Mapping of genomes and plastomes of subsection *Oenothera* with molecular marker technologies

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“Nothing in Biology makes sense, except in the light of evolution.”
Theodosius Dobzhansky (1973)

This dissertation is dedicated to my father,
† 21.01.1991 Franz Willi Rauwolf

ABBREVIATIONS

ABI	**A**pplied **B**iosystems
AFLP	**a**mplified **f**ragment **l**ength **p**olymorphism
am	**am**mophila
APS	**a**mmonium **p**er**s**ulphate
ATP	**a**denosine 5´-**t**ri**p**hosphate
atro	**atro**virens
biM	**bi**ennis **M**ünchen
BLAST	**b**asic **l**ocal **a**lignment **s**earch **t**ool
bp	**b**ase **p**air(s)
BSA	**B**ovine **S**erum **A**lbumin
CAPS	**c**leavable **a**mplified **p**olymorphic **s**equence
CIAP	**c**alf **i**ntestinal **a**lkaline **p**hosphatase
CMS	**c**ytoplasmatic **m**ale **s**terility
Col	**Col**mar (chicaginensis Colmar)
DAPI	4’,6-**D**i**a**midino 2-**p**heny**i**ndole
DM	**D**obzhansky-**M**uller
DMI	**D**obzhansky-**M**uller **i**ncompatibility
DNA	**d**eoxyribo**n**ucleic **a**cid
DSB	**D**ouble **S**trand **B**reak
DTT	**D**i**t**hio**t**hreitol
dV	**d**e **V**ries
EDTA	**e**thylene**d**iamine-**t**etraacetic **a**cid
e.g.	***e**xempli **g**ratia*
EST	**e**xpressed **s**equence **t**ag(s)
et al.	***et a**lia*
EtOH	ethanol
F1	**f**ilial generation **1**
F2	**f**ilial generation **2**
G	**G**rado (suaveolens Grado)
g	**g**ravitation force; **g**ram
h	**h**aplo(type)
h	**h**our(s)

Hz	**H**ert**z**
i.e.	***id e**st*
joh	**joh**ansen
kb (= kbp)	**k**ilo **b**ase pairs
kV	**k**ilo **v**olt
lam	**lam**arckiana
LB medium	**L**uria **B**ertani medium
LOD	**l**ogarithm of **od**ds
M	**m**olar
mRNA	**m**essenger RNA
ms	**m**illi**s**econd
µE	**m**icro**e**instein
µg	**m**icro**g**ram
µl	**m**icro**l**itre
N/A	**n**ot **a**pplicable
NaAc	sodium acetate
ng	**n**ano**g**ram
NPQ	**n**on-**p**hotochemical **q**uenching
Oe	***Oe**nothera*
P700	**p**hotosystem I primary electron donor chlorophyll *a*
p.a.	**p**er **a**nalysis
PBS	**p**hosphate **b**uffered **s**aline
PCR	**p**olymerase **c**hain **r**eaction
PGI	**p**lastome-**g**enome **i**ncompatibility
pp	**p**ages
pr(s)	**p**air(**s**)
PS	**p**hoto**s**ystem
RAPD	**r**andom **a**mplified **p**olymorphic **D**NA
RFLP	**r**estriction **f**ragment **l**ength **p**olymorphism
RNA	**r**ibo**n**ucleic **a**cid
rpm	**r**evolutions **p**er **m**inute
S	**S**weden (rr-lamarckiana Sweden)
SDS	**s**odium **d**odecyl **s**ulfate
SDS PAGE	**SDS p**oly**a**crylamide **g**el **e**lectrophoresis

SNP	**s**ingle **n**ucleotide **p**olymorphism
subsp	**subsp**ecies
Std	**St**andar**d** (suaveolens Standard)
Taq	***T****hermophilus* ***aq****uaticus*
TBE	**T**ris-**B**orate-**E**DTA
TEMED	syn. N,N,N',N'-**Te**tra**m**ethyl**e**thylene**d**iamine
tusc	**tusc**aloosa
Vol	**Vol**ume

CONTENT

1. INTRODUCTION

1.1 ENDOSYMBIOSIS AND PLASTOME-GENOME CO-EVOLUTION

The evolution of eukaryotes, which consist of cells with nuclei, presumably from the prokaryotic domain, is considered to be one of the most profound changes in the evolutionary history of organisms. In an attempt to describe the way in which this gap was bridged, scientists have proposed the serial endosymbiosis theory (Sachs 1882; Schimper 1885; Altmann 1890; Mereschowsky 1905; Margulis 1971a, b; Herrmann 1997; Martin *et al.* 2001; Kutschera and Niklas 2005). The term "endosymbiosis" specifies the relationship in which one organism lives within another (symbiont within host) in a mutually beneficial relationship. The endosymbiosis theory states that the evolution of eukaryotes from prokaryotes involved the symbiotic union of several previously independent ancestors. According to that theory, these ancestors included a host cell, presumably an archaebacterium (Brown and Dolittle; Martin and Müller 1998; Lopez-Garcia and Moreira 1999; Martin and Russel 2003), an ancestor of mitochondria, most likely an α-proteobacterium (Biagini *et al.* 1993; Andersson and Kurland 1999; Rotte *et al.* 2000; Martin *et al.* 2001; Embley *et al.* 2003), and an ancestor of chloroplasts, most likely a cyanobacterium (Cozens and Walker 1987; Nelson 1992). As the integrated progenitors then became more interdependent, an obligatory symbiosis evolved (Margulis 1993). The endoymbiosis of a eukaryotic cell, already including a mitochondrial progenitor, with a photosynthesis competent cyanobaterium, lead to the kingdom of plants (McFadden 1999).

The organelles, nucleus/cytosol and mitochondria in animals and fungi and nucleus/cytosol, mitochondria and plastids in plants, form a compartmentalized genetic system that has to be regulated in its entirety in time, quantity and spatially (Herrmann 1997; Herrmann and Westhoff 2001). Since mitochondria and plastids today possess only rudimentary genomes because they have lost a large fraction of their genes many of which by transfer to the nucleus (Herrmann 1997; Martin and Herrmann 1998; Martin 2003), much of the nuclear coding potential, in the order of 20 – 30%, is required for their management (Herrmann 1997; Leister 2003, 2005). 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; Leister 2005). The genetic mechanisms like lateral gene transfer, gene duplication, genome reduction, gene elimination and mutations in combination with natural selection have been involved in evolution and speciation processes of plants (Darwin and Wallace 1858; Mayr 1988, 1991; Herrmann 1997; Martin and Herrmann 1998; Herrmann and Westhoff 2001; Henze and Martin 2001; Herrmann et al. 2003; Huang *et al.* 2003; Martin 2003; Timmis *et al.* 2004). With regulatory dominance developed by the nucleus with time the organellar genomes lost their independency, *i.e.* mutations in one of the subgenomes can already have serious effects on the entire system (Herrmann and Possingham 1980a; Hedtke *et al.* 1999; Whitney *et al.* 1999; Rodermel 2001; Lam *et al.* 2001).

This fact explains why the individual organelles are related to each other in dependence. Any change in one of the organellar genomes leads inevitably to an evolutionary pressure and makes co-evolution of the individual organelles indispensable. Genome arrangements are thus of vital importance of the enduring genetical diversification and speciation processes (Herrmann 1997; Margulis and Sagan 2002). This becomes apparent in interspecific nuclear-plastid cybrids or hybrids. These plastome-genome exchanges between two different species often lead to serious disturbances (incompatibilities) that are a result of the missing co-evolution of organelles (Renner 1934; Stubbe 1959, 1989; Schmitz-Linneweber *et al.* 2002, 2005; Herrmann *et al.* 2003) (see more detail in Chapter 1.2.4). A broad range of these incompatible phenotypes is visible in different plastome-genome combinations within the genus *Oenothera.*

1.2 THE MODEL GENUS *OENOTHERA*

The plant model genus *Oenothera* has an outstanding genetic tradition (summarized in Lehmann 1922; Cleland 1972; Harte 1994). A comprehensive taxonomy, cytogenetics, ecology and formal genetics are available (Cleland 1972; Harte 1994; Dietrich *et al.* 1997), which was developed over more than a century. An unique combination of genetic features and the occurrence of unsaturated fatty acids (γ-linolenic acid, Omega-6) (Kies 1989; Horrobin 1990; Mol *et al.* 2001; Shimizu and Nakano 2003; Fieldsend 2007) and secondary metabolites (Taniguchi *et al.* 2002)

illustrate a special role of the genus *Oenothera* for a variety of biological, medical and pharmaceutical questions.

1.2.1 ECOLOGY, GEOGRAPHY AND A SHORT HISTORY OF RESEARCH ON *OENOTHERA*

The genus *Oenothera* is indigeneous in North and South America. The largest variety of species is found in Mexico and the Northern part of Middle America. Today, more than 300 genetically analyzed strains are known in subsection *Oenothera* (Cleland 1972; Steiner and Stubbe 1984, 1986; Stubbe and Diers 1985; Wasmund and Stubbe 1986; Wasmund 1990; Schumacher *et al.* 1992; Schumacher and Steiner 1993), which is one of the five subsections of the genus (Dietrich *et al.* 1997). In their presumable original region of distribution, *Oenothera* species are found in different habitats, varying from deserts to disturbed places and from plains to mountains. *Oenothera* species grow wild in Europe as well, in bare, abandoned places, along stony riverbanks, the sides of canals, roads and railroad tracks. Evening primroses are assumed to have invaded to European continent after 1500 A.D. (Harte 1994). The first species mentioned as cultivars in gardens of continental Europe was *Oenothera syrticola* Bart. (syn. *Oe. muricata* AUT.) in 1633 and short time afterwards a pre-form of the today well-known *Oe. biennis* L. was described. The latter is not only a long-established member of the European flora but is found meanwhile in nearly all other continents. Thus, this genus successfully distributed nearly all over the globe, with a remarkably ability of adaptation to new environmental conditions, whereby the genus can be ranked among cosmopolitan plants.

Section *Oenothera* belongs to the plant family of *Onagraceae*, which is divided into 14 sections (*Oenothera, Kleinia, Emeria, etc.*), all together including 119 species. The section of interest in the present work is *Oenothera*, further divided into five subsections, (*Eu-*)*Oenothera* (13 species), *Munzia* (45 species), *Raimannia* (11 species), *Emersonia* (four species) and *Nutantigemma* (three species) (Dietrich 1977; Stubbe and Raven 1979; Dietrich und Wagner 1988; Dietrich *et al.* 1997). Subject of analysis for my thesis is the subsection (*Eu-*)*Oenothera*.

Hugo Marie de Vries (1848-1935). At the end of the 19th century it was the Dutch scientist Hugo de Vries, professor of botany at the University of Amsterdam, who established the genus *Oenothera* as a model organism in experimental botany. Since that time, the genus is of interest not only in taxonomy but especially in genetics. Hugo de Vries rediscovered the Mendelian rules with this plant genus (de Vries 1900a, b), although today the genus *Oenothera* is considered as an example for non-Mendelian nuclear and organelle inheritance, too. Also, he proclaimed the mutation theory by observations made on *Oenothera lamarckiana* de Vries (de Vries 1901 - 1903). After this starting, a few scientists switched their interest to *Oenothera*. Further fundamental discoveries like the genetic independence of plastids (Renner 1934) and the first description of polyploidy (Lutz 1907) were also made on *Oenothera*. Otto Renner (1883 - 1960) formulated his theory of genome and chromosome complexes (Renner complexes: entire haploid genomes, which are inherited as single units) and provided first clues to solve particular problems of inheritance in *Oenothera* species (Renner 1914; 1917a, b, c; 1918a, b; 1919a, b). An example is provided by the non-Mendelian phenomenon of twin hybrids in some F1 generations (de Vries 1900c; 1907; 1913; Andrews 1910; Honing 1911) (Fig. 1-1).

1.2.2 GENERAL GENETICS OF *OENOTHERA*

An extensive knowledge about genetics in *Oenothera* was collected during the past century. Thus, today *Oenothera* is an example for genetic features, which in their combination is unique in this genus. These features are biparental transmission of plastids (*e.g.* Hagemann 2004), permanent translocation heterozygosity (PTH, syn. complex heterozygosity) (Stubbe 1960; Cleland 1972; Harte 1994; Holsinger and Ellstrand 1984; Stubbe 1989; Levin 2002; Golczyk *et al.* 2005), reciprocal translocations of chromosome arms (Blakeslee and Cleland 1930; Cleland and Blakeslee 1931; Cleland 1972; Harte 1994), a general interfertility of species, fertility of plastome-genome hybrids (Stubbe 1959; Cleland 1972; Harte 1994) and hybrid variegation (Kirk and Tilney-Bassett 1978). They allow the sexual exchange of plastids, individual (or more) chromosomes and entire genomes (Renner complexes) between species.

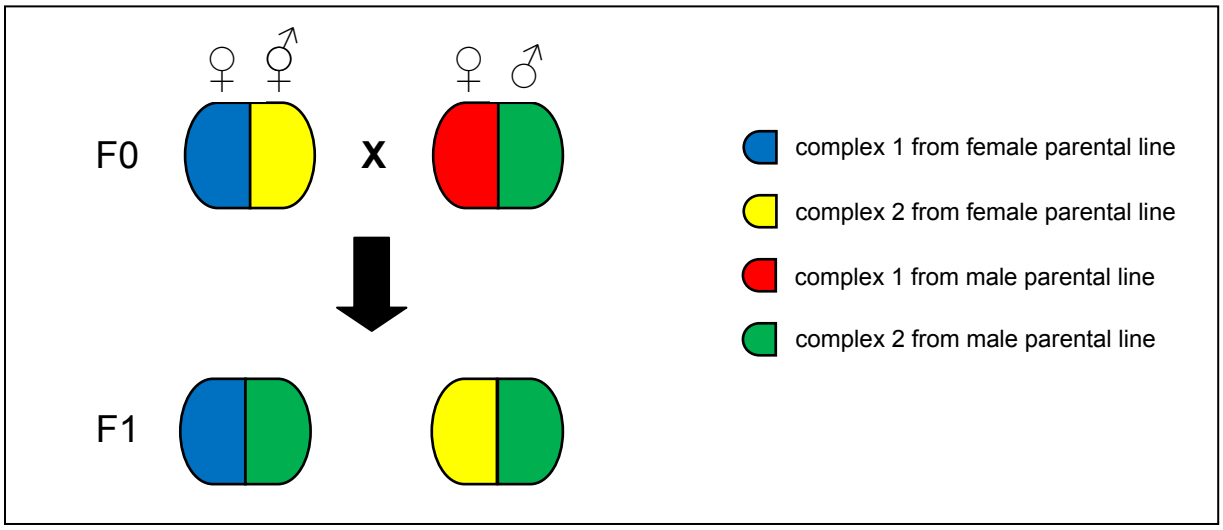


Fig. 1-1: „Twin hybrids“ were recognized with F1 generations in some *Oenothera* crossing experiments. In this example, complex 1 (blue) from the female parental line is a pure egg cell complex (♀), while complex 2 (yellow) from female parental line can exist in both, egg cell (♀) and pollen (♂). Such a species is called “half-heterogamous”. *Oenothera biennis* strain suaveolens Grado (^{G}albicans ♀ • ^{G}flavens ♀♂) is an example for such a situation. Complex 1 (red) from the male parental line is a pure egg cell complex (♀) while complex 2 (green) from male parental line is a pure pollen complex (♂). This situation is present in permanent translocation heterozygous *Oenothera* species [*e.g. Oenothera villosa* subsp. *villosa* strain bauri (laxans ♀ • undans ♂)], called “heterogamous species”. Crossing of these two species results in two different phenotypes (so called twin hybrids) in F1, contrary to Mendel´s First Rule.

The special genetical phenomenon of permanent translocation heterozygosity in genus *Oenothera* directed attention especially to the nucleus and its chromosomes. The phenomenon of reciprocal translocations of chromosome arms, in turn, reaches an endpoint in the specific system known as stable translocation heterozygosity (⊙14 = a ring-formation consisting of all 14 chromosomes, syn. terminal heterozygotes). It describes a distinct structural feature of chromosomes that usually rest on two crucial prerequisites, *i.e.* that chromatin translocations involve *entire* chromosome arms and that they are *reciprocal* (breaking points at the centromeres). This system represents the ultimate situations in linkage disequilibrium (Futuyma 1979, Úbeda and Haig 2004; 2005). In such plants each haploid complement of seven chromosomes is connected through reciprocal translocations, making nearly the entire genome behave as a single coupling group. A major feature of this evolutionary pattern is related to restriction in recombination (Cleland 1972; Raven 1979; Harte 1994). Entire haploid genomes are designated as Renner complexes and entitled with names such as ^{h}johansen, ^{h}tuscaloosa or ^{G}albicans. The genetic mechanisms that control the formation of the permanent translocation heterozygosity system were discovered and largely worked out by Otto Renner (see Cleland 1972). An

enumeration of single chromosome arms was essential as a consequence of different genome combinations resulting from reciprocal translocations. In addition to translocations, the system operates with balanced, genetically controlled lethals, in which homozygosity of non-allelic recessive lethal genes results in mortality, either sporophytic or gametophytic, when the parent plant is autogamous (Fig. 1-2). This system prevents the formation of the homozygous combinations or, as in many permanent translocation heterozygous species, renders the young embryos with homozygous complexes lethal (Muller 1917), and stabilizes the heterozygous state of species.

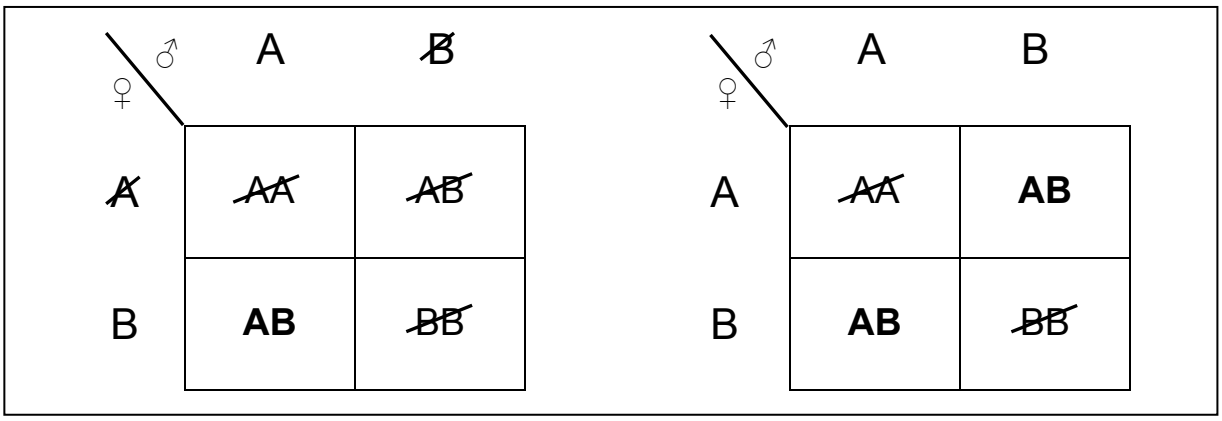


Fig. 1-2: Diagram to show the effect of balanced lethals. At the left, one complex is inactivated by one gametophytic lethal, the other complex by the other lethal. At the right, zygotes that receive the same zygotic lethal from both parents fail to develop (modified from Cleland 1972).

Nowadays it is known that approximately a quarter of the angiosperm species studied transmit plastids biparently (Corriveau and Corriveau 1988; Harris and Ingram 1991; Hagemann 1992; Zhang *et al.* 2003). First indications for biparental transmission of plastids in *Oenothera* appeared with fertilization studies. These studies showed that not only the pollen tube, containing many leucoplasts, but also generative cells contain plastids (Meyer and Stubbe 1974), which in most cases introduces considerable cytoplasm into the zygote (Ishikawa 1918). These results, in combination with observations of different phenotypes of reciprocal crosses (Renner 1924), lead to two conclusions: First, in *Oenothera* plastids can be inherited from both parents. Second, different classes of plastids exist in different lineages of the genus, which react differently in association with various genome combinations. Only few leucoplasts (around 15) are transmitted *via* the pollen (Meyer and Stubbe 1974), while egg cells possess approximately twice as many plastids (around 30). The

different plastid types, present in the zygote, segregate during development into different cells, and segmental or periclinal chimeras develop (hybrid variegation). In the next generation of this plant the division rate of different plastid types determine which plastome will dominate (determined division rate of different plastome types in Oenothera: I>III~II>V>IV) (Schötz 1954, 1974, 1975; Chiu *et al.* 1988; Chiu and Sears 1993). The existing Renner complexes determine the behavior of the nuclear genome, according to the structure present during meiosis (permanent translocation heterozygous structure or the formation of bivalents).

Permanent translocation heterozygosity has been important in the evolution of the genus *Oenothera*. The genera of tribe Onagrae possess chromosomes with highly pycnotic, condensed proximal regions that are flanked by less densely contracted distal segments. The characteristic metacentric chromosomes (Kurabayashi *et al.* 1962; Cleland 1972; Raven 1979) show ring-formation in meiosis (Cleland 1972; Holsinger and Ellstrand 1984; Stubbe 1989; Harte 1994; Levin 2002; Golczyk 2005) as a result of reciprocal arm translocations. In addition to terminal (complete) translocation heterozygotes (⊙14), there also exist naturally occurring partial translocation heterozygotic (at least one free bivalent can be formed during meiosis), and bivalent forming species. The occurrence of terminal translocation heterozygosity, the property of a genome in meiosis to distribute its maternal and paternal chromosome sets to resulting gametes without notable mixing, in combination with biparental transmission of plastids allows the exchange of plastids and haploid chromosome sets between *Oenothera* species (Fig. 1-3). This often leads to serious developmental disturbances (Stubbe 1989; Herrmann *et al.* 2003; Levin 2003). A special well-known case of such an interacting malfunction is known as Dobzhansky-Muller incompatibilities, which can probably be applied to various cases of the *Oenothera* model, too (Bateson 1909; Dobzhansky 1937; Muller 1942; Greiner *et al.* 2008a) (see Chapter 1.2.4). This way, three haploid nuclear genomes (A, B and C) in homozygous (AA, BB, CC) or heterozygous (AB, BC, AC) constitution, associated with five basic, genetically distinguishable plastomes (I - V) were identified (Stubbe 1959, 1960, 1989) (Fig. 1-4). The plastomes were recently sequenced (Greiner *et al.* 2008b).

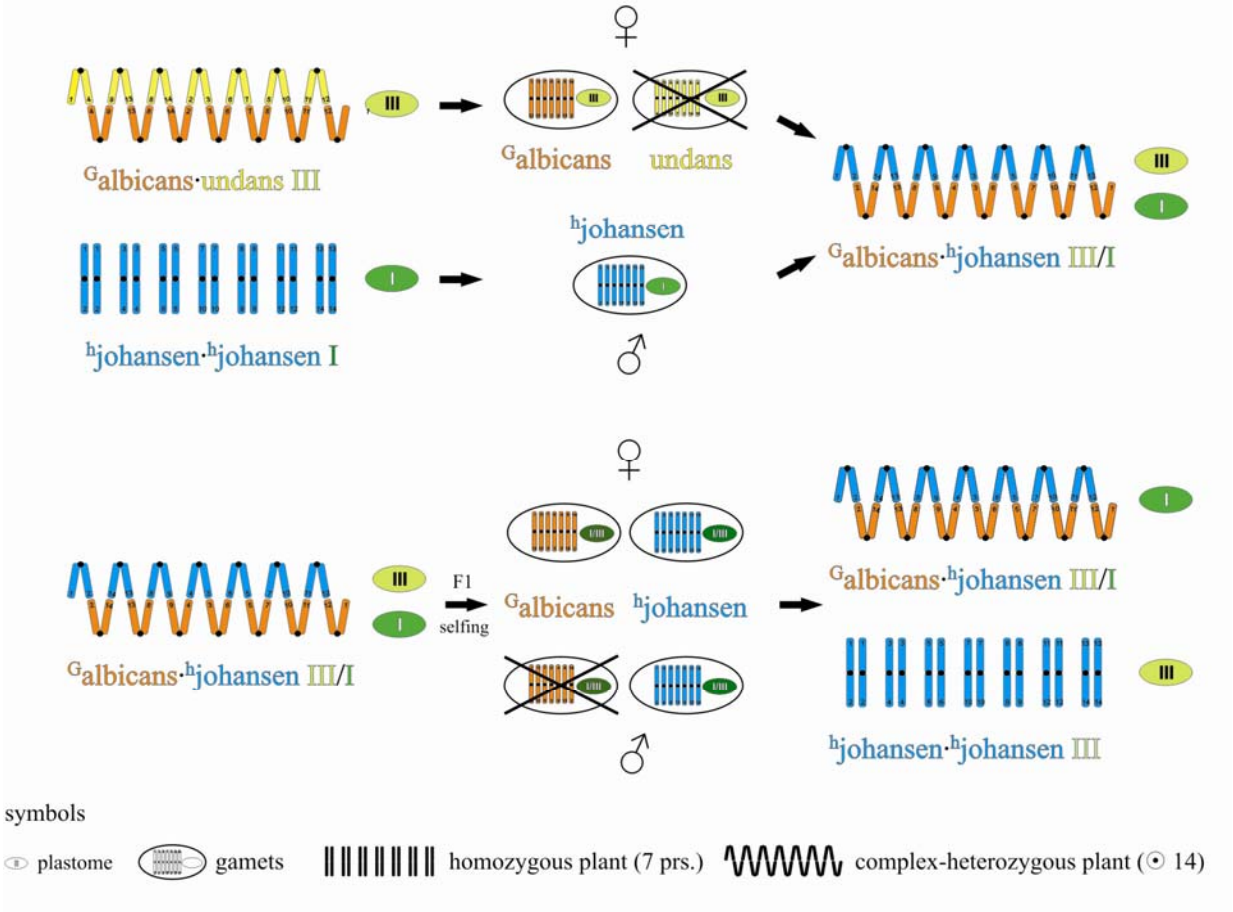


Fig. 1-3: Crossing scheme of an exchange of plastids without changing the nuclear background. Crossed pictures of gametes represent lethality because of gametophytic lethal factors. Chromosomes ordered in a "zig-zag" confirmation represent ring formation during meiosis (complex-heterozygosity).

1.2.3 DOBZHANSKY-MULLER-INCOMPATIBILITIES (DMI)

Currently, no other comparable material is available which allows an exchange of plastids and nuclei just by simple crossings. In *Oenothera,* all possible 30 plastome-genome combinations can exist and were either made by crossings or occur naturally in nature (Stubbe 1959, 1960, 1989). An exchange of plastids and nuclei, even between closely related species, often leads to serious developmental disturbances (Stubbe 1989; Herrmann *et al.* 2003; Levin 2003) (Fig. 1-4). Generally plastome-genome-incompatibilities (PGI) can cause hybrid sterility, hybrid inviability (hybrid weakness) and hybrid breakdown (Levin 2003; Stebbins 1950; Stubbe 1989; Yao and Cohen 2000; Greiner *et al.* 2008a). Incompatibilities reflect disharmonic interactions of cellular genomes resulting from a different co-evolution of gene complexes in distant species (Dobzhansky 1970; Hermann *et al.* 2003; Levin 2003; Rand *et al.* 2004; Schmitz-Linneweber *et al.* 2005). Obviously, an exchange of the

cellular compartments disturbs a co-adapted network. The disharmonic gene pairing may result in a negative cytonuclear epistatic effect, a so-called Dobzhansky-Muller-Incompatibility (Burke and Arnold 2001; Tiffin *et al.* 2001; Turelli and Moyle 2007). The theory of DMI was independently described by Bateson (1909), Dobzhansky (1937) and Muller (1942) (summarized in Fig. 1-5). Distinct gene pairs in different, temporally or spatially separated, species can co-evolve in a different manner. This way, different alleles get fixed (in Fig. 1-5: A and B). After mating of the two populations the resulting genotype (here: Aa/Bb) can be maladaptive (incompatible), because of missing co-evolution of alleles A and B. In this instance, hybridization results in reduced fitness of the offspring and a postzygotic hybridization barrier could be established. This model is also applicable to organelle-nuclear interaction (Lamprecht 1944; Michaelis 1954; Willett and Burton 2001; Sackton *et al.* 2003; Levin 2003; Fishman and Willis 2006, Greiner *et al.* 2008a), and of interest in the genus *Oenothera*.

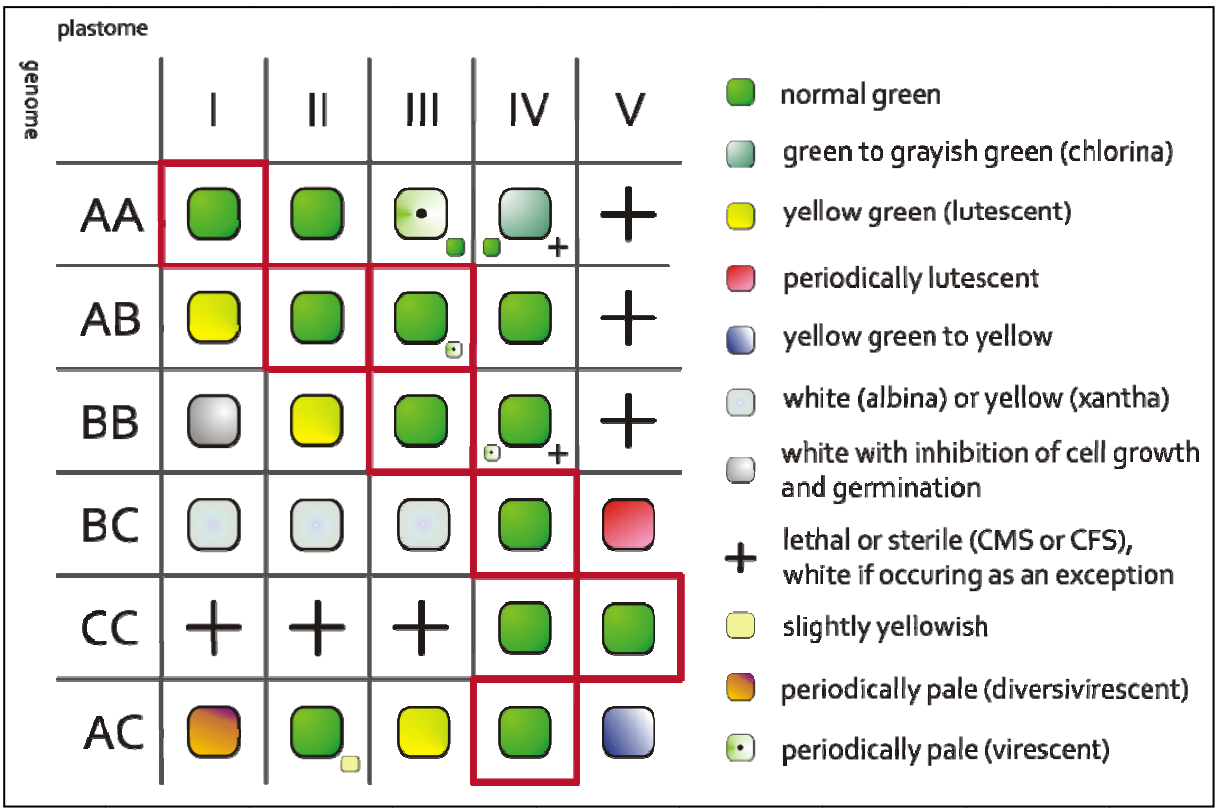


Fig. 1-4: Plastome-genome compatibility/incompatibility in subsection *Oenothera* (redrawn from Stubbe 1959; 1989). A, B and C represent the basic nuclear genotypes, I - V the five genetically distinguishable plastomes. Genotypes boxed in red represent naturally occurring species. Minor symbols indicate variances noted for some nuclear subgenotypes.

