translocations by assignment of some markers to different coupling groups. Markes which will be moved represent

translocated arms of chromosomes. The comparison of recombination frequencies of the first two genetic maps with a third one should answer another question about the impact of ring formation on the occurrence of crossing-over. The AFLP map or specific AFLP markers can also be used together with ESTs for alignment of BAC libraries and then they can be applied to physical gene mapping. When an *Oenothera* genome will be completely sequenced, *in silico* analyses would enable the direct assignment of AFLPs into the physical map (Peters et al. 2001). Understanding the basic principles of chromosome translocations and chromosomal ring conformations can uncover species formation in the *Oenothera* family and speciation processes in general.

4.3 Genome-plastome incompatibility in *Passiflora*

Passiflora is an important genus in fruit production industry and pharmacy (Perry et al. 1991; Echeverry et al. 1991). In this study, we present first evidence for hybrid bleaching in this genus. The hybrid between *Passiflora menispermifolia x Passiflora oerstedii* showed bleaching regions during development. Reciprocal crosses have also shown hybrid bleaching but also significant differences in leaf shape. Differences in reciprocal crosses may be induced by epigenetic parental dominance, which results in the dominance of the hybrid phenotype by one parent as was observed in triticale (Heslop-Harrison 1990) and in hybrids of recombinant inbred lines in maize (Kollipara et al. 2002). The epigenetic effects refer to heritable factors affecting the development or function of an organism that are not associated with its DNA sequence (Wolffe and Matzke 1999). Epigenetic processes are responsible for non-equivalent expression of maternally and paternally derived alleles in endosperm (Autran et al. 2005) and they can induce differential expression of rDNA (Komarova et al. 2004). The epigenetic processes are based on the gene suppression which is caused by increased cytosine methylation or chromatin-modifications (Kooter et al. 1999). Another explanation for reciprocal differences in *Passiflora* could be provided by *Oenothera*, respectively gametophytic or sporophytic lethal factors which could be accompanied with chromosomal ring conformations.

Since the early studies of Baur (1909) and Correns (1909) it has been known that mutations affecting plastids in higher plants can exhibit a non-Mendelian, uniparental or biparental, inheritance. The chloroplast DNA transmits uniparentally in most plant species (Hagemann 1992; Reboud and Zeyl 1994; Birky 1995). Uniparental transmission is a consequence of plastid degradation or exclusion in the female or male gamete before or during gametogenesis, plastids dissipation from gametes before fertilization or DNA degradation in the zygote after fertilization (Birky 2001). The majority of angiosperm species inherits plastids maternally, but various exceptions have been found in many taxa which show rare paternal (*Cicer arietinum*, *Oryza sativa*, *Nicotiana tabacum*, etc.), biparental (*Oenothera*, *Pelargonium*, *Secale cereale*, etc.) and paternal (*Daucus* and *Medicago sativa*) inheritance (reviewed in Reboud and Zeyl 1994). In contrast to angiosperms, the majority of gymnosperms transmits cpDNA predominantly paternally (Yang et al. 2000). It was found that nuclear genomes can influence the transmission of cpDNA (Cornu and Dulieu 1988; Chiu and Sears 1993) as well as different plastomes involved in crosses (Schötz 1974; Chiu et al. 1988), but certain genes responsible have not yet been identified. The first molecular analyses on cpDNA of *Passiflora* discovered that (i) plastids are inherited biparentally, and that (ii) the *P. menispermifolia* plastome is incompatible with the F1 hybrid genome. Therefore, the incompatibility in *Passiflora* is dominant as well as the incompatibility in *Oenothera* AB-I. The phenomenon, genome-plastome incompatibility, has already been described from hybrids of *Oenothera*, *Zantedeschia*; and in cybrids in *Solanaceae* (Stubbe 1959; Kushnir et al. 1991; Babiychuk et al. 1995; Yao and Cohen 2000; Zubko et al. 2001) but this is the first evidence in *Passiflora*. There are indications that genome-plastome incompatibility results in alteration in processing of chlorophyll *a/b* binding proteins (Babiychuk et al. 1995), inhibition of plastid reproduction and development, cell division and gametophytic development, as well as in quantitative differences in chlorophyll and carotenoid content (Schötz and Bathelt 1964; Kutzelnigg and Stubbe 1974; Tilney-Bassett 1978; Stubbe and Herrmann 1982; Kushnir et al. 1990; Babiychuk et al. 1995). The genome-plastome incompatibility in *Passiflora* is completely different from that of *Oenothera* AA-III and AB-I. *Passiflora* incompatibility as well as *Oenothera* AB-I has a heterozygotic nuclear background instead of a homozygotic one in *Oenothera* AA-III. The