Generally, organelles are preferentially maternally inherited in angiosperms. However, in 25% of the cases studied it is biparental (Hagemann 2004). Thus, DMIs formed by organelles are often observable only in one crossing direction (asymmetric). A very prominent example of such an asymmetric hybridization barrier is the commercially important cytoplasmatic-male-sterilty (CMS) (Chase 2007). Theoretical models on population genetics and speciation forces exist for both, symmetric and asymmetric DMIs (*e.g.* Turelli and Orr 2000; Coyne and Orr 2004; Turelli and Moyle 2007). Nuclear Dobzhansky-Muller gene pairs were identified in *Drosophila* (Brideau *et al.* 2006; Presgraves and Stephan 2007).

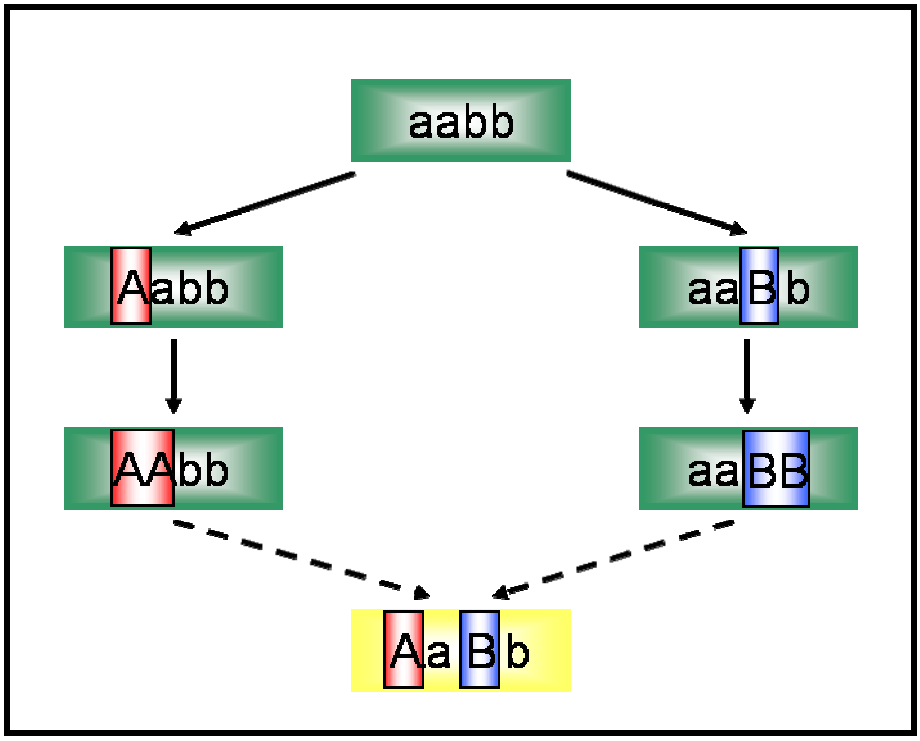


Fig. 1-5: The Dobzhansky-Muller model assumes a reproductive barrier based on gene interaction. Alleles A and B did not co-evolve with each other. After mating of the two populations the resulting genotype AaBb is maladaptive (incompatible; hybrid inviability) (Greiner *et al.* 2008a).

PGI promises an easier access to molecular determinants involved in speciation than the identification of nuclear Dobzhansky-Muller-gene pairs. PGI was so far described from 14 plant genera (Greiner 2008). The occurrence of hybrid variegation (reflecting at least two plastome types) is essential and the only reliable way to correlate a plastome to a bleached phenotype (PGI). In some plastome-genome incompatible combinations in *Oenothera* (Fig. 1-4) much milder effects on hybrid fitness are

observed than in the predominantly studied hybrid sterility. The availability of the entire sequences for the five genetically distinguishable plastome types from *Oenothera* (Greiner *et al.* 2008b) allowed for the first time a bioinformatical comparative analysis to deduce determinants causing incompatibility (Greiner *et al.* 2008c). This way, one gene involved, a "plastome factor" of a "Dobzhansky-Muller gene pair", has been identified, that could be corroborated by biochemical and biophysical approaches. The corresponding nuclear gene ("nucleus factor") can either be identified by mapping approaches, by gel shift approaches, or by information already available from other plants about interacting partner genes (*e.g.* of complexes) of the previously identified "plastome factor". With mild PGIs good chances exist to pinpoint primary effects of speciation.

1.2.4 MEIOSIS RESEARCH IN *OENOTHERA*

In earlier *Oenothera* research, *i.e.* during the first three quarters of the 20th century, analysis of *Oenothera* meiosis suffered from methodical limitations of cytological resolution and phenotypic observations. Theoretically, two possibilities existed how chromosomes of permanent translocation heterozygotic *Oenothera* species behave during meiosis. It was proposed that chromosome arms either fit together just at their telomeric parts, forming a "ring" or that entire arms pair and form a "star" (Fig. 1-6) (Stubbe 1980; Harte 1994). The latter version was finally excluded because of assumed sterical problems caused by centromere ordering and because no such structure could be observed cytologically. In the following years, a "run" on chromosomal formulas began and all possible chromosome configurations were

Fig. 1-6: Graphic of a suspected „star" confirmation of chromosomes in meiotic state metaphase I (stable translocation heterozygosity; ⊙14). Such a chromosome arrangement was excluded because of sterical problems caused by centromere ordering. Maternal (black) and paternal (white) chromosomes are ordered in an alternate way and in truth connected at the telomeres [ring-formation (see Fig. 1-7)] (Stubbe 1980).

found (Table 1-1), either in naturally occurring *Oenothera* species or in hybrids generated by appropriate crosses. On the basis of cytological investigations of chromosome configurations of hybrids, chromosome formulas were postulated, taking the chromosome formula of *Oenothera elata* subsp. hookeri de Vries as a standard (^{h}hookeri = 1•2 3•4 5•6 7•8 9•10 11•12 13•14). For instance, the arms of chromosomes 1•2 and 3•4 can display three different segmental arrangements, also 1•4 3•2 and 1•3 2•4. Since all 14 chromosome arms are involved in this process, 91 arm combinations in individual chromosomes are possible [$\frac{n!}{k!\,(n-k)!} = \binom{n}{k}$; n = objects, k = selected objects (without attention of a particular order and without "putting back")]. All these combinations have been found, but do not appear or prevail with equal frequency, *e.g.* chromosome 1•2 occurs frequently, but 1•3 is rare.

Table 1-1: Possible chromosome configurations in homozygous/heterozygous plants with 2n = 14

⊙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

All possible chromosome configurations were observed in either natural occurring *Oenothera* species or hybrids (Cleland 1972).

Rates of recombination events, observed with just a few phenotypical markers (summarized and listed in Cleland 1972), were nearly impossible to be determined in the entire dimension because of special genetical attributes: First, appearance of balanced lethals within some *Oenothera* species; second, appearance of megaspore

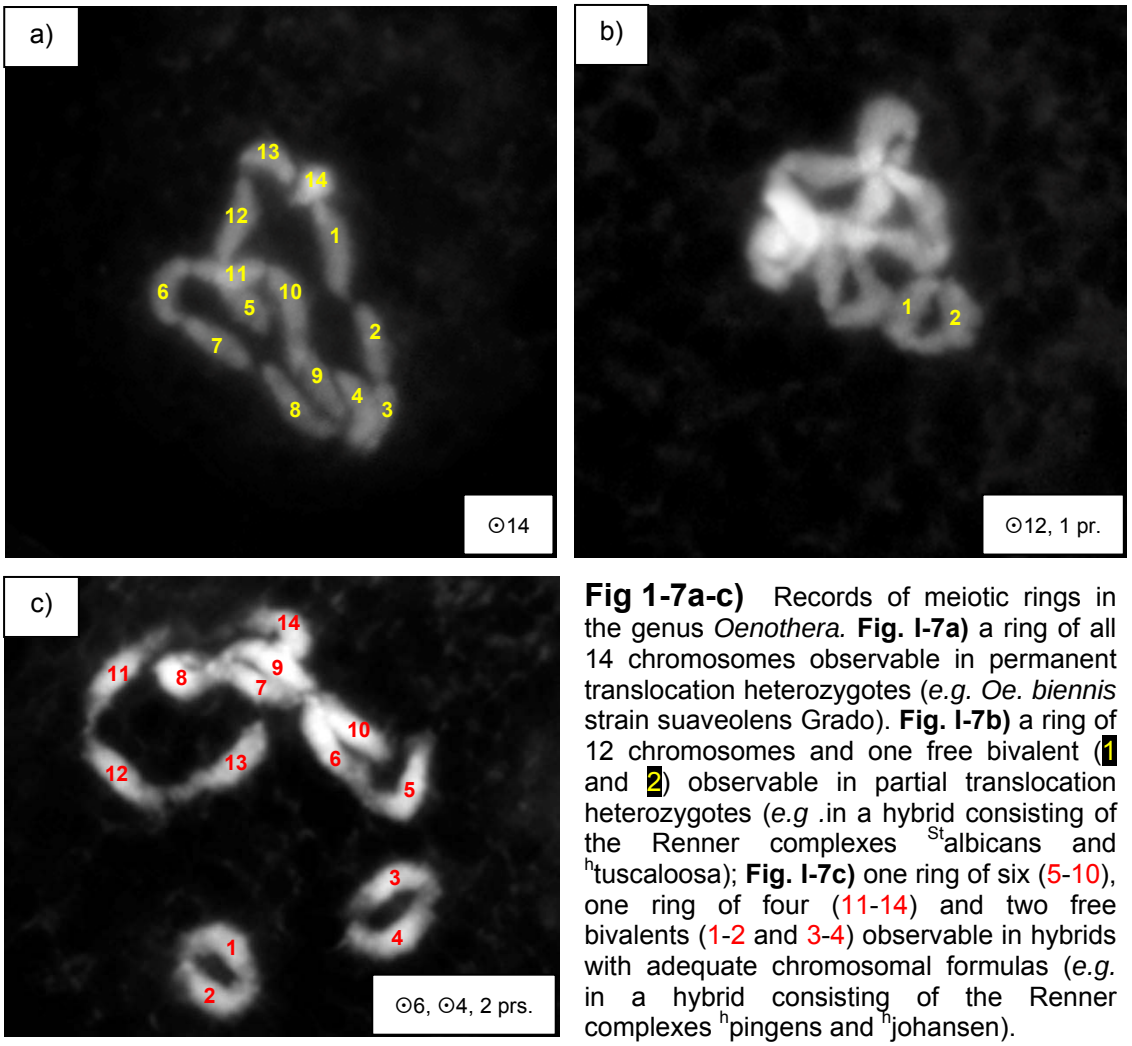


Fig 1-7a-c) Records of meiotic rings in the genus *Oenothera.* **Fig. I-7a)** a ring of all 14 chromosomes observable in permanent translocation heterozygotes (*e.g. Oe. biennis* strain suaveolens Grado). **Fig. I-7b)** a ring of 12 chromosomes and one free bivalent (1 and 2) observable in partial translocation heterozygotes (*e.g* .in a hybrid consisting of the Renner complexes Stalbicans and ^{h}tuscaloosa); **Fig. I-7c)** one ring of six (5-10), one ring of four (11-14) and two free bivalents (1-2 and 3-4) observable in hybrids with adequate chromosomal formulas (*e.g.* in a hybrid consisting of the Renner complexes ^{h}pingens and ^{h}johansen).

and embryo sac competition of Renner complexes (Harte 1994; Renner 1921a); third, appearance of pollen tube competition of Renner complexes (Renner 1917a); fourth, a limited number of available phenotypical markers. All this can lead to deviations from typical Mendelian segregation, *e.g.* to altered, atypical segregation ratios (Cleland 1972). A further complication arises, since genes often appear to be differently linked in different hybrids, depending upon the chromosome configurations present. It is relevant to mention that homologous recombination is expected to be substantially reduced and limited to telomeric regions in translocation heterozygous hybrids, because bivalent formation is lacking (Stubbe 1989; Levin 2002). Despite their sequence diversity (Lamb *et al.* 2007), plant telomeres are not known to possess genes. The crossovers that occur in telomeric regions of evening primrose are therefore neutral in that they do not generate genetic diversity. The situation in naturally occurring, bivalent forming *Oenothera* species is unclear. Segregation of

some phenotypic markers has been observed and, consequently, the homologous recombination machinery is present in *Oenothera*, in translocation heterozygotes and in bivalent formers.

1.3 HOMOLOGOUS RECOMBINATION AND EVOLUTION OF SEX

It is important to understand how homologous recombination works, last not least because of its perspectives for plant genome engineering. The ability to modify plant chromosomes through homologous recombination (gene targeting) has been a long idea and goal of plant biology (*e.g.* reviewed by Reiss 2003), but the mechanisms are not yet fully understood.

Rates of recombination events generally depend on the degree of homology between pairing chromosomes. Crossing-over events are, in most cases, essential for mapping markers (exception: HAPPY mapping; Thangavelu *et al.* 2003). During the past decades genetic maps were generated from a number of materials using inter- or intraspecific crosses. In these cases, the degree of polymorphism between the parental lines ranged up to 60.7% (Tan *et al.* 2001). In the meiosis of yeast, one of the best studied organisms, efficient recombination even occurs between 1 kb blocks of perfect homology embedded in non-homologous regions (Haber *et al.* 1991). Thus, for homologous recombination small homologous segments on chromosomes appear to be sufficient to enable crossing-over events.

Although the molecular processes and advantages of recombination are not yet fully understood, studies in yeast and other organisms have led to the Double-Strand Break Repair (DSBR) model (Szostak *et al.* 1983; Sun *et al.* 1989). It involves a single pathway of DNA intermediates that produces both crossovers (COs) and non-crossovers (NCOs). Statistical and experimental evidence suggests that *Arabidopsis thaliana*, like the budding yeast *Saccharomyces cerevisiae* and humans, has two recombination pathways: one that exhibits crossover interference (CO prevents additional COs from occurring nearby) and another that does not (Copenhaver *et al.* 2002; de los Santos *et al.* 2003; Housworth and Stahl 2003; Malkova *et al.* 2004). This is in contrast to organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, in which all COs are thought to be subject to interference (Zhao *et al.*

1995; Meneely *et al.* 2002), or to fission yeast *Saccharomyces pombe,* in which all COs are subject to an interference-insensitive pathway (Kohli and Bahler 1994). In the organisms studied to date with both interfering and non-interfering COs, the majority of events are thought to be generated by the primary, interference-sensitive pathway.

Meanwhile, various components and intermediates are known, especially from yeast (Smith and Nicolas 1998; Keeney 2001), but more and more homologues could be identified in *Arabidopsis* (*e.g.* reviewed by Mézard *et al.* 2007). These components were also assigned to different steps in the procedure of homologous recombination, as there are, for instance, condensation/decondensation of chromatin, DSB formation, end processing, strand invasion and many genes involved in interfering crossover pathway, non-interfering crossover pathway or non-crossover pathway.

Homologous recombination is an event occurring in most species with sexual reproduction. During evolution, sex is thought to have been developed from an asexual ancestor. But why? A substantial number of observations and tests were performed to approach the evolution and sense of sex (*e.g.* Stebbins 1950; Maynard-Smith 1978; Rice 2002; Nielsen 2006). A useful basic approach to tackle that question was to compare asexual with sexual reproduction. Sexual reproduction has often been related to sexual recombination, without taking note of permanent translocation heterozygous species. The most popular advantage of sexual recombination is faster adaptation to changing environments compared to asexual reproduction (Fisher-Muller model; Fischer 1930; Muller 1932). It further allows natural Darwinian selection to propagate more efficiently (Rice and Chippindale 2001; Nielsen 2006; Paland and Lynch 2006). However, also other hypotheses have been favored. (1) Ecological theories, *e.g.* the pathogen ratchet theory, defined as the reduction of similarity of resistance loci between parents and offspring (Rice 1983) and (2) genetic theories, *e.g.* mutational load, defined as the reduction in fitness of a population due to an accumulation of deleterious mutations (Muller´s ratchet) (Muller 1964; Kimura and Maruyama 1966; Kondrashov 1984, 1988; Moran 1996; Rice 1998, 2002; Race *et al.* 1999; Keightley and Eyre-Walker 2000, Hillis 2007). But opinions about the individual points diverge. For instance, it has been reported that

new alleles are of benefit only if they accumulate in a prescribed order. Only asexual reproduction allows some overlap of successive allele replacements (Kondrashov and Kondrashov 2001). Furthermore, it was shown that in species with short generation times, sex is not maintained by its capacity to purge the genome of deleterious mutations (Keightley and Eyre-Walker 2000). Thus, the question upon the advantage of sex remains, particularly with regard to the disadvantage of the "two-fold" cost of producing males ("two-fold" cost of meiosis) (Maynard-Smith 1978; Rice 2002; Nielsen 2006). Parthenogenetically reproducing females arising in a sexual population should have a twofold fitness advantage because, on average, they provide twice as many gene copies to the next generation. But parthenogenesis does not play a dominant role in nature. If sexually reproducing individuals do not have an immediate selective advantage in otherwise asexual populations, it is difficult to imagine how populations can ever evolve from asexual to sexual reproduction. Asexual lineages are found among most of the major plant and animal groups (Stebbins 1950; White 1978). Persistance over geological time, that is for million(s) of years (Maynard-Smith 1978; White 1978), means by definition not to be an evolutionary dead-end, as individuals without homologous recombination are usually seen. The bdelloid rotifers (Welsh and Meselson 2000), some small vertebrates (Judson and Nomark 1996; Butlin *et al.* 1998) and permanent translocation heterozygous species do not confirm this idea, whereby the question about the sense of sex remains unresolved and cannot be justified apparently just by homologous recombination.

1.4 GOALS OF THE PROJECT

In this work I established and utilized Amplified Fragment Length Polymorphism (AFLP) analyses for four basic goals: (1) to establish genomic markers to generate first comprehensive linkage maps for two genomes (A and B) of the genus *Oenothera*; (2) to clarify the situation in bivalent forming *Oenothera* species concerning their behavior during meiosis; (3) to evaluate both, intra- and interspecific, diversity (phylogenetic relationship) with AFLP data; (4) to provide molecular biological evidence of restriction of homologous recombination to telomeric regions in constructed translocation heterozygous hybrids, generated to exchange sexually plastids or entire genomes.

The linkage maps were used to identify chromosomes responsible for the *virescent* incompatibility between the genotype AA and the plastome type III. In addition, this possible Dobzhansky-Muller incompatibility should be characterized by using bioinformatical, molecular biological and biochemical methods in order to narrow down the possible “plastome-factor” of the Dobzhansky-Muller gene pair.

Plant breeding and crossing programs within the genus *Oenothera* requires up to now knowledge of the characteristics of different Renner complexes and their genetics to be able to exchange plastids and individual or more chromosomes. To simplify and to improve such crossing programs, a marker system was established to distinguish different complexes as well as different plastome types. The PCR based markers should provide a general molecular basis for working with the genus.

Finally, DAPI based fluorescent *in situ* hybridization techniques with chromosome arm specific probes should be established in cooperation with Dr. Hieronim Golczyk (Jagiellonian University, Krakow, Poland). This approach provided substantial progress in determing chromosome arm combinations, which are a result of relatively frequently occurring reciprocal translocation events that lead to variations of chromosome formulas within *Oenothera* species.

2. MATERIAL & METHODS

2.1 MATERIAL

2.1.1 CHEMICALS

Chemicals which are not listed were purchased either from Roth (Karlsruhe), Merck (Darmstadt), New England Biolabs (Beverly, USA), Roche (Mannheim) or Sigma Chemical Company (Munich). The chemicals used were of p.a. quality unless otherwise is mentioned.

Reagents	Manufacturer
AccuGel™ 40% w/v (29:1)	National Diagnostics, Atlanta, USA
Agarose	Biozym, Oldendorf or Bio&Sell, Nürnberg
Ammoniumpersulfate (APS)	Ambresco, Solon, USA
Ampicilline	Boehringer, Mannheim
ATP	Roche, Mannheim
Bacto-Tryptone (Bacteriological Peptone)	Amersham Biosciences, Uppsala, Schweden
Biotin (syn. Vit. B_7,Vit. H, Coenzym R)	Roche, Mannheim
Biotin-Nick Translation Mix	Roche, Mannheim
Bovine Serumalbumin (BSA)	New England Biolabs, Beverly, USA
4',6-Diamidino 2-phenyindole (DAPI)	Sigma-Aldrich, Munich
Digoxigenin-11-dUTP	Roche, Mannheim
DIG-Nick Translation Mix	Roche, Mannheim
deionised Formamide	Serva, Heidelberg
FITC-conjugated sheep anti-digoxigenin	Roche, Mannheim
Fluorescein (FITC) AffiniPure Donkey Anti-Sheep IgG	Jackson Immunoresearch, Suffolk, UK
Karmin acetic acid	Merck, Darmstadt
Long Ranger ® Gel Solution	Cambrex Bioscience, Rockland, USA
Salmon Sperm DNA (blocking DNA)	Roche, Mannheim
Streptavidin-Alexa Fluor® 488	Invitrogen, Karlsruhe
Tetramethylrhodamine-5-dUTP	Roche, Mannheim
Tween 20	AppliChem, Darmstadt
Rhodamine (TRITC)	Roche, Mannheim
ROX Standard GeneScan®-500 ROX™	Applied Biosystems, Foster City, USA
TRITC-sheep anti-digoxigenin	Boehringer, Mannheim
Tween 20	AppliChem, Damstadt
Vectashield® mounting medium H-1000	Vector Laboratories, Grünberg
Yeast RNA	Ambion, Austin, USA

2.1.2 MOLECULAR BIOLOGICAL "KITS"

Molecular biological "kits"	Manufacturer
DNeasy Plant Mini Kit	Qiagen, Hilden
Thermo Sequenase™ Dyenamic Direct Sequencing Kit	Amersham Biosciences, Uppsala, Schweden
Fluorescent Antibody Enhancer Set for DIG Detection	Roche, Mannheim
DIG-Nick translation Mix	Roche, Mannheim
QIAGEN Plasmid Mini Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
Ultrafree-DA Centrifugal Filter Device	Millipore, Eschborn

2.1.3 ENZYMES

Enzymes	Manufacturer
Restriction enzymes: *AluI, ApeKI, BamHI, BclI, BseRI, BsrI, BsuRI (HaeIII)* *DdeI, EarI, EcoRI, HhaI, HindIII, HpyCH4III, MseI,* *NaeI, NotI, PflFI, PstI, RsaI, SacI, TaqI, XhoI*	New England Biolabs, Beverly, USA,
Cellulase (from Aspergillus nigra)	Fluka, Sigma-Aldrich, Buchs, Suisse
Cellulase Onozuka R-10 (from Trichoderma viride*)*	Serva, Heidelberg
Calf intestinal alkaline phosphatase (CIAP)	New England Biolabs, Beverly, USA
Pectinase (from Aspergillus niger*)*	Sigma-Aldrich, Buchs, Suisse
Pepsin	Merck, Darmstadt
Phusion™ High-Fidelity DNA Polymerases	Finnzymes, Espoo, Finland
RNase A	Roche, Mannheim
T4 DNA Ligase	Qiagen, Hilden
Taq DNAPolymerase	Qiagen, Hilden

2.1.4 UNMODIFIED OLIGONUCLEOTIDES

Name	Sequence
SP6 Pro	5´-ATT TAG GTG ACA CTA TAG AAT-3´
T7 Pro	5´-TAA TAC GAC TCA CTA TAG GG-3´
M40for	5´-ACC GTC TCC TCC AAG CAC TGC-3´
M40rev	5´-TCA GCC CTT TGT CCG AAG TCG-3´

2.1.5 UNMODIFIED OLIGONUCLEOTIDES AND ADAPTORS (AFLP)

All nucleotides listed below were obtained from MWG-Biotech (Ebersberg, Germany).

Name	Sequence
MseI adaptor 1	5'–CCC AGT CAC GAC GTT GTA AAA CG–3'
MseI adaptor 2	5'–AGC GGA TAA CAA TTT CAC ACA GG–3'
MseI+C	5´–GAT GAG TCC TGA GTA A**C**–3´
MseI+CAA	5´–GAT GAG TCC TGA GTA A**CA A**–3´
MseI+CAC	5´–GAT GAG TCC TGA GTA A**CA C**–3´
MseI+CAG	5´–GAT GAG TCC TGA GTA A**CA G**–3´
MseI+CAT	5´–GAT GAG TCC TGA GTA A**CA T**–3´
MseI+CCA	5´–GAT GAG TCC TGA GTA A**CC A**–3´
MseI+CCC	5´–GAT GAG TCC TGA GTA A**CC C**–3´
MseI+CCG	5´–GAT GAG TCC TGA GTA A**CC G**–3´
MseI+CCT	5´–GAT GAG TCC TGA GTA A**CC T**–3´
MseI+CGA	5´–GAT GAG TCC TGA GTA A**CG A**–3´
MseI+CGC	5´–GAT GAG TCC TGA GTA A**CG C**–3´
MseI+CGG	5´–GAT GAG TCC TGA GTA A**CG G**–3´
MseI+CGT	5´–GAT GAG TCC TGA GTA A**CG T**–3´
MseI+CTA	5´–GAT GAG TCC TGA GTA A**CT A**–3´
MseI+CTC	5´–GAT GAG TCC TGA GTA A**CT C**–3´
MseI+CTG	5´–GAT GAG TCC TGA GTA A**CT G**–3´
MseI+CTT	5´–GAT GAG TCC TGA GTA A**CT T**–3´
MseI+GAA	5´–GAT GAG TCC TGA GTA A**GA A-3**´
SacI Adapter1	5´–CTC GTA GAC TGC GTA CAA GCT–3´
SacI Adapter2	5´–TGT ACG CAG TCT AC–3´
SacI+G	5´–GAC TGC GTA CAA GCT C**G**–3´
SacI+C	5´–GAC TGC GTA CAA GCT C**C**–3´

AFLP-„primer" (SacI-X(X) and MseI-C(XX)) generally consist of three parts: a core-sequence (CORE), the enzyme specific sequence (ENZ) and a selective extension sequence (EXT) (Zabeau and Vos 1993).

·····CORE·····	·····ENZ·····	·····EXT
SacI 5´-GAC TGC GTA CA	A GCT C	NN N-3´
MseI 5´-GAT GAG TCC TGA G	TA A	NN N-3´

2.1.6 FLUORESCENT DYE LABELED OLIGONUCLEOTIDES (AFLP ANALYSIS)

Name	Sequence
SacI +GG 5´-FAM (6-FAM)	5´–GAC TGC GTA CAA GCT C**GG**–3´
SacI +GA 5´-FAM (6-FAM)	5´–GAC TGC GTA CAA GCT C**GA**–3´
SacI +CA 5´-FAM (6-FAM)	5´–GAC TGC GTA CAA GCT C**CA**–3´
SacI +CC 5´FAM (6-FAM)	5´–GAC TGC GTA CAA GCT C**CC**–3´
SacI +GC 5´-Joe	5´–GAC TGC GTA CAA GCT C**GC**–3´
SacI +GT 5´-Joe	5´–GAC TGC GTA CAA GCT C**GT**–3´
SacI +CG 5´-Joe	5´–GAC TGC GTA CAA GCT C**CG**–3´
SacI +CT 5´-Joe	5´–GAC TGC GTA CAA GCT C**CT**–3´

2.1.7 PLANT MATERIAL

Strain	Genome/ Plastome	Renner complex(es)	Configuration in meiosis
Oenothera elata* subsp. *elata			
chapultepec	AA-I	^{h}chapultepec	7 prs.
cholula	AA-I	^{h}cholula	7 prs.
puebla	AA-I	^{h}puebla	7 prs.
toluca	AA-I	^{h}toluca	7 prs.
Oenothera elata* subsp. *hookeri			
franciscana de Vries	AA-I	^{h}franciscana d.V.	7 prs.
franciscana E. & S.	AA-I	^{h}franciscana E.S.	7 prs.
hookeri de Vries	AA-I	^{h}hookeri d.V.	7 prs.
johansen	AA-I	^{h}johansen	7 prs.
johansen	AA-III^{lam}	^{h}johansen	7 prs.
Oenothera villosa* subsp. *villosa			
bauri	AA-I	laxans (A) • undans (A)	⊙14
Oenothera biennis			
biennis de Vries	AB-II	^{dV}albicans (A) • ^{dV}rubens (B)	⊙8, ⊙6
biennis München	AB-II	^{biM}albicans (A) • ^{biM}rubens (B)	⊙8, ⊙6

Strain	Genome/ Plastome	Renner complex(es)	Configuration in meiosis
Oenothera biennis			
purpurata	AA-II	^{h}purpurata	7 prs.
suaveolens Grado	AB-II	^{G}albicans (A) • ^{G}flavens (B)	⊙14
suaveolens Standard	AB-II	^{Std}albicans (A) • ^{Std}flavens (B)	⊙12, 1 pr.
chicaginensis Colmar	BA-III	^{Col}excellens (B) • ^{Col}punctulans (A)	⊙12, 1 pr.
Oenothera nuda			
nuda	AB-II	glabrans (A) • calvans (B)	⊙14
Oen. biennis x Oen. glazio*via*na			
conferta	AB-II	convelans (A) • aemulans (B)	⊙12, 1 pr.
Oenothera glazio*via*na			
rr-lamarckiana Sweden	AB-III^{lam}	r-^{S}velans (A) • r-^{S}gaudens (B)	⊙12, 1 pr.
coronifera	AB-III	quaerans (A) • paravelans (B)	⊙12, 1 pr.
blandina	AA-II	^{h}blandina (levans)	7 prs.
deserens	A/B-III	^{h}deserens	7 prs.
decipiens	A/B-III	^{h}deserens	7 prs.
Oenothera grandiflora			
bellamy A	BB-III	^{h}bellamy A	7 prs.
B^AB^A castleberry A-4	B^AB^A-III	$^{h}B^A$ castleberry A-4	7 prs.
B^AB^A chastang 7	B^AB^A-III	$^{h}B^A$ chastang 7	7 prs.
stockton 1	BB-III	^{h}stockton 1	7 prs.
tuscaloosa	BB-III^{tusc}	^{h}tuscaloosa	7 prs.
tuscaloosa	BB-III^{lam}	^{h}tuscaloosa	7 prs.
Oenothera nutans			
elkins II	B_1B_2-III	α elkins (B_1) • β elkins (B_2)	⊙14
horsehead II	B_1B_2-III	α horsehead II (B_1) • β horsehead II (B_2)	⊙14
marienville III	B_1B_2-III	α marienville III (B_1) • β marienville III (B_2)	⊙14
mitchell	B_1B_2-III	α mitchell (B_1) • β mitchell (B_2)	⊙14

Strain	Genome/ Plastome	Renner complex(es)	Configuration in meiosis
Oenothera oakesiana			
ammophila Standard	AC-IV	^{am}rigens (A) • percurvans (C)	⊙12, 1 pr.
Oenothera parviflora			
atrovirens	BC-IV	pingens (B) • flectens (C)	⊙14
silesiaca	BC-IV	subpingens (B) • subcurvans (C)	⊙14
st. stephen	BC-IV	α st. stephen (B) • β st. stephen (C)	N/A
Oenothera argillicola			
douthat 1	CC-V	^{h}douthat 1	7 prs.
williamsville	CC-V	^{h}williamsville	7 prs.
wilson creek 1	CC-V	^{h}wilson creek 1	7 prs.

For a detailed taxonomy see Dietrich 1977 and Dietrich *et al.* 1997.

Furthermore, 244 F2 plants from the cross *Oenothera grandiflora* strain tuscaloosa with plastome III of *Oenothera glazioviana* strain lamarckiana Sweden (BB-III^{lam}) and *Oenothera elata* subsp. *hookeri* strain johansen also with plastome III from the same plant (AA-III^{lam}) and 40 F2 plants from the cross *Oenothera elata* subsp. *elata* strain cholula with plastome I^{cho} (AA-I^{cho}) and *Oenothera elata* subsp. *elata* strain puebla with plastome I^{pue} (AA-I^{pue}) were used to generate linkage maps. The crossing partners had the same chromosomal formulas to ensure homozygous hybridization. All strains used were inbred lines, which were selfed for more than 10 generations.

2.1.8 MOLECULAR WEIGHT STANDARDS

λ-DNA was used as molecular weight standard for agarose gel electrophoresis of DNA fragments. The phage DNA was digested with restriction enzymes *EcoRI* and *HindIII*. Defined sizes of fragments were obtained: 21,226, 5,148, 4,973, 4,268, 3,530, 2,027, 1,904, 1,584, 1,375, 947, 831, 564 and 125 bp.

In addition, the 2-log ladder (0,1 – 1 kb) (New England Biolabs, Beverly, USA) was used as molecular weight standard for agarose gel electrophoresis of some DNA fragments. This digested DNA included fragments ranging from 100 bp to 10 kb. Nineteen bands were obtained: 10,002 (40 ng), 8,001 (40 ng), 6,001 (48 ng), 5,001 (40 ng), 4,001 (32 ng), 3,001 (120 ng), 2,017 (40 ng), 1,517 (57 ng), 1,200 (45 ng), 1,000 (122 ng), 900 (34 ng), 800 (31 ng), 700 (27 ng), 600 (23), 517 (124 ng), 500 (124 ng), 400 (49 ng), 300 (37 ng), 200 (32 ng), 100 (61 ng) bp.

GeneScan®-500 ROX™ Size Standard (Applied Biosystems, Foster City, USA) was used as internal lane size standard for gel electrophoresis of AFLP samples. The following sizes of fragments were used for the analysis: 500, 490, 450, 400, 350, 300, 200, 160, 150, 139, 100, 75, 50 and 35 bp.

2.1.9 MEDIA USED

SOC medium	
Bactotryptone	2 g
Yeast extract	0.55 g
1 M NaCl	1 ml
1 M KCl	1 ml
$H_2O_{ultrapur}$	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.	

LB/Ampicillin plates (1 litre)	
Bactotryptone	10 g
Yeast extract	5 g
NaCl	10 g
Bacto-agar	15 g
add H_2O to 1 litre and autoclave. The bottle was cooled down until it is to room temperature. Then 1 ml 1000x *Ampicllin* (100 mg/ml stock) was added, mixed well and poured onto sterile plates.	

2.1.10 LABORATORY EQUIPMENT

Centrifuges

Sigma-202 MK with rotor 12045

Sigma 4K15 with rotor 12130-H

Beckman centrifuge Avanti^{TM} J-25 with Rotor JS-13.1

PCR-machine

PCR-Express, Thermal Cycler (Hybaid, England)

Pulse amplitude modulated fluorometer

PAM 101/103 (Walz Mess- und Regeltechnik, Effeltrich, Germany)

Semi-dry blotting apparatus

Peqlab (Erlangen, Germany)

Hamamatsu ORCA monochromatic CCD camera (Herrsching, Germany) attached to a **Zeiss Axioplan epifluorescence microscope** (Göttingen, Germany).

2.1.11 VECTORS

The two vectors shown in Figs. 2-1 and 2-2 were used:

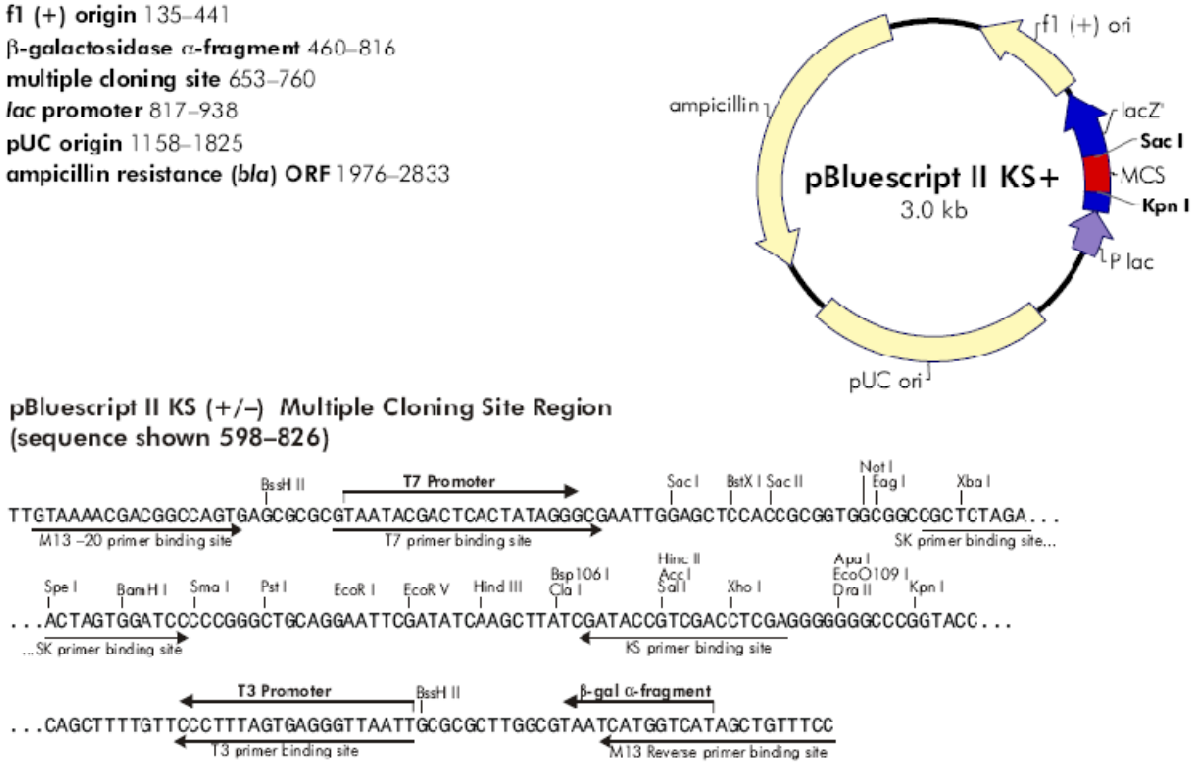
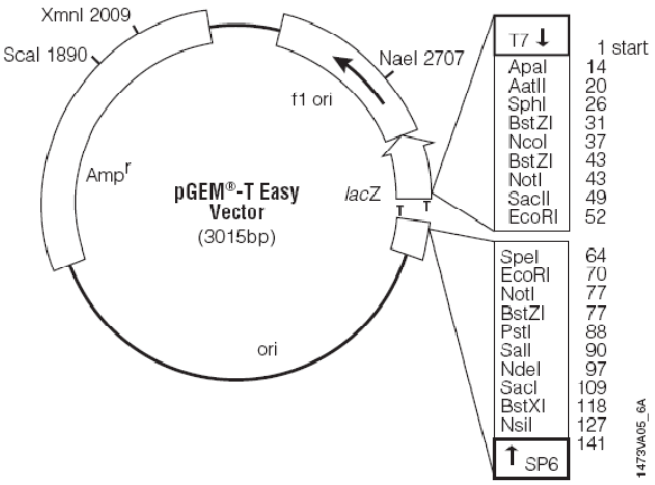


Fig. 2-1: pBluescript II KS+ vector (Stratagene, Heidelberg, Germany) was used for unspecific >10 kb DNA fragment cloning.



pGem®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning site	10-128
SP6 RNA polymerase promoter (-17 to +3)	139-158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176-197
lacZ start codon	180
lac operator	200-216
β-lactamase coding region	1337-2197
phage f1 region	2380-2835
lac operon sequences	2836-2996, 166-395
pUC/M13 Forward Sequencing Primer binding site	2949-2972
T7 RNA polymerase promoter (-17 to +3)	2999-3

Fig. 2-2: pGem®-T Easy Vector (Promega, Mannheim, Germany) was used for the cloning of PCR products.

2.1.12 BACTERIAL STRAINS

MAX Efficiency® DH5α™ Competent Cells of *E. coli* (Invitrogen, Carlsbad, California, USA)

2.1.13 SOFTWARE

- ABI Prism Collection (Applied Biosystems, Foster City, California, USA)
- Adobe Photoshop (San Jose, California, USA)
- Bioedit (Ibis Therapeutics, Carlsbad, California, USA)
- Clustal W2 (EBI, Cambridge, UK) (Chenna *et al.* 2003)
- GeneScan (Applied Biosystems, Foster City, California, USA)
- Genotyper (Applied Biosystems, Foster City, California, USA)
- JoinMap 3.0 (Kyazma B.V., Wageningen, Netherlands)
- Microsoft Access Database (Microsoft, Redmond, WA, USA)
- Microsoft Excel (Microsoft, Redmond, WA, USA)
- Microsoft Word (Microsoft, Redmond, WA, USA)
- Sequence Analysis (ABI) (Applied Biosystems, Foster City, California, USA)
- SNP2CAPS (Plant Bioinformatical Portal, Gatersleben, Deutschland) (Thiel *et al.* 2004)
- Sputnik (http://sputnik.btk.fi/)

2.2 METHODS

2.2.1 NUCLEIC ACID ANALYSIS

2.2.1.1 DNA ANALYSIS

2.2.1.1.1 ISOLATION OF TOTAL DNA

Oenothera produces high amounts of tannins and mucilage (Zinsmeister *et al.* 1965, 1970), which provide the major obstacle to DNA extraction from *Oenothera.* It was therefore necessary to avoid the co-precipitation of polysaccharides with DNA. The presence of these polysaccharides prevents complete redissolution of DNA. Furthermore, polysaccharides prevent enzyme activities, thus inhibiting processes

such as cutting with restriction enzymes, PCR or *in vitro* labeling (Barnwell *et al.* 1998). It has been proposed to use hydrolytic enzymes (Rether *et al.* 1993) or ion-exchange resins (Guillemaut and Maréchal-Drouard 1992; Maréchal-Drouard and Guillemaut 1995) to remove polysaccharides from nucleic acid solutions.

The genomic DNA was extracted from plants growing either in a greenhouse or on a field. DNeasy Plant Mini Kit in combination with a 96-well ball mill (Qiagen, Hilden, Germany) allows an efficient large-scale DNA isolation procedure for *Oenothera* tissue. The standard protocol was slightly modified: 50 - 100 mg fresh leaf material was disrupted in 2 ml reaction tubes in the presence of 400 µl of buffer AP1, 4 µl RNase A (100 mg/ml), 4 µl 10% PVP (polyvinyl pyrrolidone) and 0.4 µl 1 M sodium 1-ascorbic acid, using a Mixer Mill MM 300 (Qiagen, Hilden, Germany) (2 times 2 min at 30 Hz). The samples were subsequently incubated for 10 min at 65°C to lyse the cells. During incubation, the samples were mixed 2 - 3 times by inverting the tube. The homogenized leaf material was centrifuged (1 - 2 min at 4,000 *x* g). 130 µl of buffer AP2 was added to the lysate, mixed, and incubated for 5 min on ice to precipitate detergent, proteins and polysaccharides, followed by another centrifugation step (5 min at 20,000 *x g*). This step is crucial to DNA isolation of leaf material from *Oenothera* plants. *Oenothera* generates highly viscous lysates and large amounts of precipitates during this step. This can result in shearing DNA in the following step. Satisfactory results were obtained, because the majority of precipitates were removed by this centrifugation step. The supernatant was pipetted into the QIAshredder Mini spin column that was placed in a 2 ml collection tube, and centrifuged for 2 min at 20,000 *x g*. The column removes most of the remaining precipitate and cell debris but still a rest passed through and formed a pellet in the collection tube. The flow-through fraction was carefully placed in a new 2 ml collection tube without disturbing the cell-debris pellet. 1.5 volumes of buffer AP3/E was added to the lysate and mixed immediately by pipetting. The mixture was transferred into the DNeasy Mini spin column (a silica-based membrane) that was placed in a 2 ml collection tube and centrifuged for 1 min at ≥ 6,000 *x g.* The flow-through was discarded and the column was placed into a new 2 ml collection tube. 500 µl of buffer AW was added and centrifuged for 1 min ≥ 6,000 *x g.* This step was repeated twice, followed by another centrifugation step (2 min at 20,000 *x g*) to dry

the membrane and remove the complete ethanol that is the essential element in buffer AW. The column was transferred to a 1.5 ml tube and 30 - 50 µl of buffer AE were directly pipetted onto the DNeasy membrane. After an incubation (5 min at room temperature), the column was centrifuged (1 min 6,000 *x g*) to elute DNA. All columns and buffers used were components from DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentration and quality was estimated by spectrophotometry and ethidium bromide stained agarose gel electrophoresis.

2.2.1.1.2 PCR PRODUCT PURIFICATION

PCR products were purified using either "*QiaQuick Purification Kit"* according to manufacturer´s manual or by standard precipitation *via* Na-acetate (pH 5.2) and 100% EtOH.

2.2.1.1.3 PLASMID TRANSFORMATION

50 - 100 µl MAX Efficiency® DH5α Competent Cells (Invitrogen, Karlsruhe, Germany) (Bethesda Research Laboratories 1986) were carefully unfrozen on ice. The required volume of DNA (vector) from the ligation reaction was added and the suspension gently mixed with a pipette. Subsequently to an incubation period for 30 min on ice, cells were exposed to a heat shock (40 - 50 sec at exactly 42°C in a water bath) followed by an incubation for 5 min on ice again. 0.2 – 0.4 ml of SOC medium (Sambroock and Russell 1989) were added and the suspension was shaken at 225 rpm at 37°C for 1 h. 50 µl of the cells were plated on LB medium (Bertani 1951) plates with ampicillin and incubated for 14 – 18 h at 37°C

2.2.1.1.4 PLASMID ISOLATION

Plasmids were isolated using the "Qiagen Plasmid Mini Kit" according to the manufacturer´s protocol.

2.2.1.2 AUTOMATED SEQUENCING ON THE ABI PRISM 377 DNA SEQUENCER

Table 2-1: Pipetting scheme of sequencing gels	
H_2O_{bidest}	22.8 ml
Urea	18 g
10x TBE	6 ml
Accugel 29:1 40%	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. The DNA was labeled with "Thermo Sequenase™ Dyenamic Direct sequencing kits". The following conditions were used: 1 cycle of 95°C denaturation for 2 min; 99 cycles at 95°C (10 sec), 49 - 55°C (5 sec), and 1 cycle 60°C (4 min). The samples were cleaned up by ethanol precipitation to remove unincorporated fluorescent. The pellet was resuspended in 4 µl loading buffer (80% formamide, 10 mg/ml Dextran Blue and 5 mM EDTA), followed by a denaturation step at 80°C for 2 min. After denaturation the sample can be stored at 4°C. The sequencing gel was prepared as described in Table 2-1. The sequence run was performed using 48 cm glass plates, 0.2 mm spacers and a 36 well comb. Settings for electrophoresis conditions were 2.5 kV, 45°C and 11 h using 1x TBE electrophoresis buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3).

2.2.2 PROTEIN ANALYSIS

2.2.2.1 MEMBRANE PROTEIN ISOLATION

The entire procedure was performed at 4°C. Four g fresh *Oenothera* leaf material were homogenized in 25 ml isolation buffer (2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES-KOH (pH 7.6), 330 mM sorbitol, 10 mM NaF and 2 – 5 mM L-ascorbic acid) in a mixer. The homogenized material was filtered piece by piece through 2 layers of Miracloth (Calbiochem, Darmstadt, Germany) respectively, and

centrifuged (4°C, up to 3,000 g). The supernatant containing soluble proteins was discarded and the pellet was washed 5 - 8 times with isolation buffer. The washing step was repeated until the supernatant was not viscous anymore. The pellet containing membrane proteins was then resuspended in minimum volume of sample buffer (100 mM Na_2CO_3, 10% (w/v) sucrose and 50 mM DTT).

2.2.2.2 TOTAL PROTEIN ISOLATION

Fresh *Oenothera* material (young leaves) was ground in liquid nitrogen. The powder was then resuspended in approximately 6 ml isolation buffer (see Chapter 2.2.2.1), until the suspension is not viscous anymore. The homogenized material was filtered through 2 layers of Miracloth (Calbiochem, Darmstadt, Germany) and centrifuged for 10 min at 15.600 g. The supernatant containing soluble proteins was transferred to another collection tube and incubated for 10 - 15 min at 80°C in a heating block. The samples were placed on ice and a tip of a spatula ascorbic acid was added.

2.2.2.3 CHLOROPHYLL ABSORPTION MEASUREMENTS

Chlorophyll concentrations were measured according to Arnon (Mackinney 1941; Arnon 1949) applying the formula:

$$\mu g \text{ chlorophyll/ml} = \frac{(A_{645\ nm} \times 20.2) + (A_{663\ nm} \times 8.02)}{1000}$$

One µl protein suspension (thylakoids or total proteins) was diluted with 999 µl 80% acetone.

2.2.2.4 SEPARATION OF PROTEINS WITH SDS PAGE

A stacking gel of 8% acrylamide and a separating gel of 15% acrylamide were used to separate proteins by SDS polyacrylamide gel electrophoresis. Protein samples were mixed with the adequate volume loading buffer (4x) (0.25 M Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (w/v) glycerol, 20% (v/v) β-mercaptoethanol and 0.016% (w/v) Bromophenol Blue), and loaded onto gels after denaturation for 1 - 2 min at 80°C.

2.2.2.5 SEMI-DRY ELECTROBLOTTING

A semi-dry blotting apparatus was used to transfer proteins from SDS gels onto PVDF membranes (Amersham, Freiburg, Germany). The method was performed according to Khyse-Andersen (1984) with minor modifications. Three layers of thick filter papers soaked in cathode buffer (40 mM aminocaproic acid, 0.01% SDS) were placed onto the cathode pole, followed by the polyacrylamide gel. The PVDF membrane was preincubated in 100% methanol and placed on top of the gel. Two layers of filter papers soaked in anode buffer II (25 mM Tris, 20% methanol, pH 10.4) and three layers of filter papers soaked in anode buffer I (300 mM Tris, 20% methanol, pH 10.4) completed the blot. The blot was run at 0.8 mM/cm^2 for 2 h.

2.2.2.6 IMMUNODETECTION OF PROTEINS BY WESTERN BLOT ANALYSIS

Membranes were blocked for 1 h at room temperature or overnight at 4°C by incubation in blocking solution (20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 0.75% (v/v) Tween, 5% (w/v) skimmed milk) to prevent unspecific binding of the primary antibody. After washing in TBS-T (20 mM Tris/HCl (pH 7.4), 137 mM NaCl, 0.75% (v/v) Tween) for 15 min, the membrane was incubated for at least 1 h at room temperature or overnight at 4°C in antisera diluted to the desired concentration. The first antibody was removed by washing the membrane with TBS-T for 2x 15 min. Anti-rabbit antibodies were diluted in blocking solution and then incubated with the membrane for 1 h or overnight at 4°C. The secondary antibody was diluted 1:10,000. Finally, the membrane was washed 4x with TBS-T for 10 min, and detected directly after the final washing step. For visualizing the signal, an enhanced chemiluminiscence detection system (Amersham, Freiburg, Germany) was used. Equal volumes of development solution 1 (100 mM Tris-HCl, pH 8.5, 1% (w/v) luminal, 0.44% (w/v) coomaric acid) and 2 (Tris-HCl, pH 8.5, 0.018% (v/v) H_2O_2) were mixed and spread onto the membrane. After an incubation time of at least 1 min, the membrane was exposed to an X-ray film (Hypofilm, Amersham, Freiburg, Germany) for varying periods.

2.2.3 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

AFLP DNA fingerprinting (Vos *et al.* 1995) is a well established molecular marker technique (basic scheme see Fig 2-3), with wide applications including population

genetics (Belaj *et al.* 2003; Woodhead *et al.* 2005; Barluenga *et al.* 2006), reconstruction of shallow phylogenies (Kardolus *et al.* 1998; Després *et al.* 2003; Perrie *et al.* 2003), population assignment (Campbell *et al.* 2003), linkage mapping (*e.g.* van Eck *et al.* 1995; Alonso-Blanco *et al.* 1998; Yang *et al.* 2000; Saliba-Colombani *et al.* 2000; Tan *et al.* 2001; Hayashi *et al.* 2001; Peters *et al.* 2001; Rauwolf *et al.* 2008a), parentage analyses (Gerber *et al.* 2000), measuring genetic diversity (Mariette *et al.* 2002; Nybom 2004), identifying hybrids (Goldman *et al.* 2004) and cultivars (McGregor *et al.* 2000), and single-locus sequence-characterized amplified region (SCAR) marker development (McLenachan *et al.* 2000; Brugmans *et al.* 2003; Nicod and Largiadèr 2003; Shirasawa *et al.* 2004; Bussell *et al.* 2005). The method is based on amplification of restriction fragments of genomic DNA by polymerase chain reactions (PCR) (Mullis *et al.* 1986).

DNA fingerprinting techniques rest always on one of two strategies:

- Hybridization based method (classical method):
  In this case genomic DNA becomes digested by restriction endonucleases, followed by separation of the resulting fragments by gel electrophoresis. For example, it is possible to detect fragment length polymorphisms (RFLPs) *via* Southern hybridization by introducing radioactively labeled nucleic acid probes which correspond to variable regions.

- PCR based method:
  DNA sequences are amplified *in vitro*, using specific or unspecific starter oligonucleotides and a thermostable polymerase. The amplified products become either separated *via* gel electrophoresis or are visualized *via* coloration using labeled starter oligonucleotides.

AFLP is a combination of both strategies. It contains 3 steps, including as well digestion of DNA *via* restriction endonucleases as amplifications of the resulting fragments:

- Digestion of DNA and simultaneous ligation of adapter molecules to restriction sides (AFLP I);
- Amplification of a subset of the resulting fragments (selective "pre-amplification") (AFLP II);
- Highly selective amplification of "pre-amplified" fragments (AFLP III).

Table 2-2: Comparison of different "fingerprinting" techniques

Methods	Advantages	Disadvantages
RFLP	• good replicability	• requirement of large amounts of DNA • necessity of labeled probes • low number of bands
RAPD	• amplification *via* PCR • bands detectable without labeled probes	• unsatisfactory replicability • relatively low number of bands
AFLP	**• good replicability** **• high number of bands**	

2.2.3.1 PLANT MATERIAL USED FOR AFLP ANALYSIS

The naturally occurring species *Oenothera elata* subsp. *hookeri* strain johansen with plastome I^{joh} (AA-I^{joh}), *Oenothera grandiflora* strain tuscaloosa with plastome III^{tusc} (BB-III^{tusc}), *Oenothera elata* subsp. *elata* strain cholula with plastome I^{cho} (AA-I^{cho}) and *Oenothera elata* subsp. *elata* strain puebla with plastome I^{pue} (AA-I^{pue}) were used for crosses and to judge the genetic diversity by AFLP genotyping. The incompatible (*virescent*) combination *Oenothera elata* subsp. *hookeri* strain *johansen* with plastome III^{lam} (AA-III^{lam}) from the partial translocation heterozygote *Oenothera glazioviana* strain lamarckiana Sweden was generated by combining the ^{h}johansen-complex (AA-I^{joh}) exclusively with complexes forming complete translocation heterozygous hybrids (⊙ 14) to exchange the plastids (I^{joh} to III^{lam}) (see Fig. 3-1). All plants used were selfed over many generations to reach a high degree of homozygosity and to avoid the appearance of multiple allelism. *Oenothera grandiflora* strain tuscaloosa plants (BB-III^{tusc}) were pollinated with *Oenothera elata* subsp.

hookeri strain johansen plants (AA-III^{lam}), both with the same chromosomal formula 1•2 3•4 5•6 7•10 9•8 11•12 13•14, and the resulting F1 was selfed for generation of a F2 mapping population (244 F2-plants). A second F2 mapping population was generated with *Oenothera elata* subsp. *elata* strain cholula (AA-I^{cho}) as maternal parent in an intraspecific cross with *Oenothera elata* subsp. *elata* strain puebla (AA-I^{pue}) as paternal part (40 F2-plants), again both with the same chromosomal formula 1•4 3•2 5•9 7•10 6•8 11•12 13•14. Identical chromosomal formulas are essential to ensure homozygous hybridization. Plants were either grown under green house conditions or on the field.

2.2.3.2 AFLP REACTION I (DIGESTION AND LIGATION)

The first reaction contains the digestion of the DNA by using two different restriction endonucleases. One enzyme cuts rarely (SacI = “6-base-cutter”) and the other one frequently (MseI = “4-base-cutter”). The ligation of adapter molecules takes place simultaneously. The adapters are compatible to restriction sites, generated by the restriction endonucleases SacI and MseI.

The first AFLP reaction contained 50 to 500 ng DNA, 20 nmol ATP, 2.5 U MseI, 5 U SacI, 1x NEB-1 buffer, 15 µg BSA, 1.2 Weiss U T4 DNA ligase, 50 pmol per MseI adapter and 5 pmol per SacI adapter. The reaction batch was prepared in a 1.5 ml collection tube in a total volume of 20 µl and incubated in 37°C for 3 h, followed by a 10-fold dilution with $TE_{0.1}$ buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The samples were stored at -20°C.

2.2.3.3 AFLP REACTION II (SELECTIVE “PRE-AMPLIFICATION”)

AFLP reaction II contains the amplification of a part of the restriction fragments (AFLP I) using specified starter oligonucleotides, in our case so-called +1-primers (SacI+1 and MseI+1). The oligonucleotides used are complementary to respective adapter molecules and contain an additional nucleotide at their 3´ end. The additional nucleotide determines the fragments which are amplified in the PCR reaction.

In AFLP reaction II 0.5 U Qiagen Taq polymerase, 4 µl diluted AFLP reaction I, MseI+1 primer (MseIC or MseIG) and SacI+1 primer (SacIG or SacIC) were used with standard PCR conditions. The following PCR program was used: 2 min at 72°C cycle was followed by 20 sec at 94°C, 30 sec at 56°C, 2 min 72°C cycle. The second cycle was repeated 20 times, followed by a 30 min 60°C cycle. The resulting PCR product was diluted 10-fold with $TE_{0.1}$ buffer (pH 8.0). The samples were stored at -20°C.

2.2.3.4 AFLP REACTION III (SELECTIVE AMPLIFICATION)

Table 2-3: Pipetting scheme of AFLP gels	
Long Ranger® gel solution (acylamide)	3 ml
10x TBE	3 ml
Urea	10.8 g
H_2O_{bidest}	15.6 ml
10% APS	150 µl
TEMED	21 µl

AFLP reaction III was used to reduce the number of amplified fragments in a way that it is possible to separate the resulting amplified fragments in a polyacrylamide sequencing gel. A further reduction of the number of fragments was achieved *via* starter oligonucleotides with additional selective nucleotides at their 3´ ends (SacI+2 and MseI+3 primers). Here, the first selective nucleotide corresponds to the selective nucleotide used in AFLP reaction II. The optimum number of detectable fragments range between 50 and 200. To visualize the resulting fragments one fluorescent labeled primer has to be used (in *Oenothera* SacI+2 primers were labeled). In our case, SacI+2 primers were 5´ labeled with either 6-FAM (blue) or Joe (green).

In AFLP reaction III, 0.5 U Qiagen Taq polymerase, 4 µl diluted AFLP reaction II, 0.25 µM MseI+3 primer (Tables 2-2 – 2-4) and 0.1 µM SacI+2 (Tables 2-4 – 2-6) were used with the same PCR conditions as the AFLP II reaction. The following PCR program was used: a 2 min cycle at 94°C was followed by 20 sec at 94°C, 30 sec at

66°C – 1°C each cycle, 2 min at 72°C cycle. The second cycle was repeated 10 times. The next steps was a cycle of 20 sec at 94°C, 30 sec at 56°C, 2 min at 72°C, repeated 20 times, followed by a 30 min cycle at 60°C.

2.2.3.5 INTEGRATION OF CO-DOMINANT MARKERS INTO AFLP MAPS

ESTs were chosen to generate PCR based markers. Primers were designed and used for amplification, sequencing and comparison of genomic DNA from A and B genotypes (Mráček *et al.* 2006). 75 out of 98 selected primer pairs successfully amplified from genome A and B, respectively, demonstrating a close relationship between the two species (Greiner 2008). PCR based markers, designated M02, M07, M08, M28, M38, M39, M40, M41, M43, M46, M47, M50, M58, M59, M74, M75, M86, M88, M95, M97 and M98 were detected as described (Mráček *et al.* 2006; Greiner 2008) and used for genotyping the F2 population of the parental lines AA-III^{lam} and BB-III^{lam} (detail information about markers see Table 3-5). Five of these co-dominant markers, namely M07, M08, M28, M74 and M95, were used for segregation analysis of the F2 population (BB-III^{lam} x AA-III^{lam}). The others were used for segregation analysis of a set of 40 plants being part of the 244 F2 plants (BB-III^{lam} x AA-III^{lam}).

2.2.3.6 DNA FINGERPRINT FRAGMENT DETECTION

AFLP reaction III products were mixed with an equal volume (1 µl) formamide dye (80% formamide; 10 mg/ml Dextran Blue; 5 mM EDTA) and 0.15 µl GENESCAN-500 ROX internal lane standard (Applied Biosystems, Foster City, USA). Mixtures were denatured at 90°C for 2 min and subsequently kept at 4°C prior loading. To derive a DNA fingerprint, fragments were separated on a 5% denaturing polyacrylamide gel (Fig. 2-3) on an ABI Prism 377 DNA automated sequencer (Applied Biosystems, Foster City, USA). The gel was prepared by using Long Ranger gel solution (50% stock solution) (Cambrex, Rockland, USA). The gel finally 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% APS and 21 µl TEMED were added. Electrophoresis conditions were the following 2.5 kV, 51°C, 4 h using 1x TBE as buffer. Parental genotypes were run in the outer lanes on a subset of gels for each primer combination. The MATRIX file was generated with the dyes 6-FAM, ROX, NED and Joe. Gel images were captured

Tables 2-4, 2-5 and 2-6: List of primer combinations used for AFLP analyses.

Table 2-4

primer combinations	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	sm385	sm267	sm387	sm388	sm389	sm390	sm265	sm392	sm298	sm264	sm297	sm299	sm300	sm266	sm303	sm301
SacI GC - Joe	sm401	sm271	sm403	sm404	sm405	sm406	sm269	sm408	sm284	sm268	sm283	sm285	sm286	sm288	sm289	sm287
SacI GG - FAM	sm417	sm263	sm419	sm420	sm421	sm422	sm261	sm424	sm291	sm260	sm290	sm292	sm293	sm295	sm296	sm294
SacI GT - Joe	sm433	sn275	sm435	sm436	sm437	sm438	sm273	sm440	sm277	sm272	sm276	sm278	sm279	sm281	sm282	sm280

Table 2-5

primer combinations	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI CA - FAM	sm441	sm442	sm443	sm444	sm445	sm446	sm447	sm448	sm449	sm450	sm451	sm452	sm453	sm454	sm455	sm456
SacI CT - Joe	sm457	sm458	sm459	sm460	sm461	sm462	sm463	sm464	sm465	sm466	sm467	sm468	sm469	sm470	sm471	sm472
SacI CC - FAM	sm473	sm474	sm475	sm476	sm477	sm478	sm479	sm480	sm481	sm482	sm483	sm484	sm485	sm486	sm487	sm488
SacI CG - Joe	sm489	sm490	sm491	sm492	sm493	sm494	sm495	sm496	sm497	sm498	sm499	sm500	sm501	sm502	sm503	sm504

Table 2-6

primer combinations	MseI GAA	MseI GGA	MseI GCA	MseI GTA
SacI CA - FAM	sm505	sm506	sm507	sm508
SacI CT - Joe	sm521	sm522	sm523	sm524
SacI CC - FAM	sm537	sm538	sm539	sm540
SacI CG - Joe	sm553	sn554	sm555	sm556

Primer combinations are designated with sm (restriction enzymes SacI and MseI used for AFLP analysis) and a number. Primers are designated by using name of restriction enzyme (in this case it stands for the sequence of the adaptor) and the added selective nucleotides. **Table 2-4**: MseICXX and SacIGX primers were used; **Table 2-5**: MseICXX and SacICX primers were used; **Table 2-6**: MseIGXX and SacICX primers were used; All primer combinations used for AFLP analysis in this work are marked in orange.