incompatible tissue of *Passiflora* is unable to provide photosynthesis and incapable to recover in normal greenhouse conditions, but *Oenothera* incompatibility in AA-III is restricted to a limited period and often plant recovers into a wild-type phenotype. The incompatibility of *Oenothera* AB-I has green yellow phenotype which is called as a lutescent (Stubbe 1959). The lutescent phenotype as well as AA-III is not lethal and plants are also fertile. The genome-plastome incompatibility AA-III from *Oenothera* is also able to complement a wild-type phenotype in heterozygous combination AB-III, but incompatibility in *Passiflora* and *Oenothera* AB-I is not. These differences prefer to concede that incompatibility in *Passiflora* and in *Oenothera* have different causes which are dominant in *Passiflora* and *Oenothera* AB-I and recessive in AA-III.

The analysis of cpDNA was complemented by studies of the plastid ultrastructure. The green tissue in the F1 generation has fully mature chloroplasts with thylakoids and grana, the incompatible material in F1 hybrids shows no-differentiated plastids (pro-plastids) with only rudimentary membranes. Formations of thylakoid membranes or grana structures, which are typical for mature chloroplast, were missing in the incompatible materials. This demonstrates that the nuclear background supports the development of plastids into chloroplasts, but plastids inherited by *P. menispermifolia* are not able to initiate differentiation from proplastids into chloroplasts.

An unexpected plastid ultrastructure was found in *P. menispermifolia*. The leaf from plant growing at greenhouse conditions contains plastids in different development stages including etioplasts, which normally develop from proplastids in darkness with one or more prolamellar bodies and containing vesicles (Ryberg et al. 1993). Whereas vesicle transport is a common process in eukaryotic cells, it has not been described for prokaryotes. First vesicle structures have been described inside chloroplasts by electron microscopic studies (Mühlethaler et al. 1959; von Wettstein 1959). Vesicles are rarely detected in mature chloroplasts but can accumulate in the stroma space between the inner envelope and thylakoid membranes after low temperature incubation (Morre at al. 1991; Kroll et al. 2001; Vothknecht and Westhoff 2001). A similar phenomenon has been found in animal cells, where low

temperature blocks the fusion of vesicles with their target membrane (Morre et al. 1989). Thylakoid formation requires basic material as lipids and non-lipid components. Thylakoid lipids and several non-lipid components are synthesised at the inner envelope or are imported from the cytosol (Douce 1974; Soll and Tien 1998; Keegstra and Cline 1999). The vesicle transport covers such needs. Accumulation of vesicles and probably retardation of their fusion with thylakoid membranes in *P. menispermifolia* demonstrate the formation of thylakoids and especially grana stacks directly from vesicles. Electron micrographs uncover retardation of grana formation in *P. menispermifolia* which shows that vesicles could deliver parts of thylakoid components and that they can directly participate in the formation of grana stacks. Grana formation begins when a single vesicle attaches another one or later a grana stack. Then the vesicle shape is flattening and this process continues until grana stacks are fully formed.

Expression of plastid and nuclear genes encoding chloroplast proteins has been studied in albino mutants (Rêgo et al; 1994, Mandel et al. 1996, Meurer et al. 1998) and photobleached plants (Mayfield and Taylor 1987). These studies suspected that chloroplast development requires a co-ordinated expression of plastid and nuclear genes encoding chloroplast proteins (Leon and Arroyo 1998). Interspecific hybrids of *Zantedeschia* have shown that incompatibility reduces gene expression of plastid and nuclear genes encoding chloroplast proteins (Yao et al. 2000). In the present study it has been found that in white sectors mRNA of *petA* was detected and significantly more abundant in comparison with green tissue of the F1 hybrid or parent plant. On the other hand, Western analysis has shown that all plastid encoded proteins were almost not detectable excluding D1 protein of PSII (product of *psb*A) and cytochrome *f* of the cytochrome *b6f* complex (product of *pet*A). The detected proteins of D1 and cytochrome *f* protein could represent precursors, because they are not efficiently cleaved at their C-terminus. In contrast, the 33 kDa protein of oxygen-evolving center of photosystem II, which is encoded by the nuclear gene *psb*O, shows signals equivalent to 25% of the wild-type. This illustrates that genome-plastome incompatibility causes different expression patterns of plastome and nuclear genome encoded genes and that it causes also different translation efficiency of mRNA into proteins. Different expression of genome-plastome incompatibility in various plants

shows that primary causes of this phenomenon could differ, but suggests also that a small number of genes plays a major role in genome-plastome incompatibility (Yao et al. 2000). Genome-plastome incompatibility is a phenomenon which plays a direct role in speciation processes when combinations of plastomes and nuclear genomes fail to develop fully autotrophic plants and are then eliminated by natural selection. Therefore, genome-plastome incompatibility assists as a barrier in speciation processes and in evolution.

5. SUMMARY

Interspecific genome-plastome incompatibility is a widely observed phenomenon but its primary causes are still unknown. It reflects genome-plastome interactions that play a direct role in speciation processes, such interspecific combinations of nuclear genomes and plastomes that fail to develop fully autotrophic plants which then are usually eliminated by natural selection. We have investigated two plant models displaying genome-plastome incompatibility, *Oenothera* and *Passiflora*, using strategies of molecular biology in order to contribute to an analysis of primary causes of interspecific genome-plastome incompatibility.

1. Expressed sequence tags in *Oenothera*: In this study we present the first analyzed EST data set for *Oenothera*. 3,532 cDNA sequences derived from 9-week-old *Oenothera* plantlets were the analysed and assembled into 1,621 nonredundant clusters, including 1,133 singletons and 488 multi-member unigenes which contain a total of 875,940 nonredundand nucleotides. EST sequences were analysed by Sputnik algorithm. They were also used in the development of gene-specific PCR-based co-dominant markers (SNPs, CAPS, micro-satellites). The cDNA library could be directly used for macroarray applications including gene expression studies and for physical mapping.

2. Genotyping analyses in *Oenothera* using AFLP technology: The comparison of AFLPs from *Oenothera* with AFLPs from *Arabidopsis* was used to obtain an approximation of the genome size. The genotyping data provide evidence that genome of *Oenothera* is only six times larger than that of *Arabidopsis* corresponding to a size of about 750 Mb. The AFLP markers were also successfully applied to construct first genetic maps using F2 mapping population of interspecific hybrids between *Oenothera elata* ssp. *hookeri*, line *johansen*, AA-III, x *Oenothera grandiflora* ssp. *tuscaloosa*, BB-III. The linkage maps contain 88 AFLP markers covering a total map length of 154.4 cM for dominant markers in *johansen*, AA-III and 104 AFLP markers and a total size of 155.3 cM for dominant markers in *grandiflora*, BB-III. In addition, it was possible to assign genome-plastome incompatibility locus to the margin of coupling group 2B with 13 cM distance to the next AFLP marker.

The EST project followed by genotyping analysis increases knowledge and requirements in discovering primary causes of genome-plastome incompatibility. *Oenothera* with genome-plastome incompatibility, chromosomal translocations and many chromosomal arrangements provides an elegant tool in the study of genome-plastome interactions, speciation processes and species evolution.

3. Investigation of genome-plastome incompatibility in *Passiflora*: We present the first evidence of hybrid bleaching in this genus. The hybrid between *Passiflora menispermifolia x Passiflora oerstedii* showed bleaching regions during plant development. Reciprocal crosses have also shown hybrid bleaching but as well significant differences in leaf shape. Molecular analyses of cpDNA showed that *Passiflora* plastids are inherited bi-parentally and that the *P. menispermifolia* plastome is incompatible in F1 hybrids with *P. oerstedii*. This is the first evidence of genome-plastome incompatibility in *Passiflora*, which differ from *Oenothera* incompatibilities. The analysis of plastid ultrastructure showed that green tissues in the F1 generation have fully developed chloroplasts with thylakoids and grana; the incompatible material in F1 hybrids lacks differentiated plastids and contains plastids with only rudimentary membranes. An unexpected plastid ultrastructure was found in *P. menispermifolia*. The leaf from plant growing at greenhouse conditions contains plastids in different development stages including etioplasts, which normally develop from proplastids in darkness. Electron micrographs also indicated retardation of grana formation in *P. menispermifolia* which shows that vesicles could deliver parts of thylakoid components and that they may directly participate in the formation grana stacks. Northern and Western analyses demonstrated that genome-plastome incompatibility affects both transcription and translation, but with differences for nuclear and plastome encoded genes.