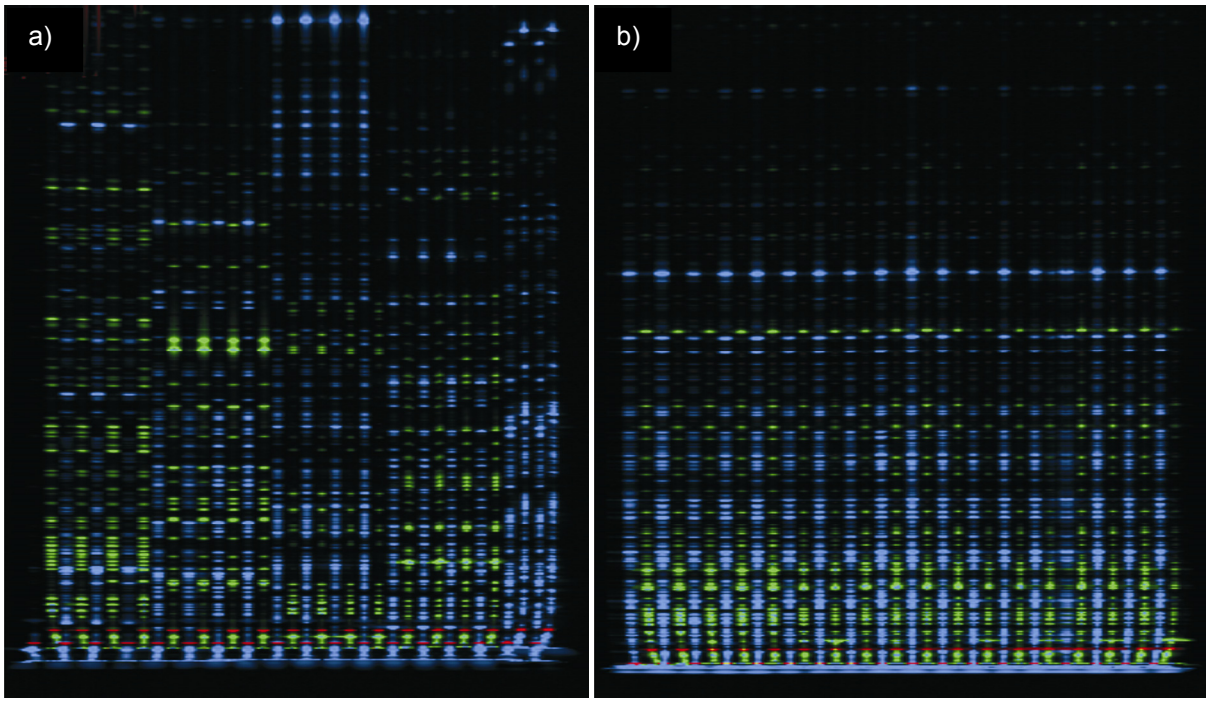


Fig. 2-3: Examples of AFLP gel images with three different dyes: ROX (red-size standard), 6-FAM (blue), Joe (green). **(a)** Gel image of parental analysis using different primer combinations, visible in clearly distinguishable lane patterns; **(b)** Gel-image of a F2 generation analysis by two different primer combinations (blue and green);

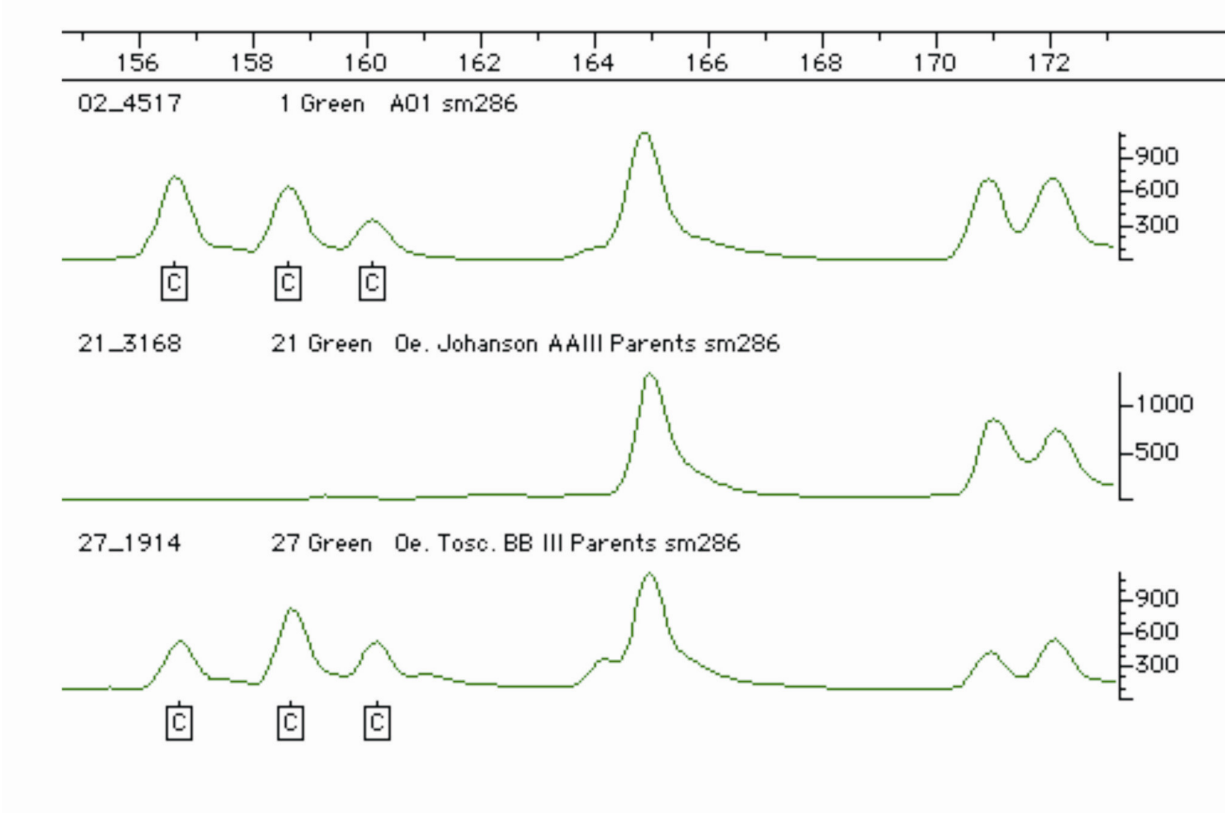


Fig. 2-4: Example of a genotyping analysis with the Genotyper program. The first chromatograph represents a plant from a F2 generation and the last two belong to parental individuals. The sm286 primer combination was used in this example.

by the GeneScan software (Applied Biosystems, Foster City, USA) (Fig. 2-4). The primer combinations were abbreviated in a matrix manner. The nomenclature for primer combinations and AFLP markers were used as described in Peters *et al.* (2001). The first two letters are initials of restriction enzymes, the next three numbers are unique IDs for primer combinations (*e.g.* sm263) (see Tables 2-4 – 2-6) and the last numbers indicate marker sizes in bp (*e.g.* sm279_102.0). Amplified fragments were visualized by fluorescence laser scanning.

2.2.3.7 COMPUTER ANALYSIS OF AFLP DATA

The GeneScan analysis software and Genotyper were used to capture genotyping and inheritance data for each polymorphic marker. Polymorphic bands were selected with 0.5 base tolerances. The data were stored in the Microsoft Access database. As statistically significant evidence of linkage a minimum LOD score threshold of 3.0 was used between at least one pair of markers in one linkage group. Segregation data were imported into JoinMap version 3.0 (Stam 1993) for initial grouping of markers. In a second step the most probable order of markers in each linkage group was determined using the Kosambi mapping function (Ott 1991). The chosen LOD is sufficiently selective to discriminate between true linkage and experimental noise.

2.2.4 A PCR-BASED NUCLEUS MARKER SYSTEM TO DISTINGUISH BETWEEN RENNER COMPLEXES

The M40 SSLP marker, derived from EST cluster C_1231-11-B04 from *Oenothera elata* subsp. *hookeri* strain hookeri de Vries encodes a chloroplast located sedoheptulose-bisphosphatase (Mráček *et al.* 2006). This marker showed a length polymorphism of 117 bp between ^{h}johansen (A genotype) and ^{h}tuscaloosa (B genotype) (Mráček *et al.* 2006). Different Renner complexes were investigated by using the M40 SSLP marker. The resulting PCR products were separated by electrophoresis on 2 – 2.5% agarose gels. PCR products in which a single band was detected were purified. In some permanent translocation heterozygotic species two bands, one for each Renner complex, were detected. The single bands were carefully excised from the agarose gel and the DNA extracted by using "*Ultrafree-DA Centrifugal Filter Device*". The resulting product was precipitated and dissolved in 15

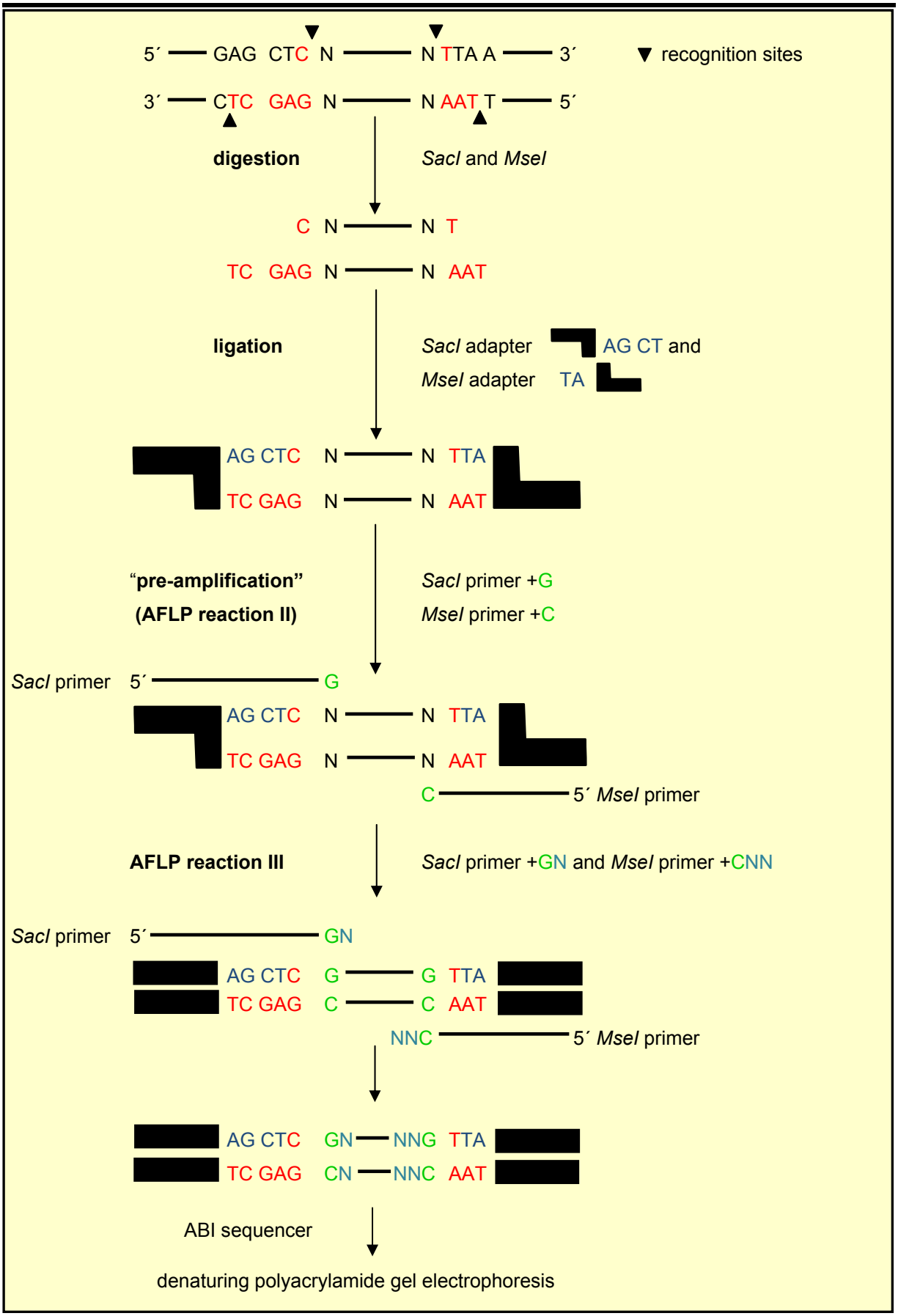


Fig. 2-5: Basic scheme of AFLP reactions used for AFLP analysis in *Oenothera*.

µl EB-buffer (component of *"QiaQuick PCR Purification Kit"*) or H_2O_{bidest} using the protocol described in Chapter 2.2.1.1.2. PCR product cloning (ligation, transformation) was performed with the "pGEM®-T easy Vector systems" (see Chapter 2.2.1.1.3). Colonies were picked, the bacteria resuspended in liquid LB/ampicillin medium in incubation tubes and incubated for 14 – 18 h at 37°C. Plasmid isolation was performed according to Chapter 2.2.1.1.4. The correctness of the inserted DNA fragment (size) was checked by PCR using primers "*SP6 Pro*" and "*T7 Pro*". The plasmids were prepared for sequencing (see Chapter 2.2.1.2). All kits were used according to manufacturer´s protocol.

2.2.5 CHROMOSOME ARM SPECIFIC LABELING BY MEANS OF FLUORESCENCE *IN SITU* HYBRIDIZATION

2.2.5.1 ISOLATION OF ≥10 KB CLONES OF *OENOTHERA*

Total DNA was isolated from *Oenothera elata* subsp. *hookeri* strain johansen as described in Chapter 2.2.1.1.1, digested with *NotI* ("8-base cutter") in 1x NEB3 buffer and 100 µg/ml BSA at 37°C for 3 - 4 h. DNA fragments were separated on a 1% low melting agarose gel by electrophoresis. Fragments ≥10 kb were extracted from the gel, using the 10 kb band of the 2-log ladder as size marker. Recovery of DNA from low melting agarose was performed according to Sambrook and Russell (1989). To check that the "correct bands" were removed, the extracted DNA was run on an agarose gel.

The vector pBluescript II KS+ was digested with *NotI* in 1x NEB3 buffer and 100 µg/ml BSA at 37°C for 3 – 4 h, followed by a CIAP treatment to dephosphorylate the restriction sites to prevent religation of the vector. A phenol/chloroform precipitation, followed by an EtOH/3M Na-acetate (20+1) precipitation was performed to purify the vector. Ligation of ≥10 kb DNA fragments and the restricted vector was performed as follows: (molar ratio of insert / vector) x amount of vector (insert: 10 kb, vector: 2,961 kb), T4 DNA ligase, T4 DNA ligase buffer, vector and DNA probe were mixed and incubated overnight at 14°C. Transformation was performed as described in Chapter 2.2.1.1.3. Colonies were picked and placed in liquid LB/ampicillin medium in tubes and incubated for 14 – 18 h at 37°C. A part of the grown colonies was mixed with an

equal volume of glycerol and stored in -70°C. Plasmid isolation was performed with the rest of the bacterial suspension as described in Chapter 2.2.1.1.4. To test the correctness of the insert, the isolated plasmid was digested using *NotI* again. Sizes of resulting fragments (~3.0 kb for the vector, ≥10 kb for the insert) were visualized by agarose gel electrophoresis (Fig. 3-19).

2.2.5.2 PREPARATION OF "METAPHASE" CHROMOSOMES FROM MITOTICALLY ACTIVE MERISTEMATIC TISSUE FROM SEEDLINGS

The cytological part was mainly done in the cytological laboratory of Dr. Hieronim Golczyk in Krakow (Poland). One to three day-old seedlings (*Oenothera elata* subsp. *hookeri* strain johansen) were stored in fixative (ethanol : glacial acetic acid 3:1) for 2 – 3 days at -20°C. The fixative was then replaced by 70% EtOH for storage. Before use, fixed root tips were washed 6x in H_2O_{bidest} for 3 min respectively and viewed under a binocular. Root tips with visible meristematic tissue (white top) were preselected, separated from the rest of the seedling and soaked in citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 6.0) for 30 min. Citrate buffer was changed every 7 – 10 min. Seedlings were treated with the enzyme mixture (40% pectinase, 40% cellulase (from *Aspergillus niger*), 2% Cellulase Onozuka R-10) for 2½ - 3 h at 37°C. Subsequently, meristems were checked again using a binocular.The enzyme mixture was removed and root tips were washed carefully 8 – 12x in citrate buffer. For staining the meristem, seedlings were covered with karmin acetic acid and incubated for 1½ - 2 h at room temperature. Karmin acetic acid was replaced by 45% acetic acid and microscopic slides were prepared. One to two seedlings was positioned in a drop of 45% acetic acid. Red colored meristematic tissue was separated from the other tissue using a binocular. Cover slips were used to cover the meristems and to squash the preparation. All preparations were examined under phase contrast and those containing well-spread mitotic metaphase chromosomes were chosen for FISH analysis. Slides were rapidly frozen in liquid nitrogen and stored in -20°C after removing the cover slips.

2.2.5.3 DIGESTION OF CYTOPLASM BY PEPSIN TREATMENT

Pepsin Solution	
Stock solution	1 mg/ml 0.01 M HCl

Microscopic slides were unfrozen before the chromosomes were treated with 100 µl 0.01 M HCl for 2 – 5 min at room temperature. HCl was removed without completely drying the slides, and 100 µl of a 1:10 dilution of pepsin stock solution was pipetted onto the chromosomes. The slides were placed in a humidity chamber and incubated for 30 min at 37°C. Pepsin was removed by washing with H_2O_{bidest}. Slides were positioned in an incubation chamber, filled with H_2O_{bidest} for 3 – 5 min at room temperature. Afterwards, the slides were dehydrated by incubation with 70% EtOH followed by another incubation step with 100% EtOH for 5 min at room temperature, respectively. The preparations were checked by DAPI treatment.

2.2.5.4 LABELING OF PROBES BY NICK TRANSLATION

DNA polymerase I possesses three activities, a 5´→3´ DNA polymerase activity, a 5´→3´ exonuclease activity and a 3´→5´ exonuclease activity. The nick translation method (Rigby *et al.* 1977) is based on the ability of DNase I to introduce randomly distributed nicks into DNA at low enzyme concentrations in the presence of $MgCl_2$. This polymerase can sequentially replace the removed nucleotides with isotope- or hapten-labeled deoxyribonucleoside triphosphates. This way, unlabeled DNA can be converted into labeled DNA.

A “DIG-Nick Translation Mix” was used to perform nick translation reactions. DIG-Nick translation mix contains digoxygenin-11-dUTP that will be inserted into the sequence. The reaction was performed according to manufacturer´s protocol. One µg DNA template (≥10 kb clone) was used for the reaction. Probes were checked by agarose gel electrophoresis.

Nick-translation products were purified with 1/10 Vol 3 M NaAc (pH 5.2) and 2.5x 100% EtOH (-20°C), incubated for 20 min at -20°C and a centrifugation step (18,800

g, 30 – 60 min, 4°C). The supernatant was discarded and the pellet washed twice with 70% EtOH (18.800 g, 5 – 10 min, 4°C). The pellet was dissolved in 10 µl EB buffer (component of *"QiaQuick PCR Purification Kit"*).

2.2.5.5 FLUORESCENCE *IN SITU* HYBRIDIZATION

Formaldehyde (1.1%)	
10x PBS	5 ml
37% Formaldehyde	1.5 ml
H_2O_{bidest}	43.5 ml

The method essentially as described in Hasterok *et al.* (2002) was used. Chromosome preparations were covered with 100 µl of RNAse A solution and incubated in humidity chamber for 1 h at 37°C. Afterwards, the slides were washed 3x with 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 5 min at room temperature. After removing SSC, the slides were incubated with 1.1% formaldehyde at room temperature for 10 min and washed again 3x with 2x SSC for 5 min. A dehydration step followed, for which slides were placed in incubation chambers filled with 70% EtOH, 90% EtOH and 100% EtOH, respectively, for 3 min each at room temperature. Subsequently, the slides were air-dried for 20 – 60 min. The critical step in this procedure is the following denaturation step of the chromosomes prepared. Therefore slides were positioned for exactly 5 min in a 75°C preheated incubation chamber filled with 70% formamide (in 2x SSC). Immediately after this step the slides were dehydrated again by incubation in icecold (4°C) 70% EtOH, 90% EtOH and 100% EtOH respectively, for 5 min. The slides were then air-dried for at least 20 min to remove all ethanol. The hybridization mixture used was (Table 2-7).

The hybridization mix was denatured for 4 min at 83°C and then incubated for 4 min at 4°C. The microscopic slides were shortly preheated at 40 – 45°C on a heating plate. 38 µl of the hybridization mix were placed on the chromosome preparations and incubated in a humidity chamber for 16 – 24 h at 42°C. The slides were then

Table 2-7: Pipetting scheme of the hybridization mixture for FISH analysis	
100% deionized formamide	50%
50% (w/v) Dextran Sulfate	10%
20x SSC	2x
Blocking DNA (sonicated Salmon Sperm DNA)	25 – 100 x probe
H_2O_{bidest}	add to 40 µl
Probe (DIG labeled)	75 – 200 ng
total volume	40 µl

washed twice in 2x SSC for 2 min, twice in 20% (v/v) formamide (in 0.1x SSC) for 5 min and three washing steps with 2x SSC for 3 min, all at 42°C. The slides were incubated again 3x in 2x SSC for 3 min at room temperature. Immunodetection was performed with the “Fluorescent Antibody Enhancer Set for DIG Detection” according to the manufacturer´s protocol: The slides were washed in 1x PBS (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2HPO_4, pH 7.4) and 1 ml 1x blocking solution was pipetted onto the slides, followed by an incubation for 30 min at 20 - 25°C. After blocking the slides were drained on a Whatman paper. 50 µl of anti-DIG solution (monoclonal antibody) was pipetted onto the slides and then incubated for 60 min at 37°C in a humidity chamber. They were then rinsed 3x briefly at 37°C in 2x SSC. Afterwards, 50 µl of anti-mouse-Ig-DIG solution (second antibody) was added and specimens were incubated for 60 min at 37°C in a humidity chamber, followed by three washing steps with 2x SSC for 1 min at 37°C. Then 50 µl of anti-DIG-fluorescein solution was pipetted onto the specimens and incubated for 60 min at 37°C in a humidity chamber. Finally, the slides were washed thoroughly 3 – 4x for 5 min at 37°C in 2x SSC and stained with DAPI. The signals were detected with a fluorescence microscope (Hamamatsu ORCA monochromatic CCD camera attached to a Zeiss Axioplan epifluorescence microscope).

2.2.6 PULSE AMPLITUDE-MODULATED FLUOROMETER MEASUREMENTS

2.2.6.1 CHLOROPHYLL *A* FLUORESCENCE ANALYSIS

Chlorophyll *a* fluorescence was measured *in vivo* on single leaves, using a PAM 101/103 fluorometer and a personal computer using the Wincontrol version 1.72 software for chlorophyll *a* fluorescence data collection as described (Schreiber *et al.* 1986, Varotto *et al.* 2000). Plants grown under identical conditions were used. The following settings were used: measure light intensity 6 (= 28 µE), gain 5 and damping 1 ms. Actinic light intensity was set to 6 (= 70 µE) and saturating pulses to 4 (= 4.000 µE/m^2s light intensity) with 1 sec duration. The saturation pulses were applied to determine the maximum fluorescence in the dark (F_M) and the ratio $(F_M – F_0)/F_M = F_V/F_M$. The quenching parameters qP (photochemical quenching = $(F_M´ - F_S)/(F_M´ - F_0)$), qN (non-photochemical quenching = $(F_M – F_M´)/F_M´$) and Φ_{PSII} (quantum yield = $(F_M´ - F_S)/F_M´$ were estimated in the steady state as described in Meurer *et al.* (1996). Under conditions of actinic light a stable level F_S was reached.

2.2.6.2 LIGHT-INDUCED CHANGES OF THE P_{700} REDOX STATE

Redox changes of P_{700} were measured by monitoring the absorbance changes at 830 nm with the PAM 101/103 chlorophyll fluorometer connected to a Dual Wavelength ED_P700DW emitter-detector unit (Schreiber *et al.* 1988). The same settings as in chlorophyll *a* measurements (Chapter 2.2.6.1) were used with the following additional parameter: Actinic red light intensity was set to 6 (= 76 µE).

3. RESULTS

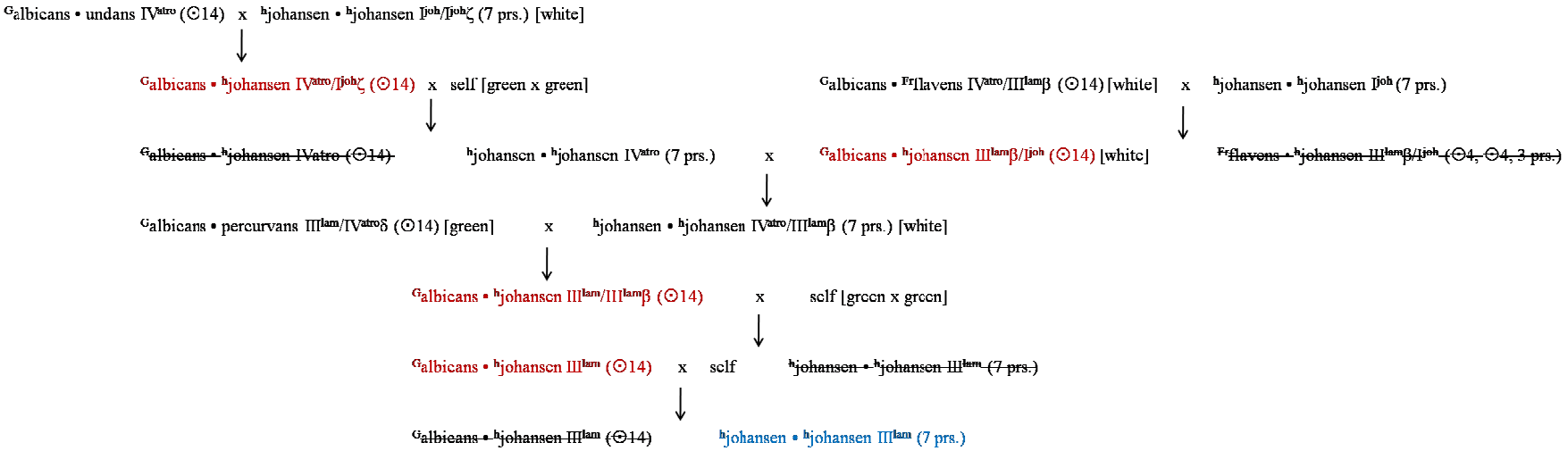
3.1 ESTABLISHMENT AND PERFORMANCE OF AFLP ANALYSES

3.1.1 GENERATION OF THE INCOMPATIBLE AA-III^{LAM} AND COMPATIBLE BB-III^{LAM} AS CROSSING PARTNERS

The basic features of the genetics of *Oenothera*, *i.e.* biparental transmission of plastids, self-fertile hybrids and translocation heterozygosity, combined with a rich resource of lines collected over about a century and for the most parts now maintained at the University in Munich allow to combine haploid genomes (complexes) from different species and to equip the resulting hybrids with chloroplasts of interest. The haploid complexes can subsequently be separated again by crosses, to generate the original parent plant containing the chloroplast foreign to the species (Fig. 1-3). This is possible because homologous recombination in the genus is suppressed and entire haploid chromosome sets in permanent translocation heterozygous species are inherited (Cleland 1972; Stubbe 1989; Harte 1994).

The naturally occurring, bivalent forming *Oenothera elata* subsp. *hookeri* strain johansen (A = haplotype johansen) and *Oenothera grandiflora* strain tuscaloosa (B = haplotype tuscaloosa) both with the chromosomal formula 1•2 3•4 5•6 7•10 9•8 11•12 13•14 were chosen as parental lines to generate a first genetic map within the genus *Oenothera*. Both were equipped with plastome III from *Oenothera glazioviana* strain lamarckiana Sweden. The artificial incompatible plastome-genome combination AA-III^{lam} (*virescent* phenotype) was a gift of Prof. Wilfried Stubbe, who established this line in 1983 and propagated it by continuous selfing. Its history of origin is shown in Fig 3-1. Therefore the complex ^{h}johansen (A) was exclusively combined with other complexes forming permanent translocation heterozygous hybrids (⊙14). The same is valid for BB-III^{lam}. Both were subsequently selfed over many generations to reach >99.99% homozygosity.

To check the purity of AA-III^{lam}, the final inbred line was compared with the naturally occurring *Oenothera elata* subsp. *hookeri* strain johansen with plastome I^{joh} (AA-I^{joh})



Symbols

Galbicans – egg cell or α-complex of *Oe. biennis* strain suavcolens Grado (Galbicans • Gflavens) [α-complex ♀; β-complex ♀♂]

Frflavens – pollen or β-complex of *Oe. biennis* strain suaveolens Friedrichshagen (Fralbicans • Frflavens) [α-complex ♀; β-complex ♀♂]; the complex if found facultative in egg cells

hjohansen – alethal complex of *Oe. elata* subsp. *hookeri* strain johansen (hjohansen • hjohansen) [haplo-complex ♀♂]

percurvans = pollen or β-complex of *Oe. oakesiana* strain ammophila (amrigens • percurvans) [α-complex ♀; β-complex ♂]

undans – pollen or β-complex of *Oe. villosa* subsp. *villosa* strain bauri (laxans • undans) [α-complex ♀; β-complex ♂]

Ijoh – basic plastome type I from *Oe. elata* ssp. *hookeri* strain johansen

IIIlam = basic plastome type III from *Oe. glazioviana* strain lamarckiana Sweden

IVatro – basic plastome type IV from *Oe. parviflora* strain atrovirens

Ijohζ – plastome mutant ζ from plastome Ijoh; bleached phenotype

IIIlamβ – plastome mutant β from plastome IIIlam; bleached phenotype

IVatroδ = plastome mutant δ from plastome IVatro; bleached phenotype

7 prs. = chromosome configuration of 7 bivalents (pairs) in diakinesis

⊙4, ⊙4, 3 prs. – chromosome configuration of two rings of four chromosomes and three free bivalents (pairs) in diakinesis

⊙14 – ring of 14 chromosomes in diakinesis of the hybrid; free segregation of chromosome and homologous recombination is suppressed

white or green – plastome mutants are used to mark plastome types in these crosses; due to somatic segregation sorting out to plastome types happens during morphogenesis; in crosses either white brachens (carrying exclusively the mutated plastome) or green brachens (with no mutated plastome) were used

Fig. 3-1: Crossing scheme to generate the hybrid *Oe. elata* subsp. *elata* strain johansen with plastome III from *Oe. glazioviana* strain lamarckiana Sweden (^{h}johansen·^{h}johnasen III^{lam}). The final hybrid is marked in blue. The Renner complex ^{h}johansen was combined four times with ^{G}albicans during the crossing program (marked in red) (Greiner 2008).

Table 3-1: Primer combinations used for the comparative AFLP analysis of AA-I^{joh} and AA-III^{lam}.

primer combinations	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	sm385	sm267	sm387	sm388	sm389	sm390	sm265	sm392	sm298	sm264	sm297	sm299	sm300	sm266	sm303	sm301
SacI GC - Joe	sm401	sm271	sm403	sm404	sm405	sm406	sm269	sm408	sm284	sm268	sm283	sm285	sm286	sm288	sm289	sm287
SacI GG - FAM	sm417	sm263	sm419	sm420	sm421	sm422	sm261	sm424	sm291	sm260	sm290	sm292	sm293	sm295	sm296	sm294
SacI GT - Joe	sm433	sn275	sm435	sm436	sm437	sm438	sm273	sm440	sm277	sm272	sm276	sm278	sm279	sm281	sm282	sm280

Primer combinations are designated with sm (restriction enzymes SacI and MseI used for AFLP analysis) and a number. Primers are designated using the names of restriction enzymes (in this case it stands for the sequence of the adaptor) and the added nucleotides. Primer combinations marked in orange were used for analysis of Oe. *elata* subsp. *hookeri* strain johansen with plastome I^{joh} (AA-I^{joh}) and artificial Oe. *elata* subsp. *hookeri* strain johansen with plastome III^{lam} (AA-III^{lam}).

Table 3-2: Results of the comparative AFLP analysis of AA-I^{joh} and AA-III^{lam}.

primer combinations	total bands AA-I^{joh}	total bands AA-III^{lam}	polymorphic bands between AA-I^{joh} and AA-III^{lam}
sm261	75	75	0
sm263	78	78	0
sm267	108	108	0
sm276	49	49	0
sm279	47	48	1
sm280	80	80	0
sm281	54	54	0
sm285	65	65	0
sm290	66	66	0
sm299	88	88	0

The total number of bands was counted. All bands detected in AA-I^{joh} were also present in AA-III^{lam}. In AA-III^{lam} a single band was detected, which was not present in AA-I^{joh}. It was detected with primer combination sm279 (marked in yellow). So, just one band of a total of 711 (0.14%) in AA-III^{lam} is a band arisen from the linkages with the Renner complex ^{G}albicans (from *Oe. biennis* strain suaveolens Grado = ^{G}albicans • ^{G}flavens) while exchanging the plastome (see Fig. 3-1).

by AFLP marker analysis. Ten primer combinations designated as sm261, sm263, sm267, sm279, sm280, sm281, sm285, sm290, sm299 were analyzed in both materials (Table 3-1). Just a single polymorphic band of a total band number of 711 (710 in AA-I^{joh}) was detected (Table 3-2). These data proof for the first time by molecular analysis that homologous recombination was probably dramatically reduced throughout crossing experiments *via* stable translocation heterozygous hybrids, although in the chosen example the johansen complex was four times combined with another complex during the crossing program (Fig. 3-1). Most probably, the limited homologous recombination events were restricted to telomeric regions, otherwise a much higher degree of contamination from other genomes would have been expected.

3.1.2 DEVELOPMENT OF AFLP MARKERS

First, polymorphic markers between the two designated parental lines *Oenothera elata* subsp. *hookeri* strain johansen (AA-III^{lam}) and *Oenothera grandiflora* strain tuscaloosa (BB-III^{lam}) were generated by AFLP analysis. For this purpose, 120 primer combinations were analyzed. *In toto*, 10,245 bands were detected. 4,075 of them (33.96 polymorphic bands per primer combination on average) were polymorphic between the two lines, whereas both shared 6,170. This way, 39.78% polymorphism between the two lines was determined (Table 3-3a). Sizes of markers detected ranged between 45 to 500 bp, with an average of 242 bp. There was considerable variation in the number of bands produced with different primer sets. Those primer combinations that generated the largest number of polymorphic markers were selected for further analysis (Tables 3-4a-c).

Tables 3-3a and 3-3b: Statistics of the linkage maps and analyses of genetic diversities.

Table 3-3a	number of investigated primer combinations	total detected polymorphic bands	average number of polymorphic bands per primer combination	detected shared bands	number of total bands (shared bands + polymorphic bands)	**percentage of polymorphism**
^{h}johansen (AA-III^{lam}) and **^{h}tuscaloosa (BB-III^{tusc})**	120	4,075	**33.96**	6,170	10,245	**39.78%**
^{h}cholula (AA-I^{cho}) and ^{h}puebla (AA-I^{pue})	14	120	**8.57**	1,100	1,220	**10.91%**

Table 3-3b	coupling group 1	coupling group 2	coupling group 3	coupling group 4	coupling group 5	coupling group 6	coupling group 7
number of markers detected (**^{h}johansen)**	134	131	116	127	91	109	114
number of markers detected (**^{h}tuscaloosa)**	142	134	100	113	94	115	125
number of markers detected (**^{h}cholula)**	4	3	1	2	1	4	2
number of markers detected (**^{h}puebla)**	3	3	1	2	3	1	1
total genetic distance detected (in cM) (**^{h}johansen** and **^{h}tuscaloosa**)	10	20	14	12	18	7	2
total genetic distance detected (in cM) (**^{h}cholula** and **^{h}puebla**)	1	1	0	0	0	0	0
segregation ratio detected of single coupling groups in F2 [**^{h}johansen (A) and ^{h}tuscaloosa (B)]**	AA:AB:BB **1 : 4,5 : 3,5**	AA:AB:BB **1 : 2,3 : 1,5**	AA:AB:BB **1,5 : 2,5 : 1**	AA:AB:BB **1 : 2,3 : 1**	AA:AB:BB **1,2 : 2,3 : 1**	AA:AB:BB **1 : 3,2 : 2,7**	AA:AB:BB **1 : 2,5 : 1,2**
segregation ratio detected of single coupling groups in F2 [**^{h}cholula (A) and ^{h}puebla (A´)]**	AA:AA´:A´A´ **1,1 : 2,4 : 1**	AA:AA´:A´A´ **1,6 : 4 : 1**	AA:AA´:A´A´ **1 : 3,7 : 1**	AA:AA´:A´A´ **1 : 2,2 : 1,3**	AA:AA´:A´A´ **1,9 : 2,4 : 1**	AA:AA´:A´A´ **1 : 1,3 : 1,3**	AA:AA´:A´A´ **1 : 2,6 : 1,4**

Table 3-4a (1-3): Primer combinations used for AFLP analysis to generate the first genetic maps of the ^{h}johansen (A) and ^{h}tuscaloosa (B) complex.

Table 3-4a (1)																
primer combinations	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	sm385	sm267	sm387	sm388	sm389	sm390	sm265	sm392	sm298	sm264	sm297	sm299	sm300	sm266	sm303	sm301
SacI GC - Joe	sm401	sm271	sm403	sm404	sm405	sm406	sm269	sm408	sm284	sm268	sm283	sm285	sm286	sm288	sm289	sm287
SacI GG - FAM	sm417	sm263	sm419	sm420	sm421	sm422	sm261	sm424	sm291	sm260	sm290	sm292	sm293	sm295	sm296	sm294
SacI GT - Joe	sm433	sn275	sm435	sm436	sm437	sm438	sm273	sm440	sm277	sm272	sm276	sm278	sm279	sm281	sm282	sm280

Table 3-4a (2)																
primer combinations	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI CA - FAM	sm441	sm442	sm443	sm444	sm445	sm446	sm447	sm448	sm449	sm450	sm451	sm452	sm453	sm454	sm455	sm456
SacI CT - Joe	sm457	sm458	sm459	sm460	sm461	sm462	sm463	sm464	sm465	sm466	sm467	sm468	sm469	sm470	sm471	sm472
SacI CC - FAM	sm473	sm474	sm475	sm476	sm477	sm478	sm479	sm480	sm481	sm482	sm483	sm484	sm485	sm486	sm487	sm488
SacI CG - Joe	sm489	sm490	sm491	sm492	sm493	sm494	sm495	sm496	sm497	sm498	sm499	sm500	sm501	sm502	sm503	sm504

Table 3-4a (3)				
primer combinations	MseI GAA	MseI GGA	MseI GCA	MseI GTA
SacI CA - FAM	sm505	sm506	sm507	sm508
SacI CT - Joe	sm521	sm522	sm523	sm524
SacI CC - FAM	sm537	sm538	sm539	sm540
SacI CG - Joe	sm553	sn554	sm555	sm556

Primer combinations were designated with sm (restriction enzymes SacI and MseI used for AFLP analysis) and a number. Primers were designated with name of restriction enzyme (in this case it stands for the sequence of the adaptor) and the added nucleotides. 3-4a (1) MseICXX and SacIGX primers were used; 3-4a (2) MseICXX and SacICX primers were used; 3-4a (3) MseIGXX and SacICX primers were used. All primer combinations used for AFLP analysis of *Oe. elata* subsp. *hookeri elata* strain johansen with plastome III^{lam} (AA-III^{lam}) and *Oe. grandiflora* strain tuscaloosa with plastome III^{tusc} (BB-III^{tusc}) are marked in orange. Primer combinations marked in red gave no satisfactory results.

Table 3-4b (1-3): All bands detected in ^{h}johansen (A) and ^{h}tuscaloosa (B).

Table 3-4b (1)																
total bands (AA-III^{lam} and BB-III^{tusc})	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	156	152	-	142	-	136	121	-	-	69	105	115	106	140	117	-
SacI GC - Joe	76	53	53	50	39	58	45	55	41	30	58	81	60	51	56	87
SacI GG - FAM	119	100	71	98	88	81	99	-	56	51	81	-	118	77	69	94
SacI GT - Joe	54	-	61	-	73	76	40	72	55	51	69	40	68	71	60	113

Table 3-4b (2)																
total bands (AA-III^{lam} and BB-III^{tusc})	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI CA - FAM	148	94	113	115	105	93	91	104	90	47	81	62	123	105	98	113
SacI CT - Joe	121	104	113	110	101	102	85	98	123	41	62	46	84	84	76	117
SacI CC - FAM	-	64	-	98	75	110	85	101	69	67	81	91	89	83	75	64
SacI CG - Joe	91	91	113	123	123	96	109	122	63	64	78	72	75	99	91	56

Table 3-4b (3)				
total bands (AA-III^{lam} and BB-III^{tusc})	MseI GAA	MseI GGA	MseI GCA	MseI GTA
SacI CA - FAM				
SacI CT - Joe	99			
SacI CC - FAM	60			
SacI CG - Joe	62			

The number of total bands (AA-III^{lam} + polymorphic bands from BB-III^{tusc} or BB-III^{tusc} + polymorphic bands from AA-III^{lam}) was counted. The number of bands ranged from 30 (sm268) to 156 (sm385). An average of 85.38 total bands per primer combination was detected.

Table 3-4c (1-3): Polymorphic bands detected between the complexes ^{h}johansen (A) and ^{h}tuscaloosa (B).

Table 3-4c (1)

polymorphic bands (AA-III^{lam} and BB-III^{tusc})	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	47	79	-	29	-	50	27	-	-	19	48	49	20	32	33	-
SacI GC - Joe	28	8	13	8	7	17	12	16	15	8	20	25	31	14	12	19
SacI GG - FAM	35	43	8	30	35	35	39	-	18	27	35	-	38	22	24	20
SacI GT - Joe	16	-	19	-	34	29	15	20	26	20	37	11	40	34	26	60

Table 3-4c (2)

polymorphic bands (AA-III^{lam} and BB-III^{tusc})	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI CA - FAM	53	34	69	31	35	63	46	41	41	15	44	28	40	65	33	69
SacI CT – Joe	18	38	50	57	22	47	41	23	26	14	11	28	35	19	24	63
SacI CC - FAM	-	43	-	52	33	72	21	38	35	25	47	63	61	56	27	13
SacI CG – Joe	54	43	50	46	76	42	18	29	40	36	30	34	43	26	54	15

Table 3-4c (3)

polymorphic bands (AA-III^{lam} and BB-III^{tusc})	MseI GAA	MseI GGA	MseI GCA	MseI GTA
SacI CA - FAM				
SacI CT - Joe	68			
SacI CC - FAM	33			
SacI CG - Joe	47			

Polymorphic bands between AA-III^{lam} (^{h}johansen • ^{h}johansen with plastome III^{lam}) and BB-III^{tusc} (^{h}tuscaloosa • ^{h}tuscaloosa with plastome III^{lam}) were counted. Between 7 (sm405) and 79 (sm267) (average 33.96) polymorphic bands per primer combination were detected. Primer combinations marked in black were used for the generation of the "basic maps" (244 F2 plants, see Chapter 3.1.3). Primer combinations marked in black and blue were used for the generation of the "extension maps" (40 F2 plants, see Chapter 3.1.6) of ^{h}johansen (A) and ^{h}tuscaloosa (B).

3.1.3 GENERATION OF THE FIRST AFLP MAPS (BASIC MAPS)

A F1 population was generated using BB-III^{lam} (^{h}tuscaloosa • ^{h}tuscaloosa with plastome III^{lam}) (Fig. 3-2b) as maternal parent plant in the interspecific cross with incompatible AA-III^{lam} plants (^{h}johansen • ^{h}johansen with plastome III^{lam}) (Fig. 3-2a). Both materials possess the same chromosomal formula to ensure homozygous hybridization. The F1 generation possesses the plastome-genome combination AB-III^{lam} and is fully compatible and uniform (green) according to Stubbe (1989). F1 plants were selfed and 244 F2 plants were used to generate a first genetic map (basic map). On this occasion bivalent forming was checked cytologically to prove the correctness of the chromosomal formulas (Fig. 3-3). Green and different kinds of incompatible F2 plants were observed (Fig. 3-4). The segregation ratio of incompatible plants in F2 could not be reliably determined because of a large variation of incompatible phenotypes (Fig. 3-4) and because of changing ratios during the first weeks of development, due to lethality and/or the different periodical appearance of incompatible phenotypes (most only 8 – 10 days after the germination) (Schötz 1958). At least two loci, possably three, cause the incompatibility in the plastome-genome combination AA-III^{lam}. Ten primer combinations of the 120 previously analyzed, designated sm261, sm263, sm267, sm276, sm279, sm280, sm281, sm285, sm290 and sm299 were applied to F2 plants (Table 3-4c (1)). Only the best polymorphic bands of these primer combinations, fulfilling the criteria (1) indisputably detectable bands, and (2) a gapless dataset in F2 plants, were used (222 of 441).

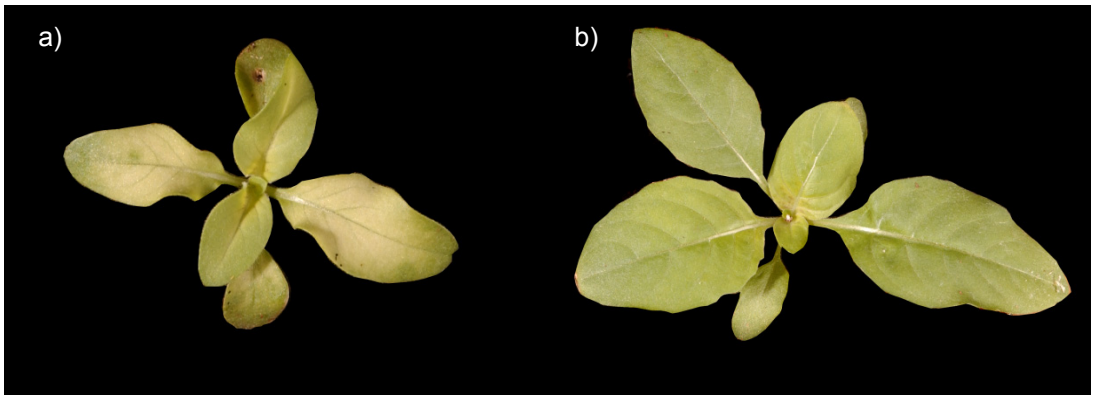


Fig. 3-2: Phenotype of parental plants used for mapping approaches. **(a)** Incompatible phenotype (*virescent*) of AA-III^{lam} plants. **(b)** Compatible green phenotype of BB-III^{lam} plants.

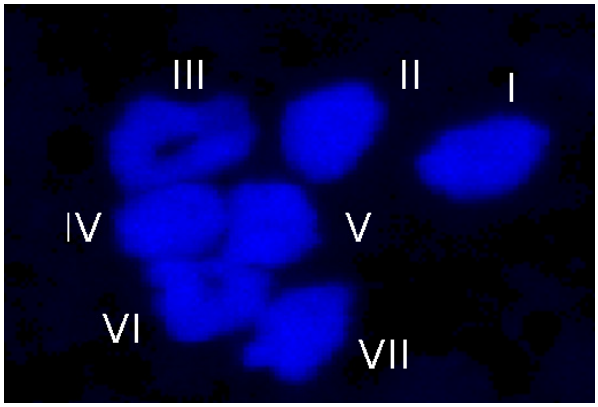


Fig. 3-3: Metaphase plate with seven bivalents in the F1 generation ^{h}johansen • ^{h}tuscaloosa (DAPI staining). The bivalent forming chromosomes are numbered I to VII.

In this way, two genetic maps, one for AA-III^{lam} (^{h}johansen) and another one for BB-III^{lam} (^{h}tuscaloosa), were calculated (Fig. 3-5a, 3-5b). Each contained indeed seven coupling groups, corresponding to the seven chromosomes of the evening promrose genome. The minimum LOD score was set to >3.0. The LOD score calculated by JoinMap for the recombination frequency is based on the G^2 statistic for independence in a two-way contingency table: $G^2 = 2 \Sigma o \log(o/e)$ (o = observed number of individuals; e = expected number of individuals; Σ = sum over all cells) (van Ooijen and Voorrips 2001). The LOD score, and thus the significance of the data, is predominantely dependent on the number of plants used for generating the genetic map (Koorneef and Stam 1992). The linkage map of the A genome consists of 100, the linkage map of the B genome of 122 dominant AFLP markers. Around 90% of the markers located on a coupling group showed clustering on the respective chromosomes, just flanked, if at all, by a few markers. Single clusters of markers suggested that homologous recombination in bivalent forming *Oenotheras* is strikingly limited to telomeric regions on the chromosomes, as in translocation heterozygotes. The order of single markers, positioned in the cluster was impossible to determine, because in anyone of the F2 plants investigated (in total approximately 300) homologous recombination could be detected with none of the markers. To summarize, it appeared that *single chromosomes* can freely segregate, even if not all chromosomes show the expected Mendelian ratio 1:2:1 (Table 3-3b). This is explainable with competition of chromosomes in megaspore, embryo sac or pollen tube, described from *Oenothera* species (Renner 1917a; Renner 1921a; Harte 1994).

The few markers which displayed recombination, showed more or less the same Mendelian or “disturbed” segregation ratios as the whole chromosomes, they are located on.

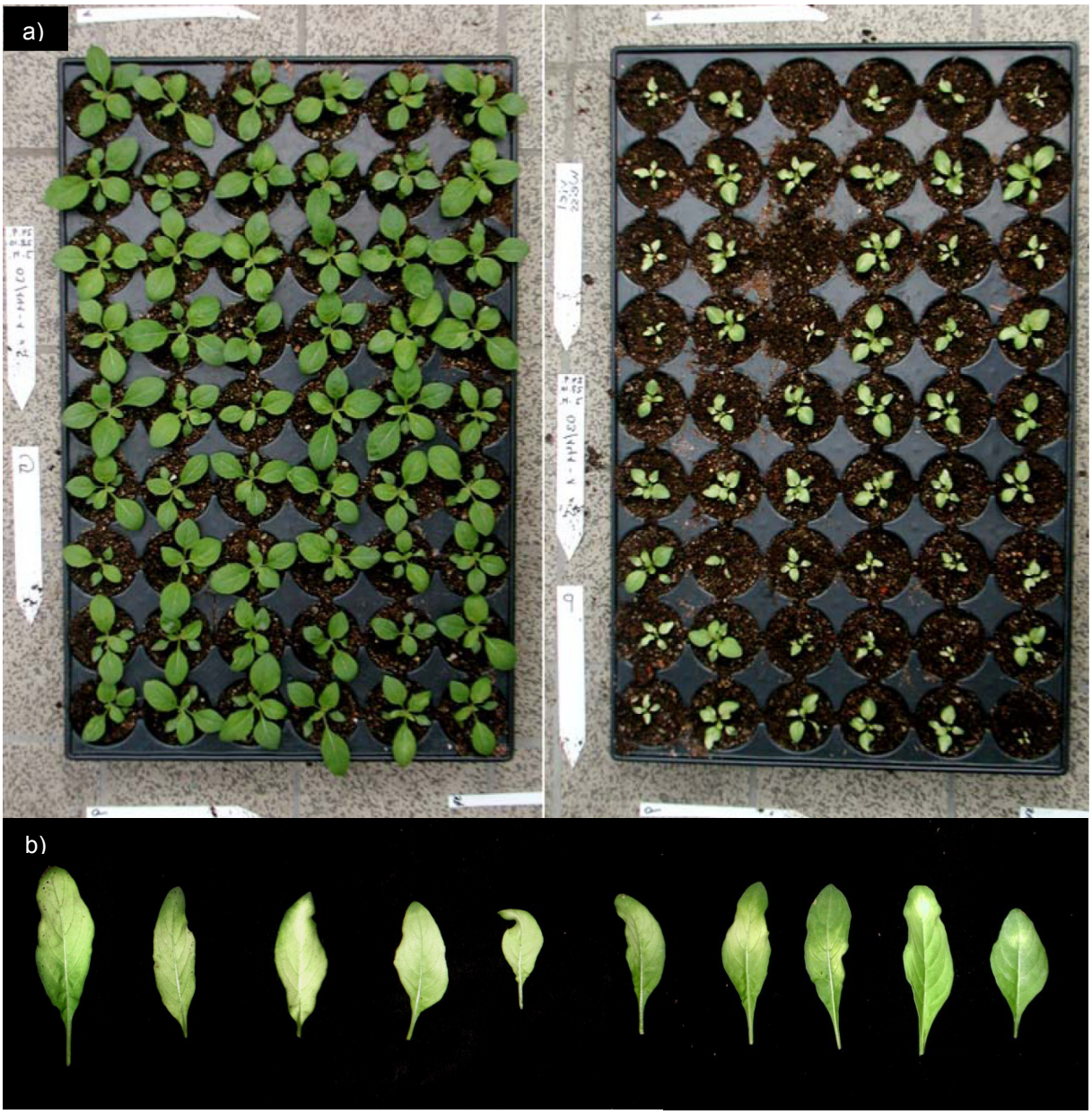


Fig. 3-4: Different phenotypes observed in the F2 generation of the cross BB-III^{lam} x AA-III^{lam}. **(a)** Compatible (left) and incompatible (right) approximately two-week-old F2 seedlings after germination. **(b)** Different strengths of incompatible phenotypes observed in the F2 generation of the cross BB-III^{lam} x AA-III^{lam}.

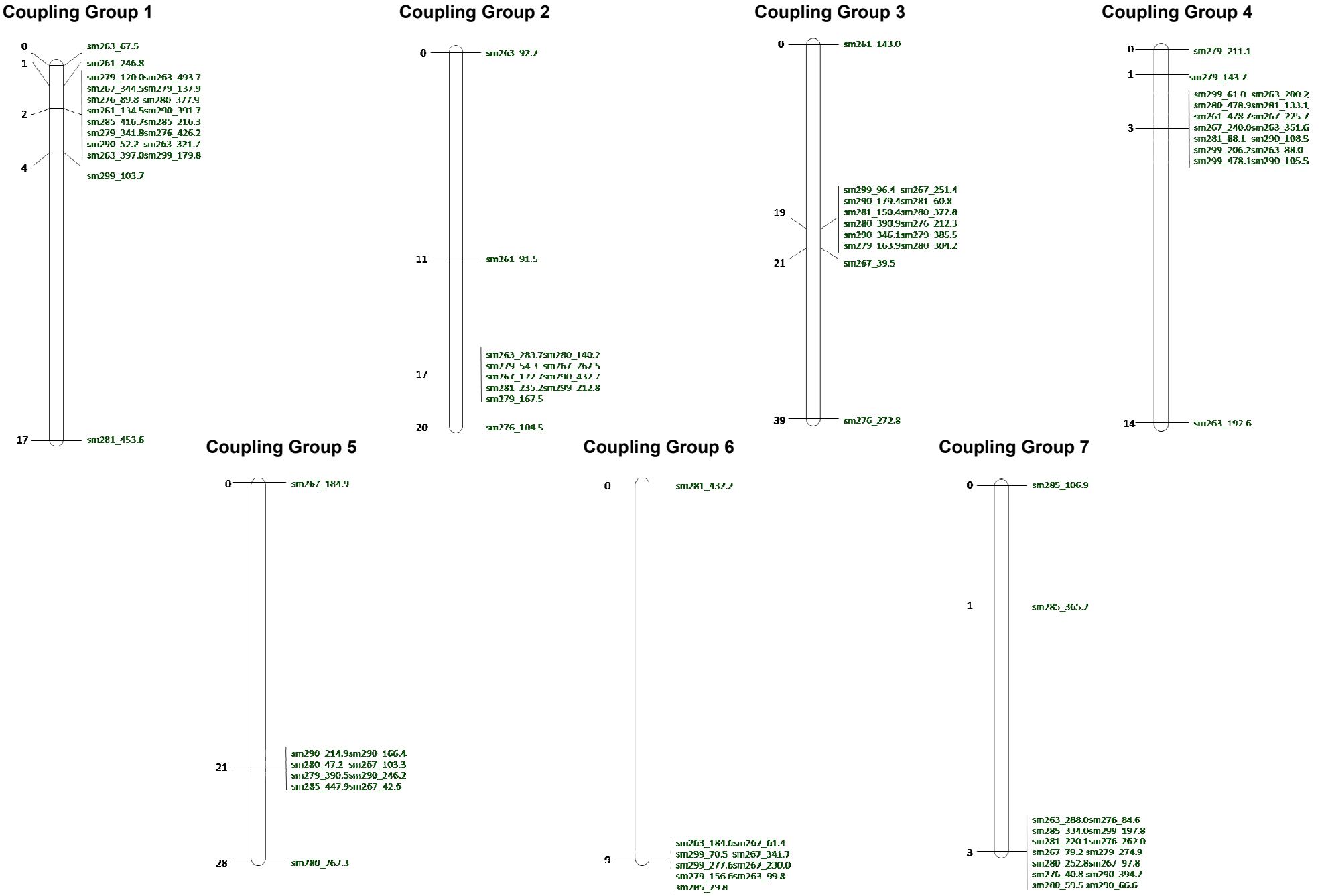


Fig. 3-5a: Genetic map of *Oe. elata* subsp. *hookeri* strain johansen with plastome III^{lam} (AA-III^{lam}) constructed with AFLP markers

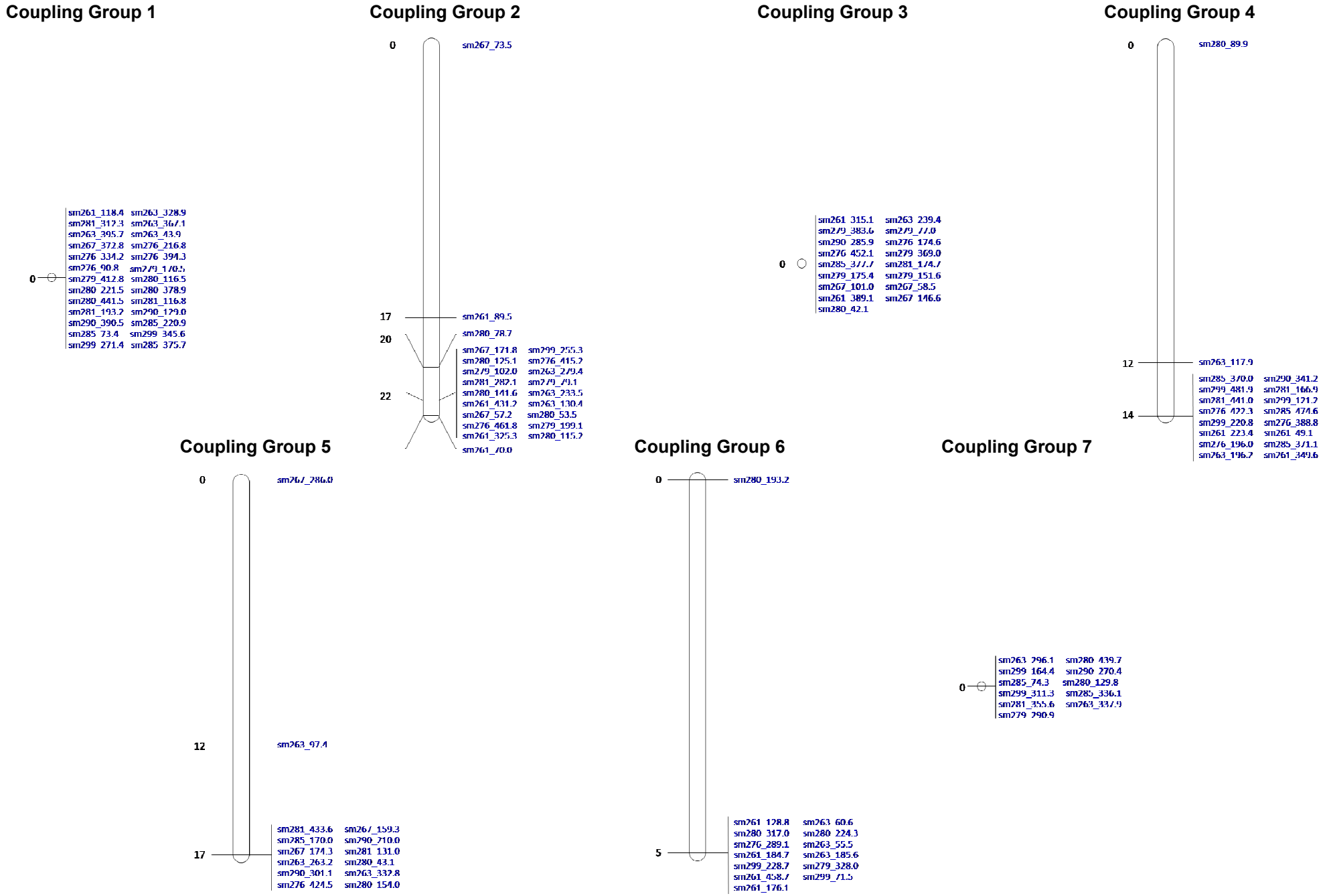


Fig. 3-5b: Genetic map of *Oe. grandiflora* strain tuscaloosa with plastome III^{lam} (BB-III^{lam}) constructed with AFLP markers

3.1.4 INTEGRATION OF THE TWO BASIC MAPS

Because of lack of homologous recombination between the two haploid chromosome sets, compatible coupling groups of the two maps were assigned. Thus, dominant, not recombining AFLP markers can be handled as co-dominant markers. The flanking markers of the respective chromosomes were compared with highest probability. This allows an integration of the two linkage maps (Fig. 3-6). A merged linkage map, consisting of 222 dominant AFLP markers, was generated. These markers were again located on seven coupling groups, corresponding to the seven chromosomes, and covered a total length of 83 cM using the Kosambi function (Ott 1991). Furthermore, two loci, evidently involved in causing AA-III incompatibility, were mapped on coupling group 4 and on coupling group 7, respectively (Fig. 3-6). Detail informations about the AFLP markers assigned to integrated genetic map are listed in the Appendix.

3.1.5 INTEGRATION OF CO-DOMINANT MARKERS INTO AFLP MAPS

Unfortunately, dominant AFLP markers cannot be used to detect allelic variation. This was one of the reasons for generating an *Oenothera* EST library (Mráček *et al.* 2006). The information obtained was used to develop the first co-dominant markers for evening primroses (Greiner 2008). Twenty-two co-dominant markers, designated M02, M07, M08, M19, M28, M38, M39, M40, M41, M43, M46, M47, M50, M58, M59, M74, M75, M86, M88, M95, M97, M98, were used for segregation analysis of selected 40 individuals of the 244 F2 plants (“expansion maps”, see Chapter 3.1.6, Fig. 3-8) with AFLP analysis. Five co-dominant markers, namely M07, M08, M28, M74 and M95, were then used for segregation analysis of the 244 plants (Table 3-5). The co-dominant markers mapped did not show differences compared to dominant AFLP markers. So, most of the markers did not show homologous recombination and were thus located in the cluster of dominant AFLP markers (Fig. 3-6 and Fig. 3-8). Only three out of the 22 co-dominant markers, designated M38, M40 and M98, showed homologous recombination (13.6%), comparable to the ratio detected with dominant markers (7.53%; see Chapter 3.1.6) considering the smaller number of co-dominant markers.