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PUBLICATIONS

Mráček, J., Cho, W.K., Rauwolf, U., Braun, M., Greiner, S., Umate, P., Altstätter, J., Stoppel, R., Mlčochová, L., Silber, M.V., Volz, S.M., White, S., Selmeier, R., Rudd, S., Herrmann, R.G., and Meurer, J. Construction, database integration and applications of an Oenothera EST library. In preparation.

Mráček, J., Rauwolf, U., Braun, M., Greiner, S., Herrmann, R.G., and Meurer, J. The first genetic map in *Oenothera* uncovers the position of genome-plastome incompatibility locus. In preparation.

Mráček, J., Mlčochová, L., Cho, W.K. Herrmann, R.G., and Meurer, J. Investigation of genome-plastome incompatibility in *Passiflora*. In preparation.

Poster presentation

Mráček, J., Gödel, M., Greiner, S., Meuer, J., and Herrmann, R.G. (2003). Genome/Plastome Interaction in Plant Development and Speciation Processes. Conference “FEBS Advanced Course on the Origin and Evolution of Mitochondria and Chloroplasts”, Hvar, Croatia.

Mráček, J., Braun, M., Rauwolf, U., Mlčochová, L., Greiner, S., Rudd, S., Herrmann, R.G., and Meurer, J. (2004). The first genetic map in *Oenothera* uncovers plastome-genome incompatibility. Conference “Botanikertagung”, Braunschweig, Germany.

ACKNOWLEDGEMENTS

This work was performed at the Botanishes Institut of the Ludwig-Maximilans-Universität in Munich, in the laboratory of Prof. Dr. R. G. Herrmann.

I am especially grateful to Prof. Dr. R. G. Herrmann for inviting me into his group and for providing the excellent working conditions. I also want to thank him for his consistent support and helpful discussions during the whole study.

I am grateful to Prof. Dr. G. Heubl for reviewing this work.

I would like to thank PD Dr. J. Meurer for his considerable support and helpful discussions during the whole study.

I would like to thank Dr. M. Silber for her considerable support during my first steps in the lab.

I would like to thanks to Uwe Rauwolf, Martha Braun, Lada Mlčochová, Martina V. Silber, Pavan Umate, and Stephen Rudd for participation on the *Oenothera* EST project.

I would like to thanks to Uwe Rauwolf and Martha Braun for participation on the *Oenothera* AFLP project.

A would like to thank my friends and colleges, especially to Lada Mlčochová, Lena Pojidaeva, Cristina Dal Bosco, Giusy Canino, Uwe Rauwolf, and Martha Braun, for great working atmosphere and a lot of fun.

I am grateful to Ms. Claudia Nickel for theoretical and practical help with protein analyses. I would like to also thank Mrs. Ingrid Duschanek for help with sequencing.

I am grateful to my parents for their considerable support during my life.

The work was supported by the Deutsche Forschungsgemeinschaft, SFB-TR1, grant B1.

CURRICULUM VITAE

Personal data

Family name: Mráček
First name: Jaroslav
Date of birth: 19.01.1977
Place of birth: Prostějov, Czech Republic
Status: single

Academic education

1995-2000	Study of agriculture at the Faculty of Agronomy of Mendel University of Agriculture and Forestry in Brno
1998-2000	Diploma Thesis: Impact of nitrogen and sulphur fertilisation on yield and seed quality of *Brassica napus*. supervisor: Prof. Ing. Rostislav Richter, DrSc.
2000-2004	Ph.D. study at the Botanisches Institut of the Ludwig-Maximilians-Universität, Munich Head of the Institute and supervisor: Prof. Dr. R. G. Herrmann
2004	Basic Gene Mapping / Linkage Analysis Course. (July 5-9, 2004) Max Delbrück Center (MDC) for Molecular Medicine Berlin, Germany.
2005-	Research scientist at Institute of Biophysics, Academy of Sciences of the Czech Republic