Table 3-5: PCR based co-dominant markers detected between the Renner complexes ^{h}johansen (above) and ^{h}tuscaloosa (below) (see also Mráček *et al.* 2006 and Greiner 2008).

marker	accession number	intron	SSLP	no. SNPs	marker type (enzyme)	predicted PCR products in [bp][1)]	predicted restriction fragments [bp][1)]	primer	primer sequence (5'-to-3')	EST/cluster accession	closest *Arabidopsis* homologue (blastX)	protein function and localization in *Arabidopsis*
M02[2)]	EU483117 EU483119	no	no	2	CAPS (*Ape*KI)	288 288	250, 38 186, 64, 38	M02for M02rev	tggccatggcgacacaagcctc cctcaacctgagccttacggag	C_4044-89-F11	At1g03130	photosystem I reaction centre subunit (PsaD2), putative, chloroplast
M07[2)]	EU483125 EU483127	no	no	2	CAPS (*Pst*I)	356 356	356 299, 57	M07for M07rev	accatacccatatacccagtgc tcaagcggcttcggtgcatctc	S_4170-90-H07	At5g64380	fructose-bisphosphatase-like protein, mitochondrium
M08[2)]	EU483129 EU483131	no	no	2	CAPS (*Bsu*RI)	282 282	247, 35 173, 74, 35	M08for M08rev	ctcagccaggaggacctcaagc gaggtgggtatcgacctcgtcg	S_3501-84-D07	At2g01290	ribose 5-phosphate isomerase, localization un-known
M19	EU447207 EU447208	no	no	1	CAPS (*Pfl*MI)	396 396	208, 188 396	M19for M19rev	aatcctaatggctgcctctaca cacactgcctcaccgaact	C_2501-25-D11	At1g29920	chlorophyll *a*/*b*-binding protein – like, chloroplast
M28	EU447211 EU447212	no	no	1	CAPS (*Bsu*RI)	278 278	182, 39, 30, 27 212, 39, 27	M28for M28rev	ggctccgacatccttgtggag gcgactaaggggacgctatcg	S_56-4-B10	At3g48560	acetolactate synthase, chloroplast
M38	EU447215 EU447216	no	no	2	CAPS (*Bsu*RI)	213 213	156, 44, 13 200, 13	M38for M38rev	ggcaaagctatggccactctc gtccgaccaagcagcgacgtt	S_1191-10-F12	At2g37220	RNA-binding protein (Cp29), chloroplast
M39	EU447217 EU447218	yes	no	2	CAPS (*Bcl*I)	680 680	374, 262, 44 374, 306	M39for M39rev	ccaaagtggtatcgcggtgtc ggaaccagtacgtagtacgttgc	S_1214-10-H11	At3g63410	MPBQ/MSBQ methyltransferase, chloroplast
M40	EU432390 EU432401	yes	117 bp	N/A	SSLP	583 500	N/A	M40for M40rev	accgtctcctccaagcactgc tcagccctttgtccgaagtcg	C_1231-11-B04	At3g55800	sedoheptulose-bisphosphatase, chloroplast
M41	EU447219 EU447220	no	no	1	CAPS (*Ear*I)	267 267	161, 106 267	M41for M41rev	acaccctcttatcaccaatggc tctccacgagagtgtccgtgg	C_1234-11-B07	At2g45290	transketolase, putative, chloroplast
M43	EU447221 EU447222	no	no	11	CAPS (*Bsu*RI)	275 275	206, 47, 22 118, 88, 47, 22	M43for M43rev	accacattcctcaaagctccg cggaagcaagaagctctttgg	S_1221-11-A06	At3g63140	mRNA binding protein precursor – like, chloroplast
M46	EU447225 EU447226	no	no	6	CAPS (*Xho*I)	194 194	108, 86 194	M46for M46rev	aaatggcgtccatggcgctta cttgggactcaagctcggcag	S_1491-13-D02	At5g12860	2-oxoglutarate/malate translocator-like protein, chloroplast
M47	EU447227 EU447228	no	no	1	CAPS (*Taq*I)	257 257	257 175, 82	M47for M47rev	tgggtgggattgccctacgtg gcgacaaccttaaccatgtcg	S_1494-13-D05	At1g42970	glyceraldehyde-3-phosphate dehydrogenase B, chloroplast
M50	EU447231 EU447232	no	no	3	CAPS (*Hha*I)	225 225	225 148, 77	M50for M50rev	ctgctccaccacaatggctgc accaacgaaccgtctagccag	C_1598-14-D12	At2g40100	photosystem II chlorophyll *a*/*b*-binding protein (Lhcb), chloroplast
M58	EU447237 EU447238	yes	11 bp	15	CAPS (*Bsr*I)	533 544	424, 109 280, 155, 109	M58for M58rev	gatccggaggatggaagtcct ctgaactgccacggctgttgg	S_2302-22-H10	At4g02510	protein import component Toc159-like, chloroplast

marker	accession number	intron	SSLP	no. SNPs	marker type (enzyme)	predicted PCR products in [bp][1)]	predicted restriction fragments [bp][1)]	primer	primer sequence (5'-to-3')	EST/cluster accession	closest *Arabidopsis* homologue (blastX)	protein function and localization in *Arabidopsis*
M59	EU447239 EU447240	yes	4 bp	51	CAPS (*Rsa*I)	703 699	322, 199, 178, 4 517, 178, 4	M59for M59rev	tgctctccgccacaatgtccg caaaccctctggtggccacac	C_2346-23-D11	At1g67090	ribulose bisphosphate carboxy-lase, small subunit (RuBisCO), chloroplast
M74	EU447243 EU447244	no	no	3	CAPS (*Hha*I)	250 250	208, 40, 2 104, 54, 50, 40, 2	M74for M74rev	aatggcggctctccagcagac tggtttcgagagtaccgttgg	C_3913-88-C06	At4g09650	ATP synthase delta subunit (AtpD), chloroplast
M75	EU447245 EU447246	no	no	1	CAPS (*Alu*I)	149 149	120, 29 64, 56, 29	M75for M75rev	gtctgttatatcgagtgctgggac cctgatcagccatgcatctgag	C_4066-89-H09	At4g14690	chlorophyll a-b binding family protein (Elip2), chloroplast
M86	EU447249 EU447250	no	no	4	CAPS (*Dde*I)	208 208	84, 66, 45, 13 150, 58	M86for M86rev	tccctcatttctctacctccagag accagccatagcaacgacgcc	C_4643-96-G12	At2g21170	triosephosphate isomerase, chloroplast
M88	EU447251 EU447252	no	no	1	CAPS (*Dde*I)	176 176	176 152, 24	M88for N88rev	accacagtctccgcagtaact tgttgagcccaatccgaggtc	C_4753-98-B06	At5g50250	RNA binding protein (rbp31), chloroplast
M95	EU447253 EU447254	yes	no	2	CAPS (*Hha*I)	315 315	299, 16 252, 47, 16	M95for M95rev	tcggactcagcaatggcgctc tggtggctgtctgtgctcgaa	C_5102-112-A10	At5g54190	NADPH:protochlorophyllide oxidoreductase A, chloroplast
M97[2)]	EU483132 EU483134	no	18 bp	23	CAPS (*Ape*KI)	357 375	246, 71, 29, 11 346, 29	M97for M97rev	atgaaagcacaaggagtcctc cgagaatgaagctgcctaaga	S_1348-12-C09	At5g47560	malate/fumarate transporter, tonoplast
M98[2)]	EU483136 EU483138	no	11 bp	24	CAPS (*Pfl*FI)	459 470	231, 184, 44 426, 44	M98for M98rev	aagccgagatcatcctgcaatgg aggcaaaataaaacggggatacagc	C_1202-10-G11	At2g06520	PsbX (photosystem II subunit X), chloroplast

[1)] Not each PCR-product was fully sequenced; length in bps was derived from the postion of the primer in the EST sequence used as template.

[2)] Polymorphisms first published in Mráček *et al.* (2006), confirmed, converted to CAPS, annotated and submitted to GenBank in this study.

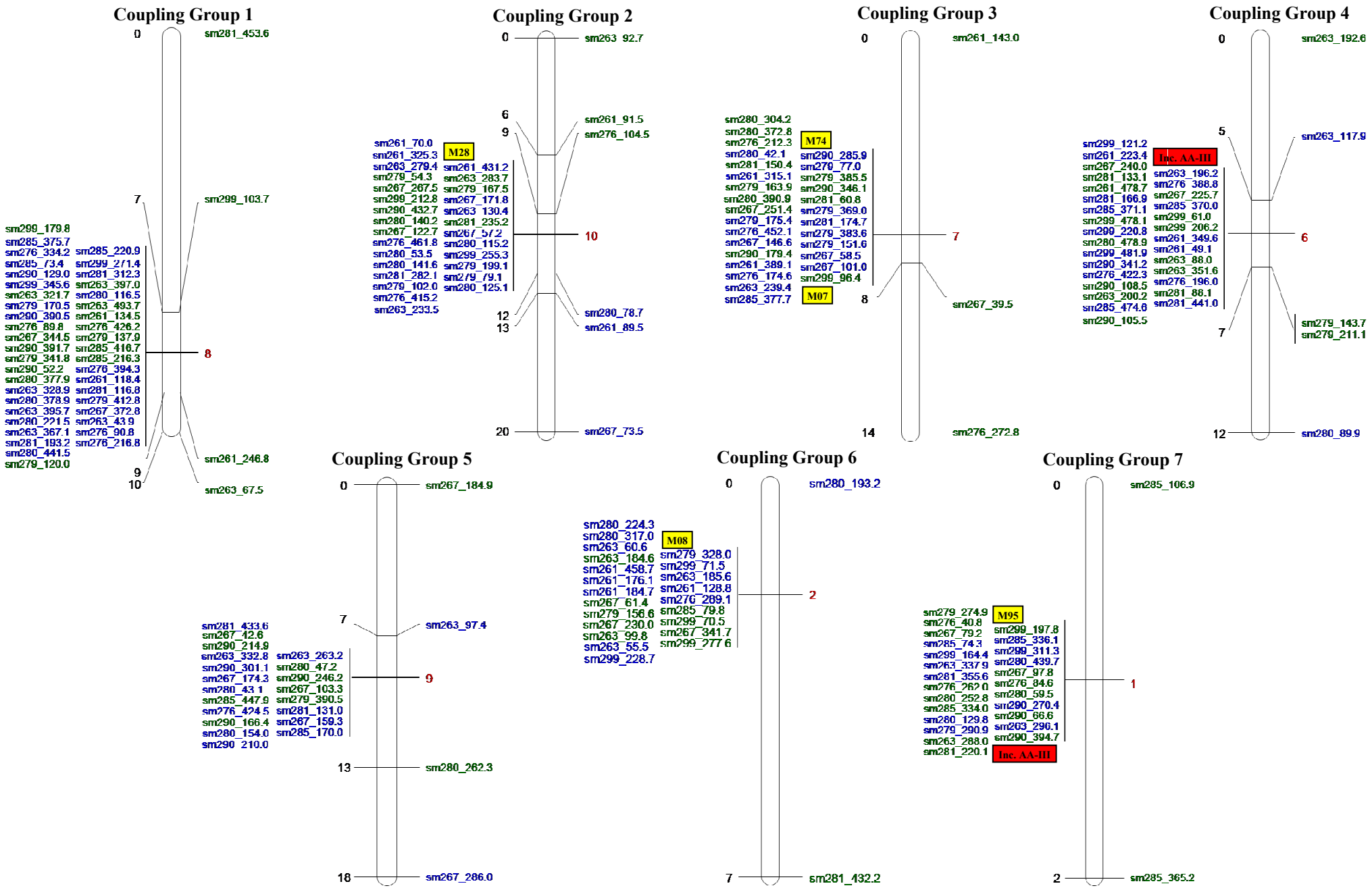


Fig. 3-6: Integrated genetic map of [h]johansen (A, markers in green marked) and [h]tuscaloosa (B, markers in blue marked). Co-dominant markers are marked in yellow. Loci mapped as potential factors for the incompatibility AA-III are marked in red.

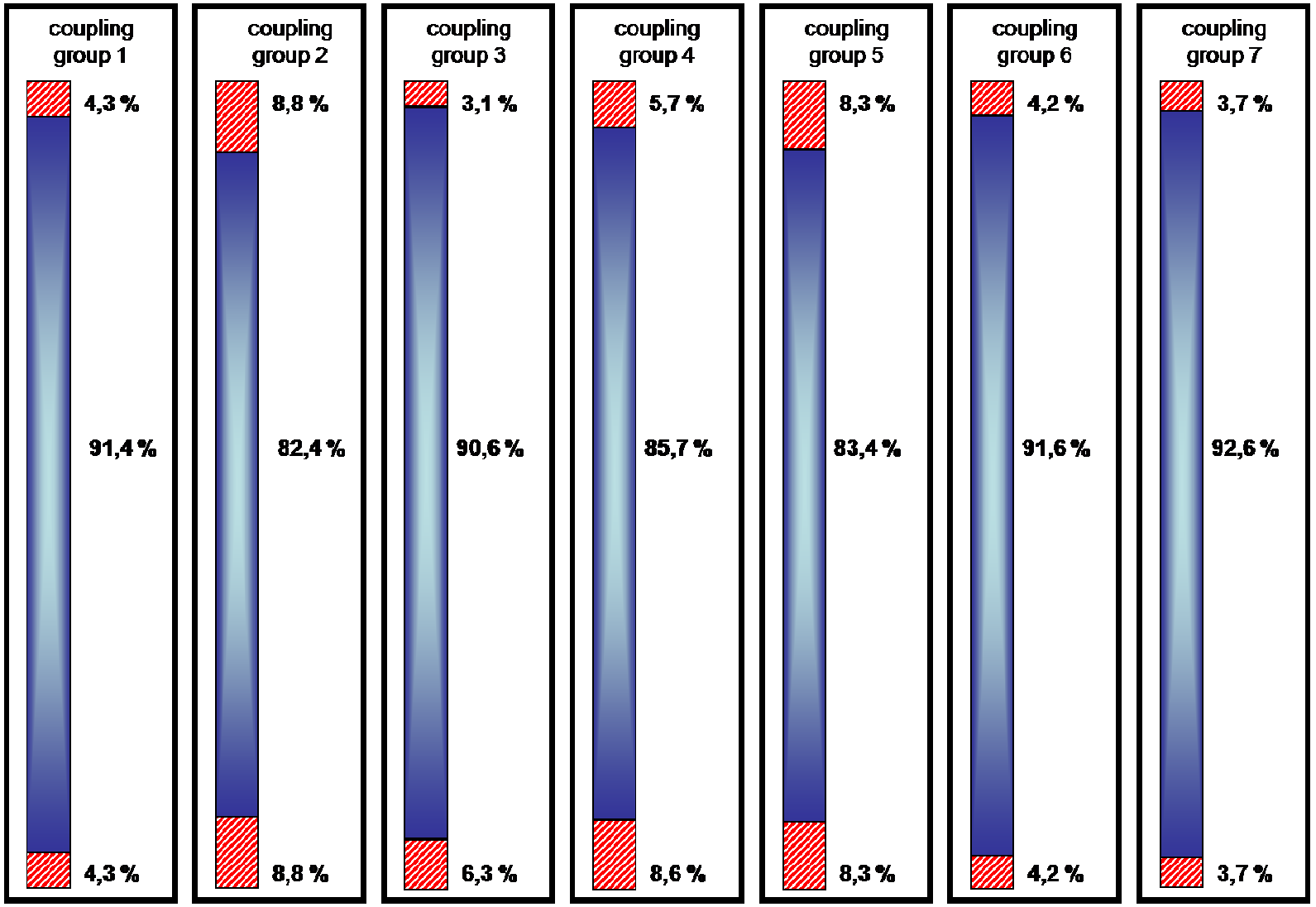


Fig. 3-7 Graphical image of the integrated map. symbolizes regions on chromosomes **with** detected recombination; symbolizes regions on chromosomes **without** detected recombination;

3.1.6 EXPANSION OF THE AFLP MAPS (EXPANSION MAPS)

Expansions of the basic maps were performed to increase the number of markers per chromosome substantially. For this, the number of F2 plants was reduced to 40, selected to be able to distinguish unequivocally between single coupling groups. A selection of F2 plants was possible because the genetic constitution of each individual chromosome was known from all F2 plants as a consequence of lack of homologous recombination. A further 35 primer combinations, implying 1,515 polymorphic markers, of which 1,384 could be mapped, were tested. So, a genetic map of the A genome, consisting of 800 markers, covering a total length of 135 cM and a genetic map of the B genome, consisting of 806 markers, covering a total length of 165 cM were calculated (Fig. 3-8). 121 (7.53%) out of 1,606 AFLP markers showed homologous recombination. These are exclusively located at telomeric regions. Even a single recombination event of one of the clustering markers would have lead to two bulks. Such splits of a cluster into two bulks could not be observed indicating complete suppression of homologous recombination along the major part of the chromosomes. 92.47% of all AFLP markers showed no homologous recombination. Thus, the largest part of the genetic information of chromosomes remains genetically stable over next generations.

3.1.7 GENERATION OF A SECOND LINKAGE MAP USING A CROSS OF SUBSPECIES

It is known that homologous recombination is dependent on the degree of polymorphism (Haber *et al.* 1991). In the interspecific cross ^{h}tuscaloosa x ^{h}johansen chosen, 39.78% polymorphism was detected between the two genomes, comparable to other species used for mapping approaches. For instance, the *Arabidopsis* lineages *Ler*/*Col* differed in 30.4%, *Cvi*/*Ler* in 34.4% and Cvi/*Col* in 33.7% of the bands (Alonso-Blanco *et al.* 1998). To exclude that the degree of divergence between ^{h}johansen (A genome) and ^{h}tuscaloosa (B genome) causes the lack of homologous recombination, a second linkage analysis was generated and studied using an *intraspecific cross* with a high genomic similarity of the crossing partners. The two lineages *Oenothera elata* subsp. *elata* strain cholula (AA-I^{cho}) and *Oenothera elata* subsp. *elata* strain puebla (AA-I^{pue}) were used, both originating from the same habitat in Mexico. To ensure again homozygous hybridization, both plants possess

Fig. 3-8a:

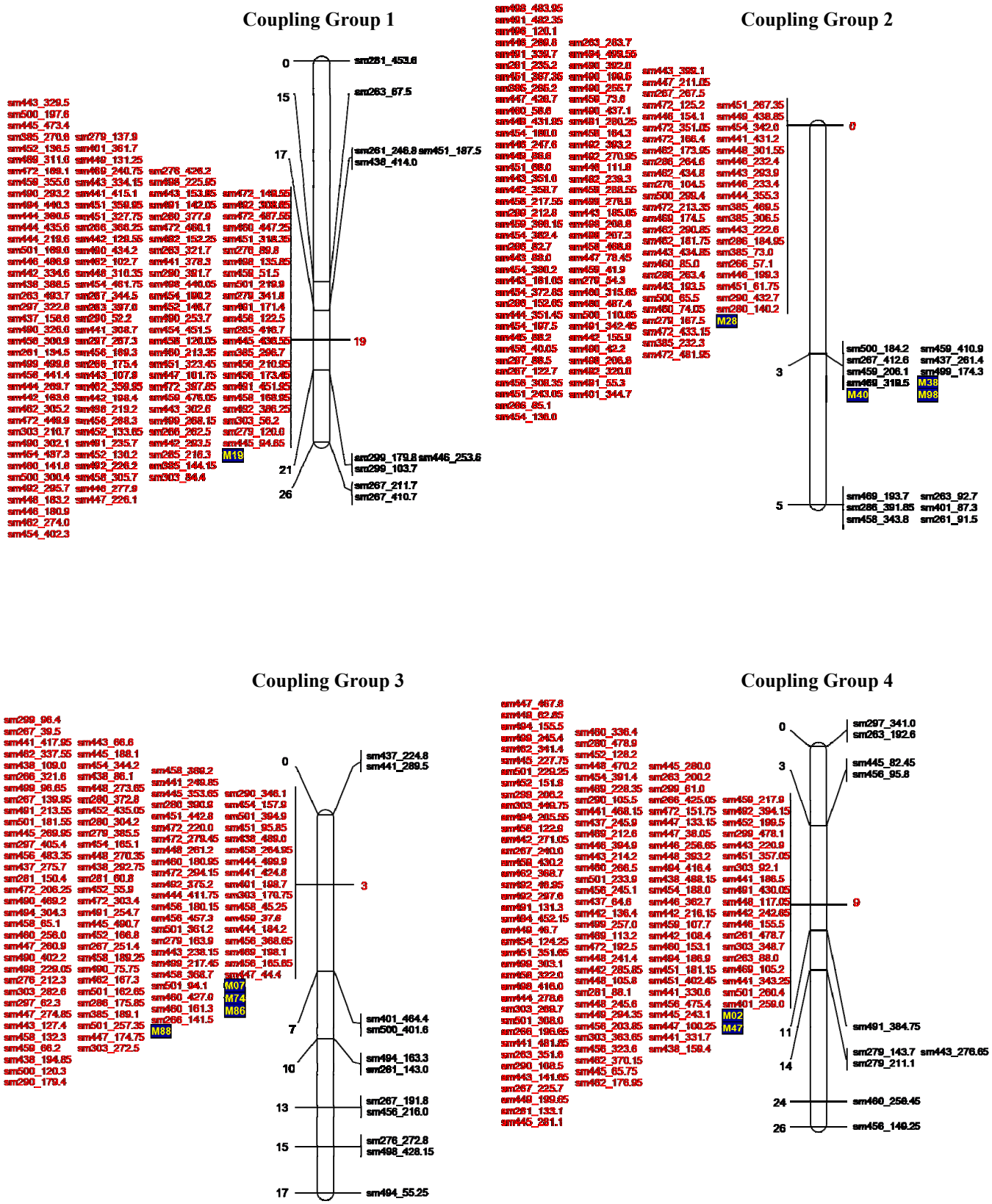

Coupling Group 1
0
15
17
19
21
26
sm281_453.6
sm263_67.5
sm261_248.8 sm451_187.5
sm438_414.0
sm299_179.8 sm446_253.6
sm299_103.7
sm267_211.7
sm267_410.7
Coupling Group 2
0
3
5
sm500_184.2 sm459_410.9
sm267_412.6 sm437_261.4
sm459_206.1 sm499_174.3
M40 M38 M98
sm469_193.7 sm263_92.7
sm286_391.85 sm401_87.3
sm458_343.8 sm261_91.5
Coupling Group 3
0
3
7
10
13
15
17
sm437_224.8
sm441_289.5
sm401_464.4
sm500_401.6
sm494_163.3
sm261_143.0
sm267_191.8
sm456_216.0
sm276_272.8
sm498_428.15
sm494_55.25
Coupling Group 4
0
3
9
11
14
24
26
sm297_341.0
sm263_192.6
sm445_82.45
sm456_95.8
sm491_384.75
sm279_143.7 sm443_276.65
sm279_211.1
sm460_258.45
sm456_149.25

Fig. 3-8 continued

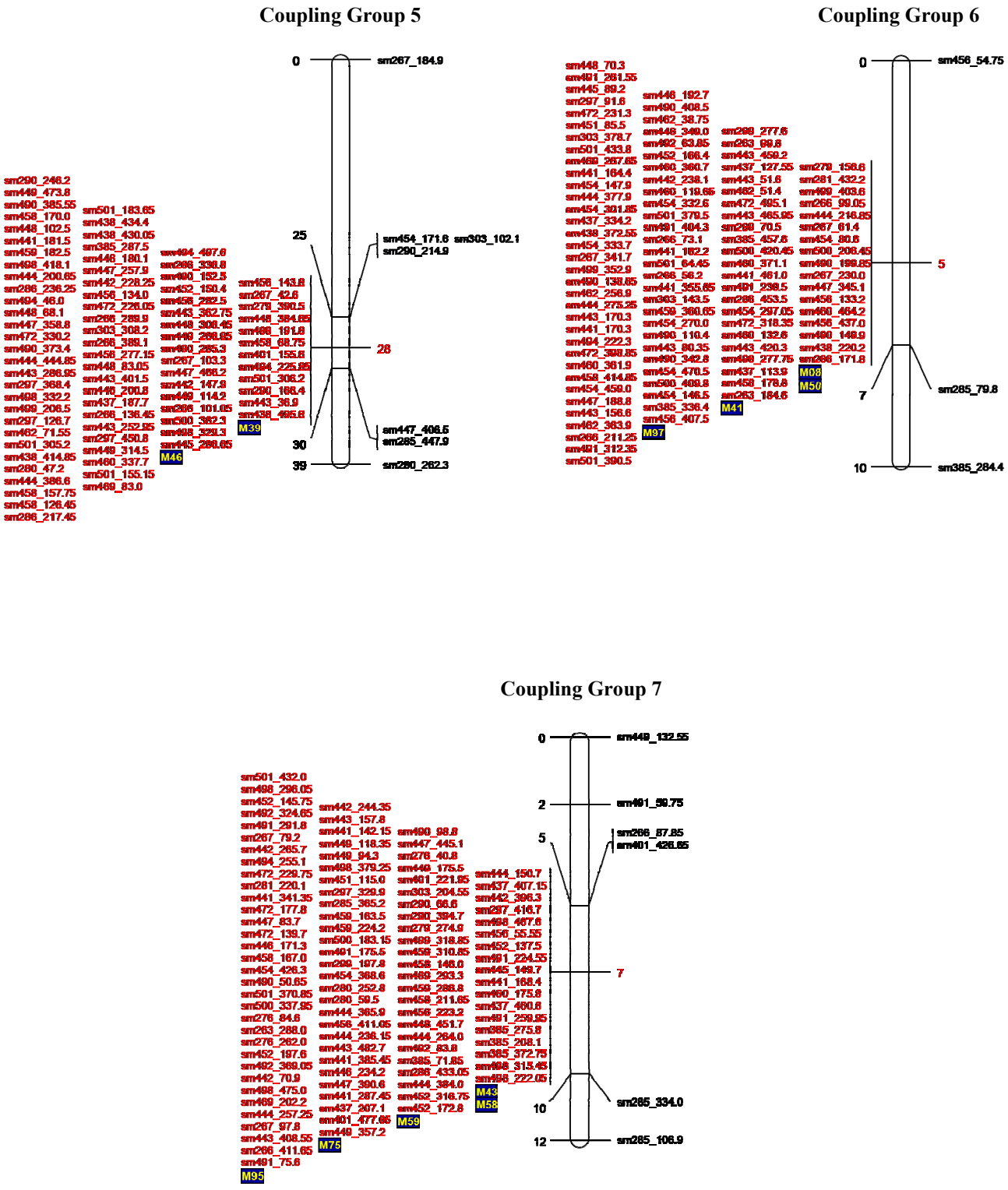


Fig. 3-8a: "Expanded" genetic map of *Oenothera elata* subsp. *hookeri* strain johansen with plastome III^{lam} (AA-III). Markers that clustered on respective chromosomes are marked in red. Co-dominant markers are marked in blue.

Fig. 3-8b:

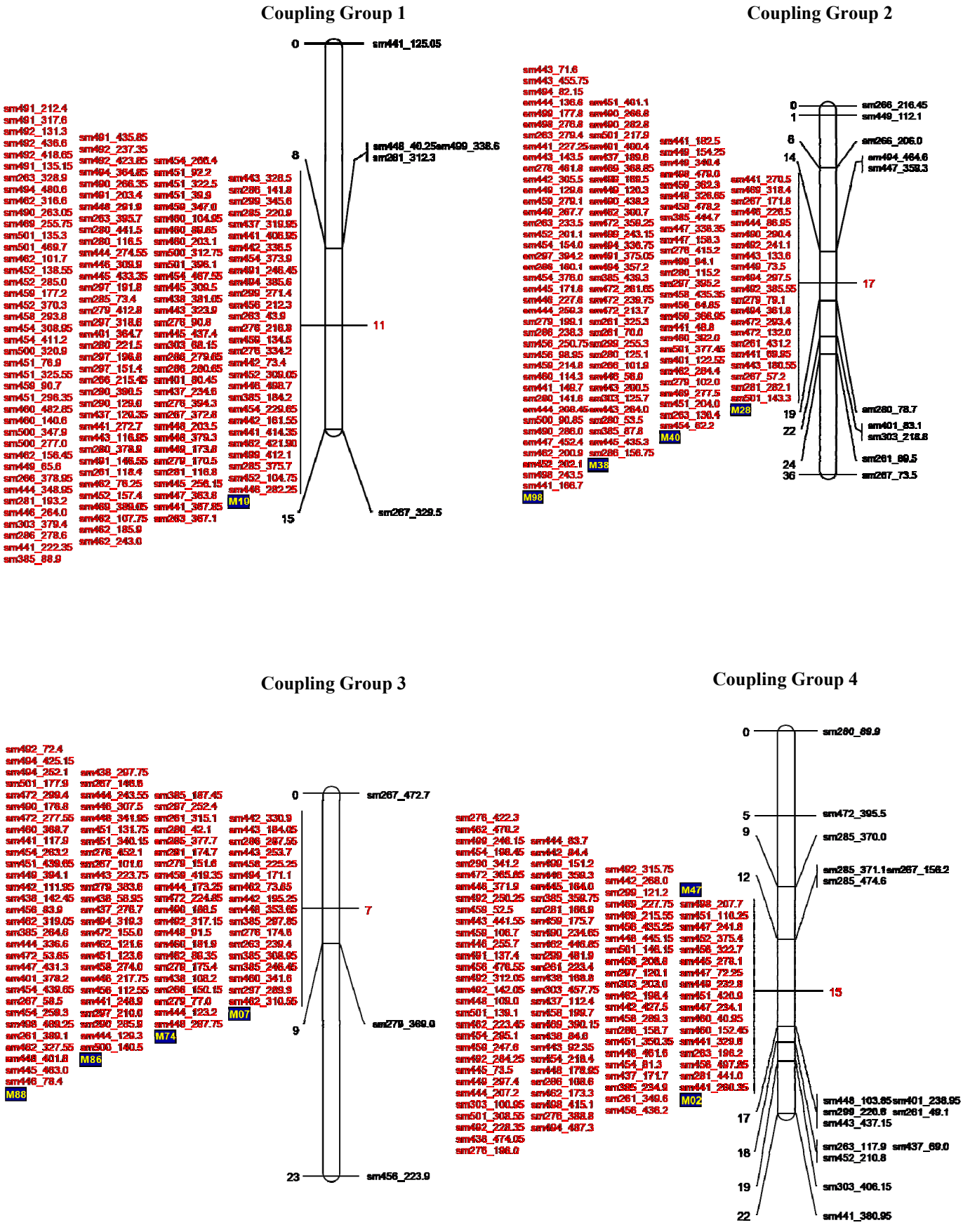
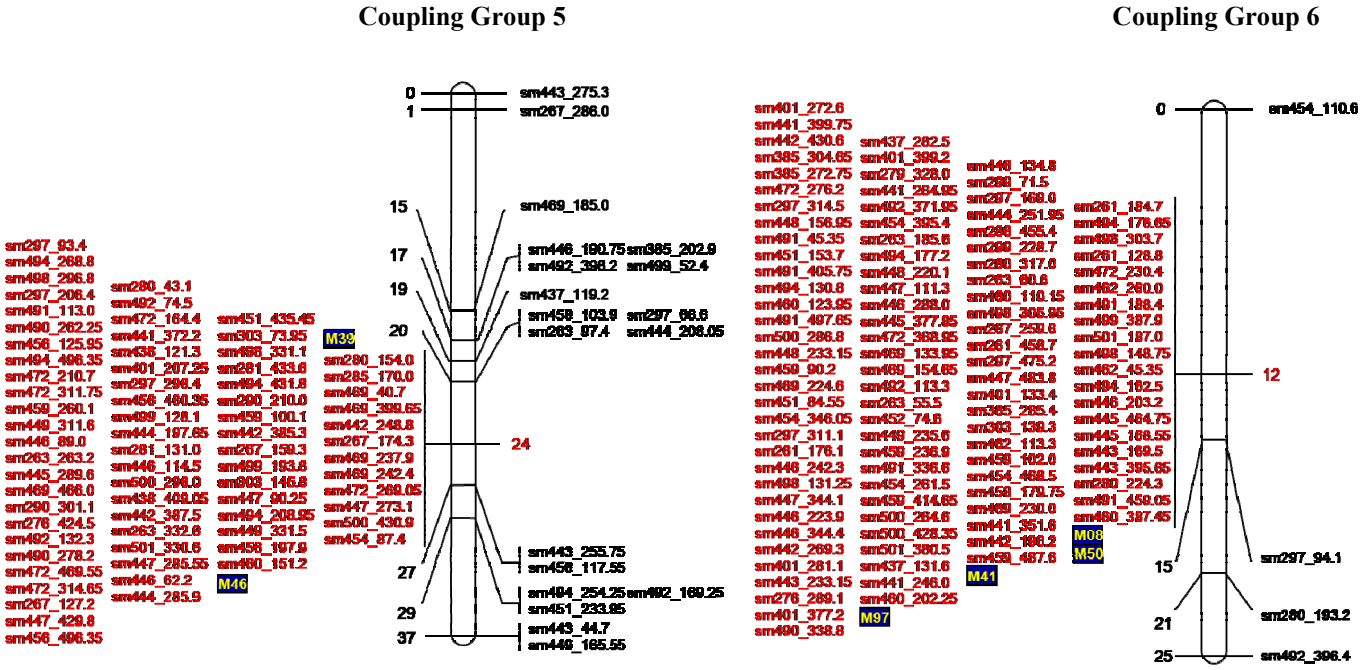

Coupling Group 1
Coupling Group 2
Coupling Group 3
Coupling Group 4
sm441_125.05
sm448_40.25sm499_338.6
sm281_312.3
sm267_329.5
sm266_216.45
sm449_112.1
sm266_206.0
sm404_464.6
sm447_359.3
sm280_78.7
sm401_83.1
sm303_218.8
sm281_89.5
sm267_73.5
sm267_472.7
sm279_369.0
sm456_223.9
sm280_89.9
sm472_395.5
sm285_370.0
sm285_371.1sm267_156.2
sm285_474.6
sm448_103.85sm401_238.95
sm299_220.8 sm261_49.1
sm443_437.15
sm263_117.9 sm437_89.0
sm452_210.8
sm303_406.15
sm441_380.95

Fig. 3-8b: continued



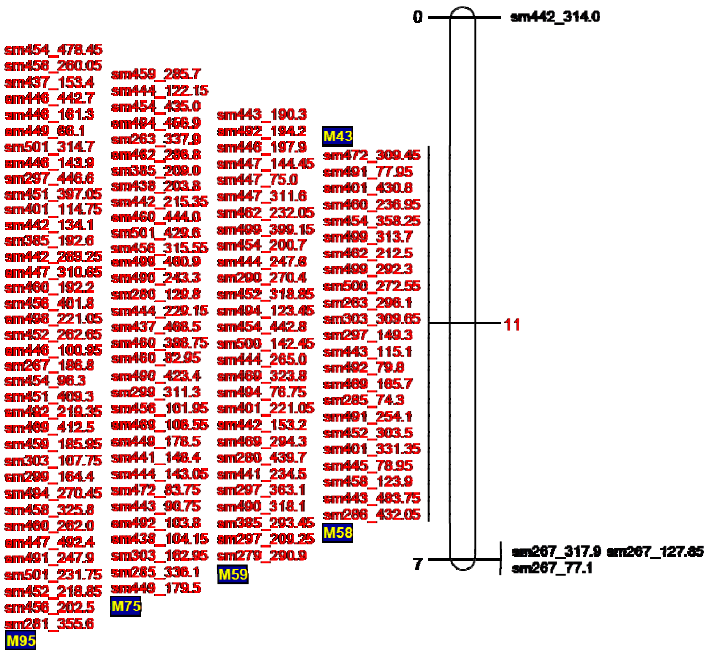


Fig. 3-8b: "Expanded" genetic map of *Oenothera grandiflora* strain tuscaloosa with plastome III^{lam} (BB-III). Markers that clustered on respective chromosomes are marked in red. Co-dominant markers are marked in blue.

the same chromosomal formula 1•4 3•2 5•9 7•10, 6•8 11•12 13•14. Fourteen primer combinations (Table 3-6) were tested at parental lines. A total band number of 1,100 was detected of which 120 (8.57 per primer combination) were polymorphic (10.91%) (Table 3-3), consistent with a close relationship. In contrast, the degree of polymorphism detected between the complexes ^{h}johansen and ^{h}tuscaloosa was 39.78%. Three primer combinations, designated as sm299, sm390 and sm406 were tested on 40 F2 plants. 31 markers were detected and coupling groups were calculated. Only two markers showed homologous recombination (6.45%), whereas the rest of the markers remained coupled (Fig. 3-9). This observation leaves no doubt that homologous recombination is almost completely repressed in all *Oenothera* species, regardless of whether they are translocation heterozygotes or homozygotes. *Thus, this work describes the first case of sexual reproduction with homologous recombination exclusively limited to telomeric regions in a natural occurring bivalent forming species.*

3.2 CYTOLOGICAL ANALYSIS OF THE MEIOTIC PROPHASE

The mapping approaches in bivalent forming *Oenothera* species revealed a lack of homologous recombination at centromeric and of large parts of the pericentromeric regions, which can extend close to telomeres. Up to now, recombination limited to telomeric parts of chromosomes were only known from translocation heterozygotic species as a result of a special ring order of the chromosomes during meiosis. Homologous recombination takes place during meiotic prophase I. Premeiotic and meiotic phases, particularly the different phases of prophase I (leptotene, zygotene, pachytene, diplotene and diakinesis), were checked cytologically with Dr. Hieronim Golczyk (Jagiellonian University, Krakow, Poland) and investigated for irregularities (Rauwolf *et al.* 2008a; Golczyk *et al.* 2008). A large fraction of chromatin showed an untypical, higher-order chromatin organization, notably condensation, before chromosomes enter the meiotic bouquet (Fig. 3-10). The overall degree of chromatin condensation at leptotene was high and the process seems to be fast, since much difference between the chromosome pairs could be observed.

Table 3-6a: Primer combinations tested at ^{h}puebla and ^{h}cholula genomes.

primer combinations	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	sm385	sm267	sm387	sm388	sm389	sm390	sm265	sm392	sm298	sm264	sm297	sm299	sm300	sm266	sm303	sm301
SacI GC - Joe	sm401	sm271	sm403	sm404	sm405	sm406	sm269	sm408	sm284	sm268	sm283	sm285	sm286	sm288	sm289	sm287
SacI GG - FAM	sm417	sm263	sm419	sm420	sm421	sm422	sm261	sm424	sm291	sm260	sm290	sm292	sm293	sm295	sm296	sm294
SacI GT - Joe	sm433	sn275	sm435	sm436	sm437	sm438	sm273	sm440	sm277	sm272	sm276	sm278	sm279	sm281	sm282	sm280

Primer combinations were designated with sm (restriction enzymes SacI and MseI used for AFLP analysis) and a number. Primers were designated by the name of restriction enzyme (in this case it stands for the sequence of the adaptor) and the added nucleotides. Primer combinations used for AFLP analysis of *Oe. elata* subsp. *elata* strain cholula with plastome I^{cho} (AA-I^{cho}) and *Oe. elata* subsp. *elata* strain puebla with plastome I^{pue} (AA-I^{pue}) are marked in orange.

Table 3-6b: Total bands detected in ^{h}cholula and ^{h}puebla.

total bands	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	148	125				117					50	68				
SacI GC - Joe	81					54					54	21	44			37
SacI GG - FAM		114									93		98			
SacI GT - Joe																

Total bands (AA-I^{cho} + polymorphic bands from AA-I^{pue} or AA-I^{pue} + polymorphic bands from AA-I^{cho}) were counted. The number of bands ranged from 21 (sm285) to 148 (sm385). An average of 78.57 total bands per primer combination was found.

Table 3-6c: Polymorphic bands detected between the complexes ^{h}cholula and ^{h}puebla.

polymorphic bands	MseI CAA	MseI CAC	MseI CAG	MseI CAT	MseI CCA	MseI CCC	MseI CCG	MseI CCT	MseI CGA	MseI CGC	MseI CGG	MseI CGT	MseI CTA	MseI CTC	MseI CTG	MseI CTT
SacI GA - FAM	13	9				18					3	10				
SacI GC - Joe	6					11					16	3	1			3
SacI GG - FAM		8									10		9			
SacI GT - Joe																

Polymorphic bands between AA-I^{cho} and AA-I^{pue} were counted. A range between one (sm286) and 16 (sm283) with an average of 8.57 polymorphic bands per primer combination was detected. Primer combinations marked in blue were used for linkage analysis of markers of AA-I^{cho} and AA-I^{pue}.

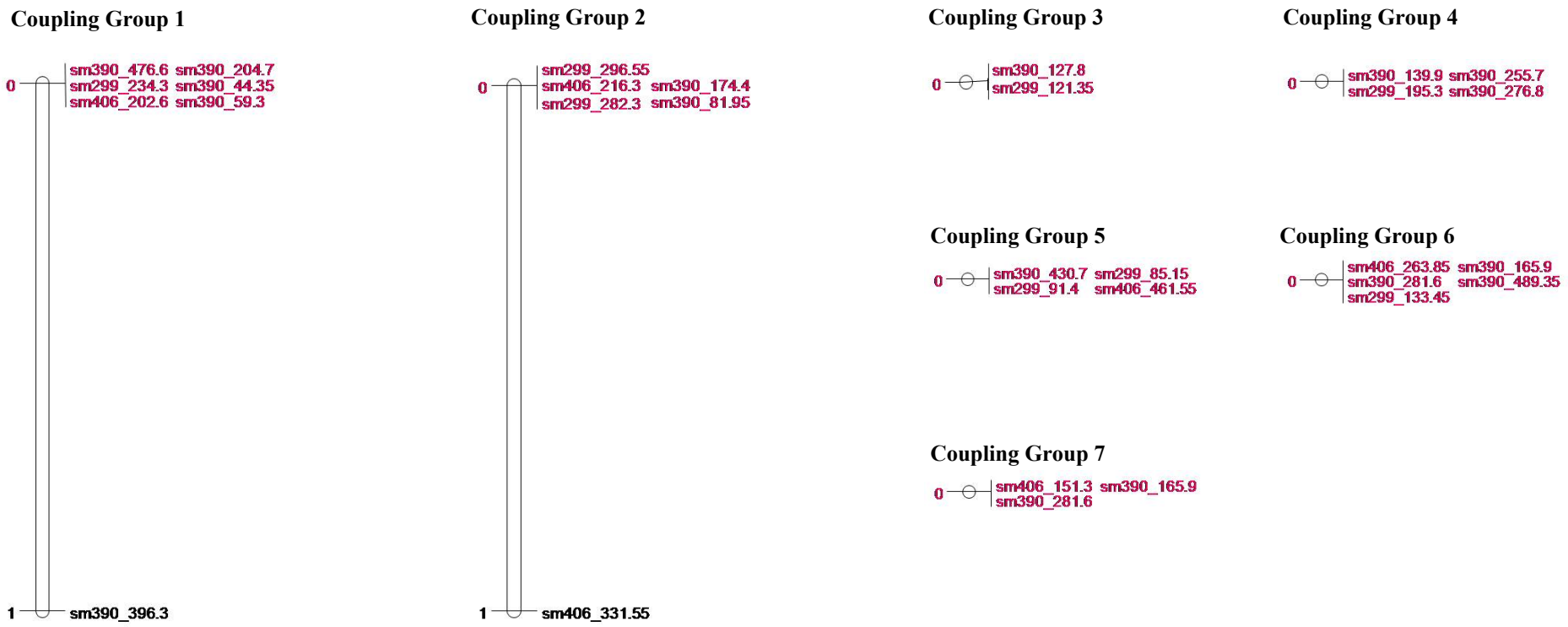


Fig. 3-9: Integrated map of *Oe. elata* subsp. *elata* strain cholula I^{cho} and *Oe. elata* subsp. *elata* strain puebla I^{pue}.

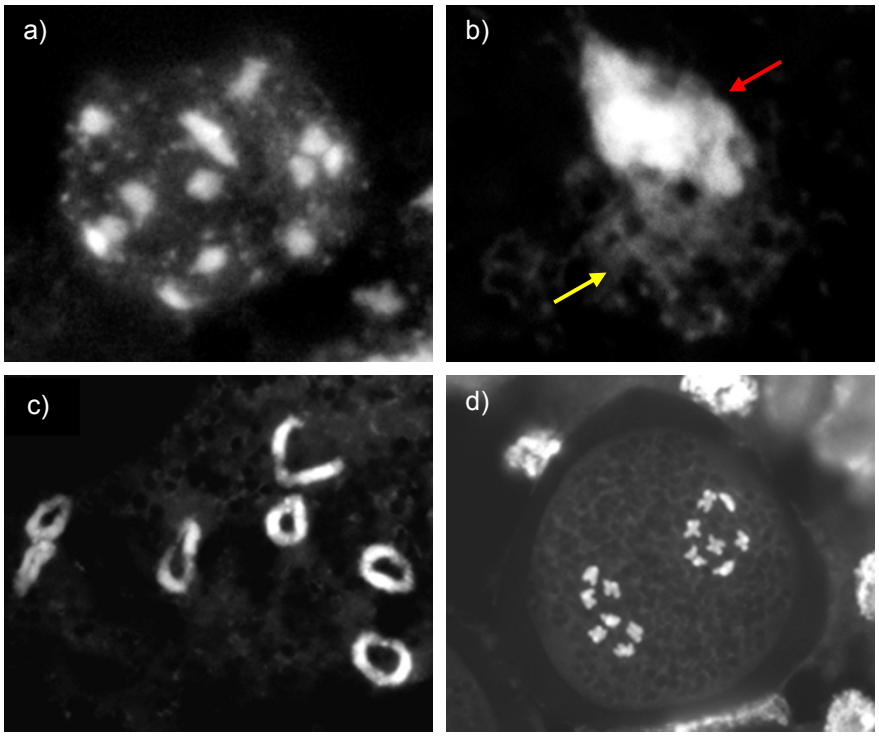


Fig. 3-10 Cytological analysis of premeiotic and meiotic phases. **(a)** Premeiotic nuclei with fourteen chromocenters (pericentromeric chromosome regions); **(b)** Rabl configuration at zygotene (Rabl 1885), arrow marked in yellow - relaxed distal chromosome segments, arrow marked in red - pericentromere pole. **(c)** Early diakinesis showed less condensed distal chromosome segments in the seven bivalents. **(d)** Chromosomes segregated regulary at late anaphase I.

3.3 A PCR-BASED MARKER SYSTEM TO DISTINGUISH BETWEEN RENNER COMPLEXES AND PLASTOME TYPES

30 different basic plastome-genome combinations are possible in the genus *Oenothera* (Fig. 1-4), not incuding the subgenomes and subplastomes, for which the numbers are unknown. So far, phenotypical markers were used to differentiate between basic nuclear and basic plastome types including plastome mutants (Cleland 1972; Stubbe and Herrmann 1982; Stubbe 1989; Dietrich *et al.* 1997). Some molecular markers were described for a limited number of strains (Mráček 2005; Larson *et al.*, 2008).

3.3.1 MARKER SYSTEM TO DISTINGUISH BETWEEN RENNER COMPLEXES

The SSLP M40 marker (Table 3-5), derived from the EST cluster C_1231-11-B04 of *Oe. elata* subsp. *hookeri* strain hookeri de Vries (Mráček *et al.* 2006), was considered

to genotype various Renner complexes. It encodes a chloroplast-located sedoheptulose-1,7-bisphosphatase that is involved in Calvin cycle and gluconeogenesis. Its gene, which possesses two introns, was highly polymorphic between the A genome of ^{h}johansen and the B genome of ^{h}tuscaloosa (Table 3-5). This promised to detect polymorphisms in further Renner complexes and gain a molecular marker for their distinction.

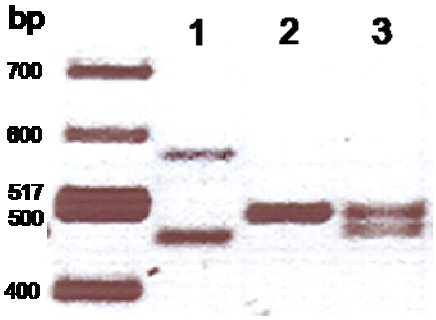


Fig. 3-11 Assignment of M40 alleles of r-^{S}velans • r-^{S}gaudens of the translocation heterozygote strain rr-lamarckiana Sweden. (1) In rr-lamarckiana Sweden two bands of 583 and 470 bp were detected; (2) in tuscaloosa a single band of 500 bp was amplified; (3) the F1 hybrid tuscaloosa • r-^{s}gaudens displays two bands of 500 and 470 bp, assigning the band of 500 bp to r-^{S}gaudens. A size standard (2-log ladder) was placed at the left.

Twenty homozygotic and five translocation heterozygotic strains were tested by amplification with M40 primers (Table 3-5). In homozygotic strains, consisting of two identical haploid chromosome sets, so-called haplo-complexes, a single band was amplified in all cases. In translocation heterozygotic strains, consisting of two different Renner complexes, two bands were detected in the presented lineages. The strain rr-lamarckiana Sweden, a partial translocation heterozygotic strain (only the chromosome 1•2 is free), consists of two different Renner complexes, designated r-^{S}velans (♀, egg cell complex) and r-^{S}gaudens (♂, pollen complex). In this case, M40 primers amplified two bands, one of 583 bp, the other one of 470 bp. Then, a F1 hybrid, consisting of the complexes ^{h}tuscaloosa • r-^{S}gaudens was used to assign the M40 allele of r-^{s}gaudens (Fig. 3-11). The hybrid was confirmed by phenotypic markers (Dietrich *et al.* 1997). “M40 analysis” of this hybrid displays two PCR products of 500 and 470 bp. Since the band of 500 bp was amplified from the haplo-

Table 3-7: Classification into groups of the different M40 alleles

M40 allele	Renner complex*	SSLP [bp]	CAPS [bp] (*Mbo*I)	CAPS [bp] (*Mps*I)	CAPS [bp] (*Spe*I)	accession number
A_1	^{G}albicans, Stalbicans, ^{h}hookeri de Vries, ^{h}franciscana de Vries, ^{h}purpurata, ^{h}blandina ^{am}rigens, ^{h}franciscana E. & S., laxans	474	210, 170, 67, 27	319, 155	249, 126, 99	EU432376, EU432377, EU432382, EU432379, EU432383, EU432378, EU432384, EU432380, EU432382
A_2	^{h}chapultepec, ^{h}cholula, ^{h}puebla, ^{h}toluca, undans	579	285, 200, 67, 27	431, 148	352, 135, 92	EU432385, EU432386, EU432387, EU432388, EU432389
A_3	^{h}johansen, r-^{s}velans	583	349, 207, 27	428, 155	352, 132, 99	EU432390, EU432391
B_1	^{h}decipiens, ^{h}deserens, r-^{S}gaudens	470	207, 169, 67, 27	315, 155	371, 99	EU432392, EU432393, EU432394
B_2	^{h}bellamy A, $^{h}B^{A}$ castleberry A-4, $^{h}B^{A}$ chastang 7, ^{G}flavens, ^{h} stockton 1	499	226, 179, 67, 27	325, 174	364, 135	EU432395, EU432396, EU432397, EU432398, EU432399
B_3	Stflavens	500	227, 179, 67, 27	325, 175	365, 135	EU432400
B_4	^{h}tuscaloosa	500	227, 179, 67, 27	325, 175	365, 135	EU432401
C_1	^{h}douthat 1, ^{h}williamsville, ^{h}wilson creek 1	496	243, 159, 67, 27	496	364, 132	EU432402, EU432403, EU432404

SSLP and restriction endonuclease patterns are shown to distinguish between the different Renner complexes.

*corresponding *Oenothera* strains and species are listed in Chapter 2.1.7

complex tuscaloosa, the band of 470 bp must have been amplified from the r-^{S}gaudens complex. This way, the bands of the translocation heterozygotic strains ammophila (^{am}rigens • percurvans), bauri (laxans • undans), suaveolens Grado (^{G}albicans • ^{G}flavens) and suaveolens Standard (Stalbicans • Stflavens) were assigned as well.

The sequences of the different marker regions are deposited in GenBank (Table 3-7). In all investigated Renner complexes two introns were found with the marker allele M40, containing microsatellites of different lengths and thus resulting in eight different SSLP in the 29 investigated Renner complexes. Furthermore, for all three basic nuclear genome types (A, B and C) at least one specific SSLP was detected that was converted into a CAPS marker (Table 3-7).

3.3.2 A MARKER TO DISTINGUISH BETWEEN PLASTOMES AND SUBPLASTOMES

In addition to the marker system, with which distinction between Renner complexes is possible, it was desirable to establish a system to differentiate also between different plastomes and even subplastomes. Therefore, the plastidic *rrn*16-*trn*I$_{GAU}$ spacer region was investigated in 41 *Oenothera* strains (Rauwolf *et al.* 2008b; Greiner 2008), using the primers 16S SEQ (+) and trnI PCR (+). Sequences of the PCR products uncovered a *Bam*HI restriction polymorphism (Table 3-8). A high degree of polymorphism in this region was already described in Hornung *et al.* (1996) and Sears *et al.* (1996). The marker alleles *rrn*16-*trn*I$_{GAU}$ I$_1$ and *rrn*16-*trn*I$_{GAU}$ I$_2$, detected in the strains chapultepec, cholula, puebla or toluca, can not be discerned *via Bam*HI, but *via* a *Bsm*BI digest (I$_1$: 619 bp, 261 bp and I$_2$: 870 bp). This work was performed in collaboration with Stephan Greiner (Greiner 2008).

Naturally occurring species were investigated to reflect both, the phylogenetic relationship and the basic plastomes. Different alleles of the investigated spacer region were detected in different strains, carrying the basic plastome type I (I$_1$ – I$_5$) (Table 3-8). In contrast to results obtained from different subplastomes of plastome I, in different species carrying the basic plastome type IV no variation among subplastomes was detected in the *rrn*16-*trn*I$_{GAU}$ spacer region. This was more or less

Table 3-8: *Bam*HI restriction and SSLP pattern of the *rrn*16-*trn*I$_{GAU}$ spacer region in *Oenothera* plastomes and subplastomes used in this study* (see also Greiner 2008).

*rrn*16-*trn*I$_{GAU}$ allel	strain	species	plastome type	SSLP [bp]	CAPS [bp] (*Bam*HI)	accession number
I$_1$	chapultepec	*Oe. elata* subsp. *elata*	I	880	322, 229, 220, 109	EU262892
I$_2$	cholula, puebla, toluca	*Oe. elata* subsp. *elata*	I	870	322, 220, 218, 110	EU262893, EU282392, EU282393
I$_3$	franciscana de Vries, franciscana E.& S., johansen	*Oe. elata* subsp. *hookeri*	I	1058	322, 224, 220, 182, 110	EU282394, EU282395, EU262894,
I$_4$	hookeri de Vries	*Oe. elata* subsp. *hookeri*	I	876	322, 247, 197, 110	EU262895
I$_5$	bauri	*Oe. villosa* subsp. *villosa*	I	891	322, 262, 197, 110	EU262896
II/III$_1$	biennis München, castleberry B-8, conferta, purpurata, suaveolens Fünfkirchen, suaveolens Grado, suaveolens Standard	*Oe. biennis*, *Oe. grandiflora*, *Oe. biennis* x *Oe. glazioviana*	II or III	977	322, 257, 216, 182	EU282396, EU282397, EU282398, EU282399, EU282400, EU262897, EU282401
II/III$_2$	coronifera, nuda	*Oe. biennis*, *Oe. glazioviana*	II	981	322, 257, 220, 182	EU282402, EU262898
II/III$_3$	bellamy A, biennis de Vries, chastang 7, chicaginensis Colmar, horsesheads 2, marienville 3, stockton 1	*Oe. biennis*, *Oe. grandiflora*, *Oe. nutans*	II or III	963	322, 318, 182, 141	EU282404, EU262899, EU282405, EU282403, EU282406, EU282407, EU282408
II/III$_4$	mitchell	*Oe. nutans*	III	980	335, 322, 182, 141	EU262900
II/III$_5$	castleberry A-4	*Oe. grandiflora*	III	845	341, 322, 182	EU262901
II/III$_6$	elkins 2	*Oe. nutans*	III	940	322, 295, 182, 141	EU262902
II/III$_7$	lawrenceville 3	*Oe. biennis*	II	781	322, 318, 141	EU262903
II/III$_8$	tuscaloosa	*Oe. grandiflora*	III	1009	364, 322, 182, 141	EU262904
II/III$_9$	blandina, decipiens, deserens, rr-lamarckiana Sweden	*Oe. glazioviana*	III	617	322, 295	EU282409, EU282410, EU282411, EU262905
IV$_1$	ammophila, atrovirens, silesiaca, st. stephen	*Oe. oakesiana*, *Oe. parviflora*	IV	961	322, 236, 221, 182	EU282415, EU262906, EU282412, EU282413
V$_1$	douthat 1	*Oe. argillicola*	V	1101	322, 236, 221, 182, 140	EU262907
V$_2$	williamsville, wilson creek 1	*Oe. argillicola*	V	1102	322, 236, 221, 182, 141	EU282414, EU262908

*corresponding *Oenothera* strains and species are listed in Chapter 2.1.7

also the case in plants carrying subplastomes of basic plastome type V (V_1 and V_2), in which just one single base pair was polymorphic (Table 3-8). The situation is different for alleles originating from subplastomes of type II or type III, naturally occurring in *Oe. biennis*, *Oe. glazioviana*, *Oe. grandiflora* and *Oe. nutans*. These two plastome types were the only ones, which did not allow distinction with *rrn*16-*trn*I_{GAU} spacer region (Table 3-8). All alleles detected in plastome II were also found in some species carrying genetically plastome III and vice versa, indicating gene flow between the four species (discussed in Greiner 2008).

3.4 CHARACTERISATION OF THE INCOMPATIBILITY IN AA-III

Plants of the genus *Oenothera* possessing the plastome-genome combination AA-III show a *virescent* phenotype (Fig. 3-2a in Chapter 3.1.3). The leaves become bleached in the center but remain green at the tips, edges, and veins (Schötz 1958; Glick and Sears 1994). The green apical half of the cotyledons as well as the partially green areas at the leaves are however sufficient, to reduce plant growth. This artificial plastome-genome combination is periodically incompatible. Developmental disturbances are only visible 8 – 10 days after germination and end dependent on the outside conditions, on average if the plants reach the “11 or 12 leaf state”, after 10 – 12 weeks (Schötz 1958). Afterwards, AA-III plants become normal green. In the chosen experiment, the plants used possessed the nuclear background ^{h}johansen • ^{h}johansen (AA) combined with the plastome III from the strain lamarckiana Sweden (III^{lam}). The establishment of the hybrid can be followed in Fig. 3-1 in Chapter 3.1.1. In the F2 leaf material used for mapping approaches of this incompatibility (see Chapter 3.1.3), a single F2 plant showing a strong incompatible phenotype looked like a revertant, since it became greenish around the rib (Fig. 3-12). Regeneration of the green tissue by tissue culture failed. Thus, further investigations with this leaf material was not possible.

Fig. 3-12 Picture of the revertant that occurred in the F2 generation of the cross ^{h}tuscaloosa x ^{h}johansen (see Chapter 3.1.3). The plant, possessed a strong incompatible phenotype, but became greenish around the rib in one leaf (red arrow).

3.4.1 COMPARISON OF PLASTOME SEQUENCES

The nuclear background AA is compatible in combination with basic plastomes I and II but incompatible with plastomes III and IV (see Fig. 1-4). The incompatible phenotype AA-IV (*chlorina*) differs from the phenotype of AA-III (*virescent*) (Stubbe 1959, 1989). Thus, plastome III must differ significantly in at least one region of the sequence from plastome I, II and IV. For this reason, the plastome sequences were searched for most significant differences between plastome III and the others. All differences in coding sequences, causing an exchange in protein sequence, as well as large deletions or insertions in non-coding sequences (repetitions of repetitive sequences were excluded in this analysis) were compared (listed in Table 3-9; Fig. 3-13). Differences in protein sequences were aligned and compared with known sequences from other plant species (Fig. 3-13), using the online program Clustal W (http://www.ebi.ac.uk/Tools/clustalw/) for multiple protein alignments (Thompson *et al.* 1994) and the program GeneDoc (Nicholas *et al.* 1997) for manual editing of alignments. The basic information multiple protein alignments provide is the identification of conserved sequence regions and, in the case of plastome III, to find out whether amino acid changes are positioned in such a potentially important region. *Clp*P1 genes were not compared with sequences from other plants, because

Table 3-9a: Intergenic differences detected in plastome III compared to plastomes I, II and IV

intergenic region	insertion [number of bps]	deletions [number of bps]
directly upstream (5´ end) of *atp*B/E operon		13
trnG_{UCC} / *trn*S_{GCU}		66
trnQ_{UUG} / *accD*	>100	
*psa*J / *rpl*33	51	
*rrn*16 / *trn*I_{GAU}	>150	

Table 3-9b: Genic differences detected in plastome III compared to the plastomes I, II and IV

Gene	exchange of amino acids	position [AS]	insertion(s) [amino acids or bps]
*atp*A		506. and 507.	KV
*clp*P1 c*lp*P1 *clp*P1	 Q (R) → K A (-) → E	24. 144. 212.	D
*ndh*A	R → G	16.	
*ndh*E	A → T	75.	
*rps*3	R → T	70.	
*rps*8 *rps*8	H → P N → S	65. 68.	
*rps*18		starting from 89.	RFKRSQSTV
*ycf*4 (photosystem I assembly protein)	N → S	47.	
*ycf*5 (cytochrome c biogenesis protein)	K → N	95.	
*rpo*C2	T → S	275.	
*pet*D Intron			12 bps
*acc*D	many differences		
*ycf*1	many differences		
*ycf*2	many differences		

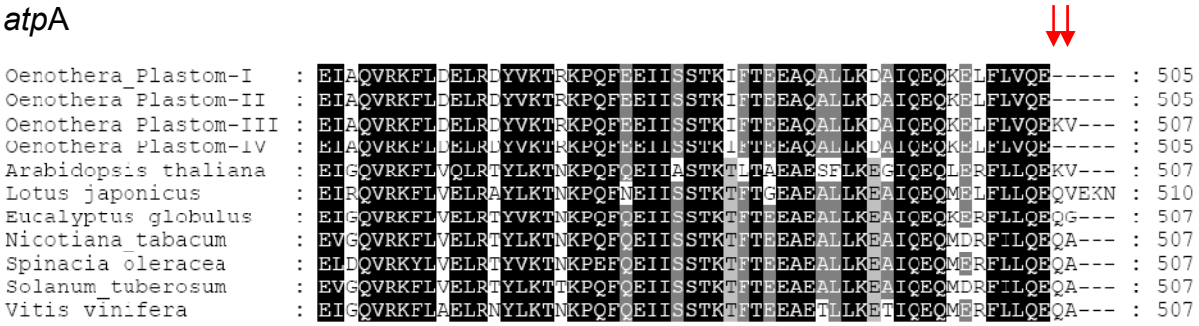
Abbreviations (symbols) used for amino acids in Table 3-9:

A = alanine (nonpolar, neutral amino acid)
E = glutamic acid (polar, acidic amino acid)
D = aspartic acid (polar, acid amino acid)
F = phenylalanine (nonpolar, neutral amino acid)
G = glycine (nonpolar, neutral amino acid)
H = histidine (polar, weakly basic amino acid)
K = lysine (polar, basic amino acid)
N = asparagine (polar, neutral amino acid)
P = proline (nonpolar, neutral amino acid)
Q = glutamine (polar, neutral amino acid)
R = arginine (polar, strongly basic amino acid)
S = serine (polar, neutral amino acid)
T = threonine (polar, neutral amino acid)
V = valine (nonpolar, neutral amino acid)

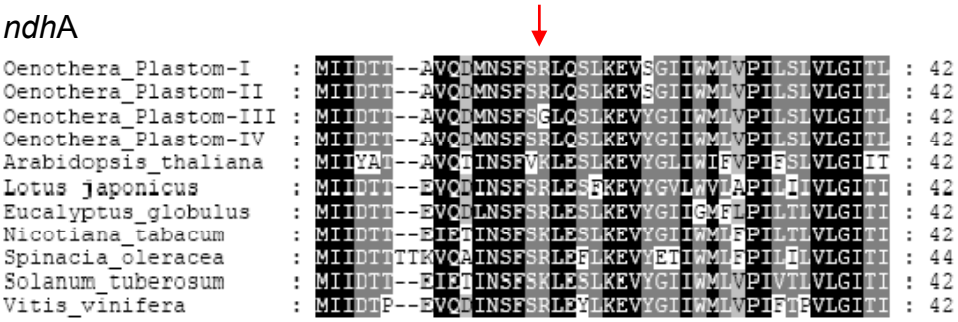
of their high degree of divergence in different plant taxa. *Ycf*1, *ycf*2 and the highly variable *acc*D N-terminus were also excluded from the analysis. The *ycf*1 and *ycf*2 genes are only moderately conserved in plastid genomes in general, and *acc*D is highly polymorphic in its N-terminal region as in the reference plastomes (Greiner *et al.* 2008b, c).

Fig. 3-13

*atp*A



*ndh*A



*ndh*E

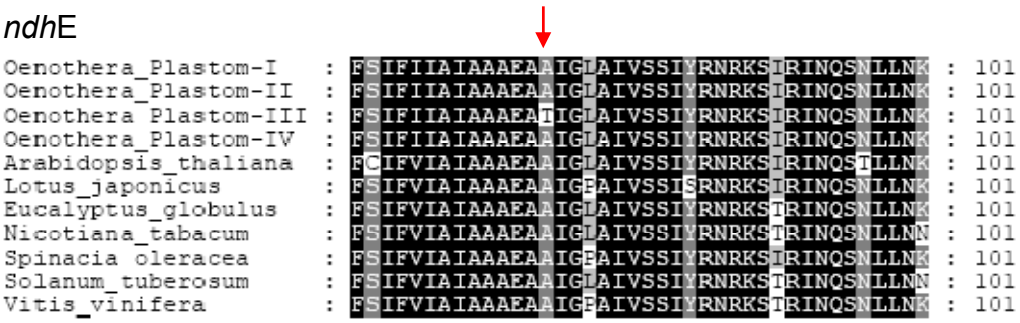
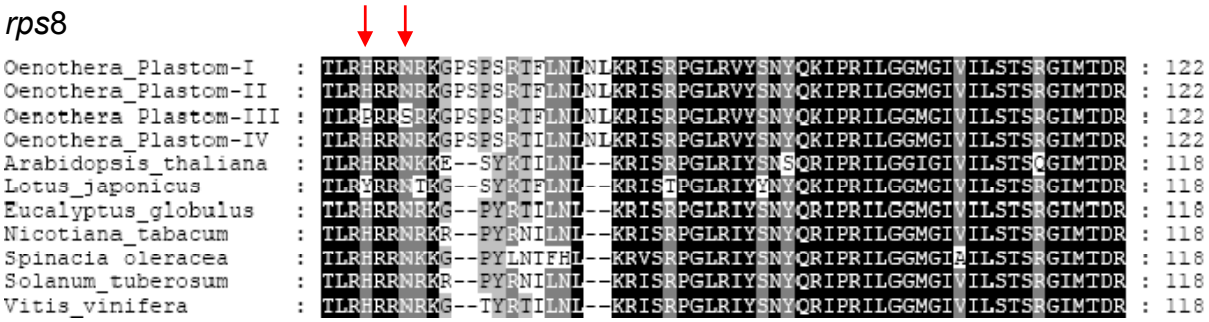


Fig. 3-13 continued

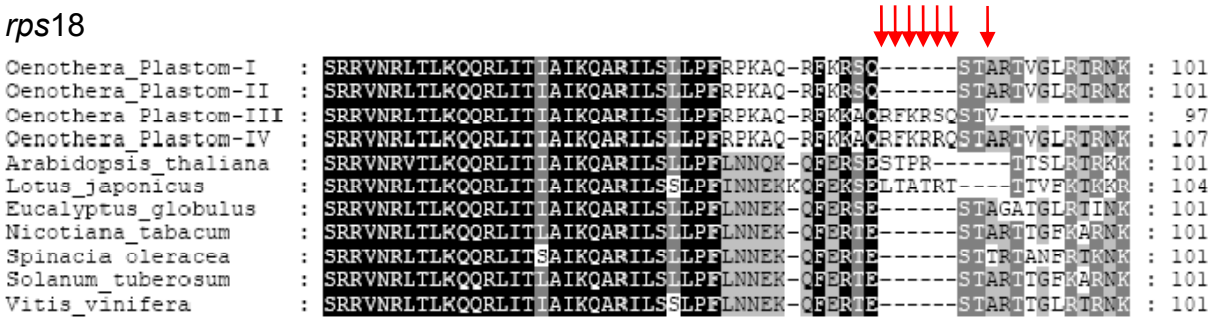
rpoC2

rps3

rps8



rps18



ycf4

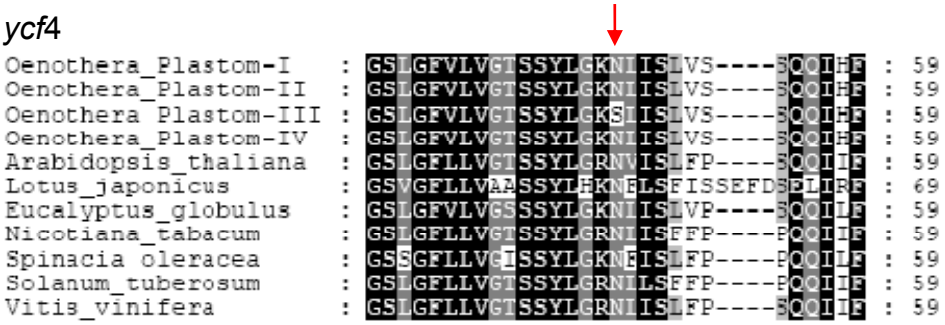


Fig. 3-13 continued

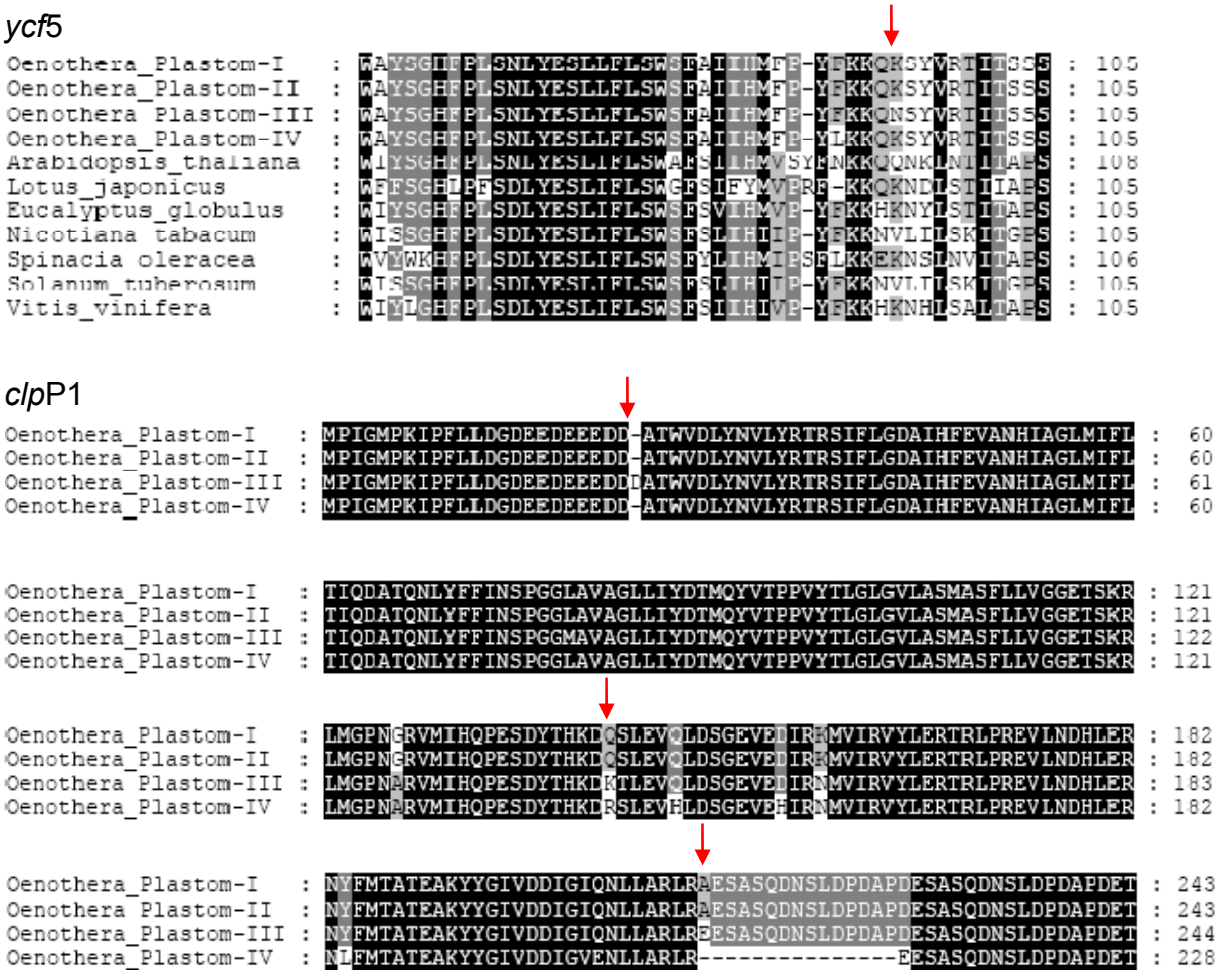


Fig. 3-13 Alignments of protein sequences, in which plastome III differs from plastomes I, II and IV in *Oenothera* (marked with a red arrow). Genes were compared with known amino acid sequences from other plant taxa to detect conserved protein sequence regions (except *clp*P1, see Text).

3.4.2 SPECTROSCOPIC ANALYSES OF INCOMPATIBLE AA-III^{LAM} PLANTS

It has been reported that thylakoids from plastome types I, II, III and IV were functionally competent in performing electron transport reactions in a AA nuclear background. In a former analysis of the incompatible AA-III combination a lower PSII activity was observed in contrast to an unchanged PSI activity (Glick and Sears 1994). To verify this observation PAM measurements of PSI and PSII were performed.

In order to measure PSI activity, light-induced changes of the P_{700} redox state were recorded by absorbance changes at 830 nm (Fig. 3-14). Furthermore, as an indication for the potential capacity of PSII, the ratio of variable fluorescence to

maximum fluorescence (F_V/F_M) was measured by the application of saturating light pulses (Fig. 3-15). F_V/F_M was found to be moderately but significantly reduced in incompatible AA-III material compared to compatible AA-I plants. Further parameters [non-photochemical (NPQ) and photochemical quenching (qP)] were measured from different stages of plant development (Table 3-10). These parameters indicate a higher NPQ and a slower transportation of the electrons in AA-III compared to AA-I. High NPQ can be due to a high proton gradient across the thylakoid membrane, as a result of malfunction of the ATP synthase or due to a metabolic effect involving ATP synthase activity. The slow transport of the electrons could be caused by deficiencies in the "downstream cascade" of the electron transport chain, *e.g.* in the cytochrome complex or in photosystem I. PAM measurements revealed pleiotropic effects and did not show distinct defects in one of the complexes of the thylakoid membrane during the first ~12 weeks of development in AA-III plants. PSI measurements did not show significant differences.

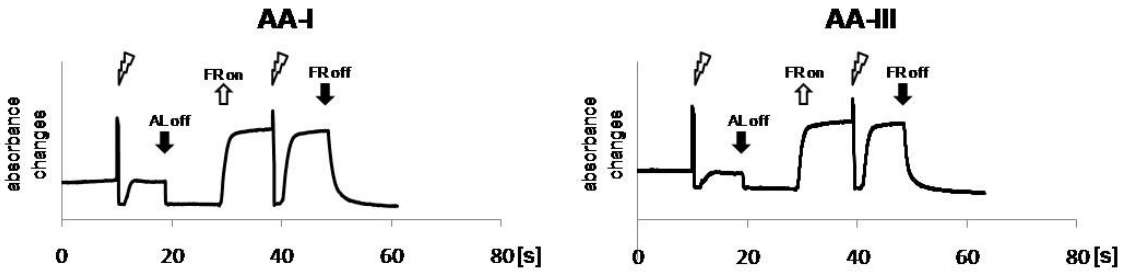


Fig. 3-14 The P_{700} oxidized state of compatible AA-I and incompatible AA-III leaves exposed to 76 µE actinic red light was recorded. Significant absorbance changes were recorded after switching off actinic light in both, indicating that PSI was oxidized. Application of FR light oxidized PSI in both compatible and incompatible leaves. Subsequent saturating light pulses on the FR background light transiently reduced PSI completely in both, compatible and incompatible leaves again (see Chapter 2.2.6.2). No significant differences were observed in P_{700} measurements. Abbreviations: AL = actininic light; FR = far red light; squiggled arrows symbolize saturating light pulses.

Table 3-10: Chlorophyll *a* fluorescence parameters for different developmental stages of compatible AA-I and incompatible AA-III plastome-genome combinations			
	F_V/F_M*	qP**	NPQ***
AA-I	0,823 ± 0,009	0,925 ± 0,094	0,306 ± 0,048
AA-III (4 weeks old leaf)	0,787 ± 0,010	0,800 ± 0,078	1,000 ± 0,101
AA-III (8 weeks old leaf)	0,791 ± 0,008	0,827 ± 0,083	0,826 ± 0,089
AA-III (12 weeks old leaf	0,803 ± 0,004	0,894 ± 0,091	0,373 ± 0,081
AA-III (16 weeks old leaf)	0,826 ± 0,010	0,946 ± 0,101	0,250 ± 0,051

*Fv / FM provides an estimate of PSII maximum efficiency within dark-adapted material.

**qP indicates quenching according to photochemistry.

***NPQ indicates quenching according to heat dissipation (ΔpH), state transition, and/or photoinhibition.

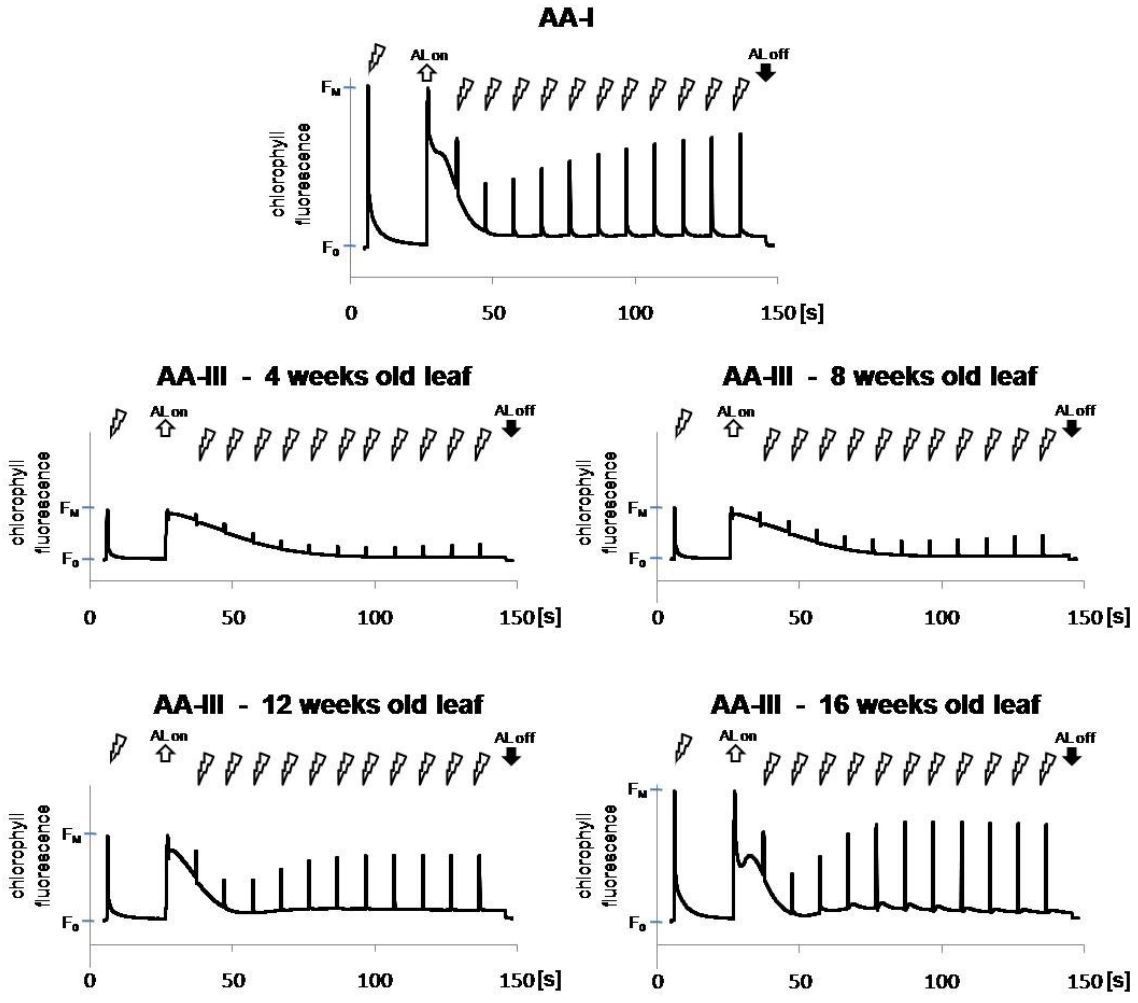
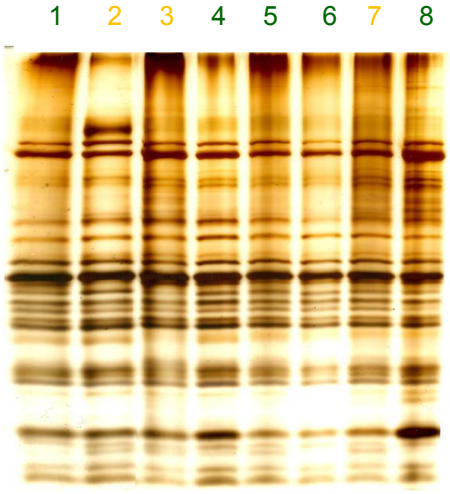


Fig. 3-15 Studies on photosystem II yield. Chlorophyll *a* fluorescence induction kinetics of compatible AA-I and incompatible AA-III leaves. Fluorescence induction traces induced by saturating white-light pulses showed the maximal fluorescence raise during the light pulse (F_M). Dark-adapted leaves were exposed to consecutive saturating light pulses during application of continuous actinic light (see Chapter 2.2.6.1). During first 12 weeks of development in AA-III plants an increased non-photochemical quenching and a slower oxidation of Q_B because of deficiencies in the "downstream cascade" of photosystem II of electrontransport chain was noted. Abbreviations: AL = actininic light; flashes symbolize saturating light pulses.

3.4.3 PROTEIN ANALYSIS OF INCOMPATIBLE PLASTOME-GENOME COMBINATIONS

Total membrane proteins were isolated from different artificial incompatible plastome-genome combinations (AA-III, AB-I, BB-IV) and from naturally occurring compatible compartmental hybrids (AA-I, AB-II, AB-III, BB-III, BC-IV), separated *via* gel-electrophoresis followed by silver staining. In the incompatible plastome-genome combination AA-III one additional high molecular band was detected. This band was not reproducible and thus is thought to represent an artifact. Furthermore, no absent or additional band was detected in artificial incompatible plants (Fig. 3-16). Furthermore, soluble proteins were checked in the same way from artificial incompatible plastome-genome combination AA-III and from green AA-I (Fig. 3-17). No significant differences were noted in either total membrane proteins or soluble proteins.



(1) *Oenothera elata* subsp. *hookeri* strain johansen (AA-I)
(2) *Oenothera elata* subsp. *hookeri* strain johansen (AA-III)
(3) ^{h}johansen • ^{h}tuscaloosa (AB-I)
(4) *Oenothera biennis* strain suaveolens Grado (AB-II)
(5) *Oenothera glazioviana* strain rr-lamarckiana Sweden (AB-III)
(6) *Oenothera grandiflora* strain tuscaloosa (BB-III)
(7) *Oenothera grandiflora* strain tuscaloosa (BB-IV)
(8) *Oenothera parviflora* strain atrovirens (BC-IV)

Fig. 3-16 Total membrane proteins in different compatible (marked in green) and artificial incompatible (marked in yellow) plastome-genome combinations

(1) *Oenothera elata* subsp. *hookeri* strain johansen (AA-I)
(2) *Oenothera elata* subsp. *hookeri* strain johansen (AA-III)

Fig. 3-17 Total soluble proteins in the compatible (AA-I; marked in green) and artificial incompatible (AA-III; marked in yellow) plastome-genome combinations

Different genes for distinct subunits of the different supramolecular complexes in the thylakoid membrane and *clp*P1 were also checked at the protein level by Western analysis (PsaF, PsbB, AtpA, and ClpP1). PsaF showed no difference at the protein level between the compatible AA-I and the incompatible AA-III, but an additional band was observed in AA-III plants, which was slightly smaller than the "normal" band of PsaF (Fig. 3-18). This band was also observed in a protein analysis in the incompatible plastome-genome combination AB-I (Greiner *et al.* 2008c). It can be assumed that PsaF is processed by ClpP protease, because in the incompatible AB-I, a large deletion of 148 bps between *psb*B operon and *clp*P1 5´ region, containing two putative *psb*B promoters and one putative and confirmed *clp*P1 promoter, was detected as most probable factor responsible for AB-I incompatibility. ClpP1 protein levels were increased in AA-III plants. Levels of PsbB (CP47) and AtpA (ATP synthase α subunit) were comparable in AA-I and AA-III (Fig. 3-18).

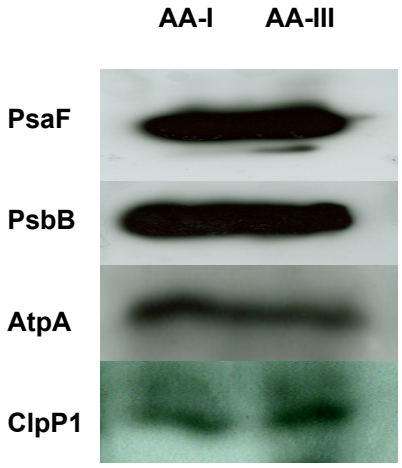


Fig. 3-18 Protein levels detected by Western analysis. No difference in protein level of the subunit psaF of photosystem I was detected, but an additional band, which was slightly smaller, could be observed. No difference in protein level was detected for psbB and atpA. ClpP1 showed increased protein level in incompatible AA-III.

3.5 CHROMOSOME ARM DETECTION BY FLUORESCENT *IN SITU* HYBRIDIZATION (FISH)

DNA fragments of ≥10 kb were isolated from *Oenothera elata* subsp. *hookeri* strain johansen leaf material (see Chapter 2.2.5.1) (Fig. 3-19). A method to isolate metaphase chromosomes from mitotically active meristematic tissue from *Oenothera*

seedlings free of overlaying cytoplasm was established (see Chapter 2.2.5.2) (Fig. 3-20). The DNA fragments were used as probes to perform FISH approaches. With two of the probes used chromosome arm specific signals were obtained but the frequencies of occurrence of the signals were still low (Figs. 3-21 and 3-22). Altogether, signals were obtained only in approximately every fifth preparation of metaphase chromosomes. Nevertheless, the approach appears to be promising to identify chromosome arms that are particularly important in *Oenothera* genetics.

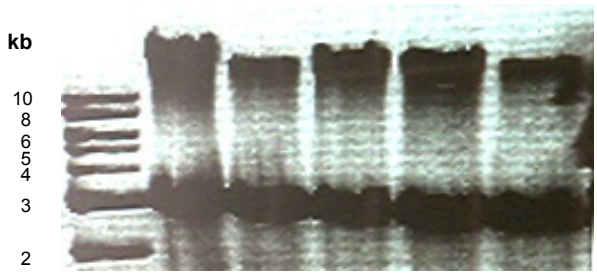


Fig. 3-19 Agarose gel of ≥10 kb clones. Size standard (2-log ladder) is placed at the left. The bands at 3 kb size are pBlueScript II KS+ vector bands. Fragments ≥10 kb reflect DNA from *Oenothera.*

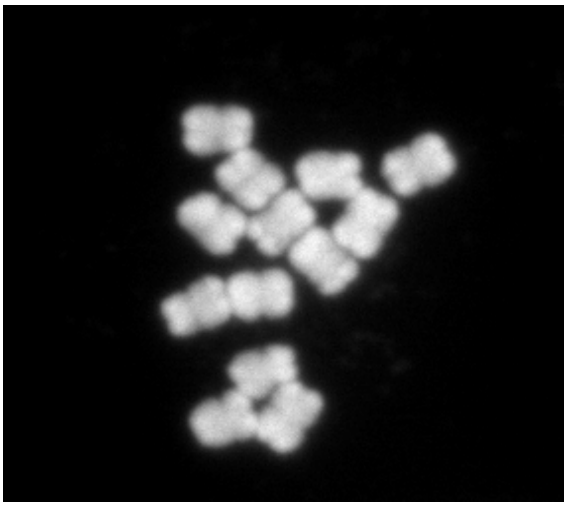


Fig. 3-20 Cytoplasm-free metaphase chromosomes prepared from mitotically active meristematic tissue from *Oenothera* seedlings.

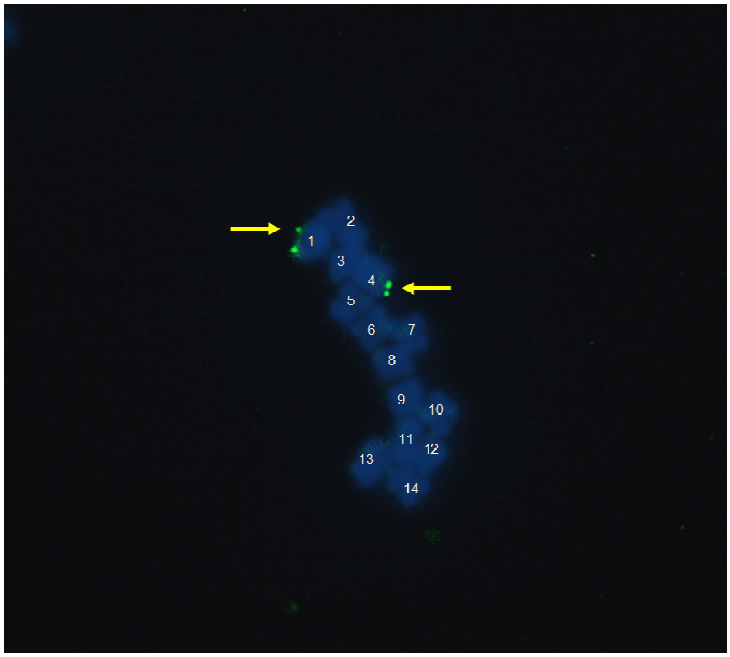


Fig. 3-21 FISH result obtained with one of the ≥10 kb probes. Signals were obtained on two chromosomes (yellow arrows) of diploid *Oenothera,* containing 14 chromosomes (numbered with white figures).

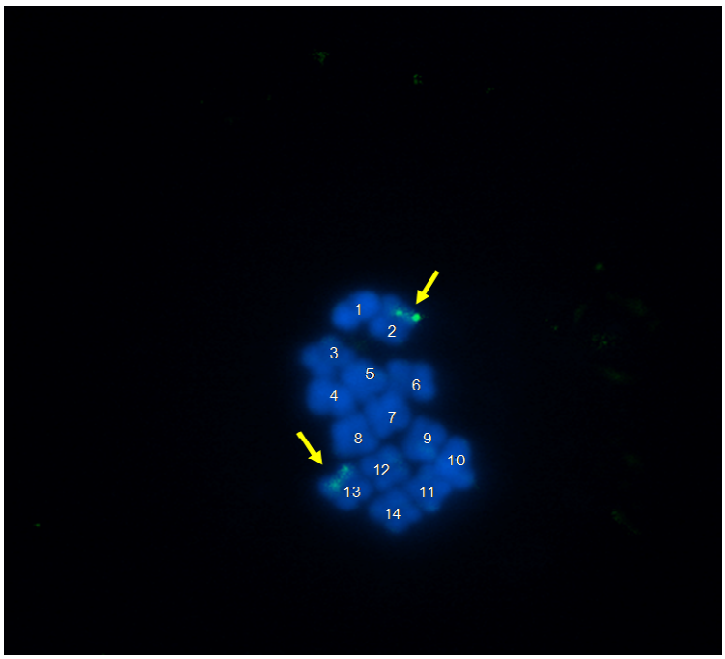


Fig. 3-22 FISH result obtained with a second ≥10 kb probe. Signals were obtained on two chromosomes (yellow arrows) of diploid *Oenothera,* containing 14 chromosomes (numbered with white figures).

4. DISCUSSION

4.1 FIRST MOLECULAR EVIDENCE FOR BASIC ASPECTS IN *OENOTHERA* GENETICS

4.1.1 BRIEF REVIEW ON FORMER WORK AND IDEAS CONCERNING *OENOTHERA* GENETICS

Oenothera species have been explored since more than a century. During this period several hundred species and lineages were collected and analyzed. In subsection *Oenothera*, next to three different genotypes (A, B and C), which occur in either homozygous (AA, BB or CC) or heterozygous (AB, AC or BC) combinations, five basic, genetically distinguishable plastome types (I – V), were detected. The latter were all sequenced and molecularly analyzed recently (Greiner *et al.* 2008b, c). Various experimental approaches were also established, notably protoplast and tissue culture, nuclear transformation (Stubbe and Herrmann 1982; Kuchuk *et al.* 1998; Mehra-Palta *et al.* 1998), and an EST library (Mráček *et al.* 2006).

In evening primroses, reciprocal translocations of entire chromosome arms lead to different chromosome formulas with consequences in meiosis. Permanent translocation heterozygosity is a phenomenon resulting as one of its consequence. In terminal translocation heterozygous species, plants consist of two different complexes and each of the parental chromosomes differ in its chromosome arm combination. Due to a lack of homologous recombination, ring-formation of these chromosomes during diakinesis is thought to restrict an exchange of genetic information, except in telomeric regions (Stubbe 1989; Levin 2002). This situation is not found in all *Oenothera* species. Various species were found, containing two identical complexes (haplo-complexes) resulting in normal bivalent formation during meiosis. Cytological methods as well as a limited number of phenotypic markers were used to examine homologous recombination events, with focus on permanent translocation heterozygotes and a few bivalent forming *Oenotheras.* In both materials, altered, atypical Mendelian segregation ratios of phenotypic markers were detected, but the number of phenotypic markers per complex available was extremely low (Cleland 1972). Disturbed Mendelian segregation ratios can be

particularly strong in reciprocal heterozygotes because of different loci conferring distinct viabilities, as could occur with genomic imprinting (Úbeda and Haig 2004; 2005). The mean fitness in such stable heterozygotes may be higher. In this case, mean fitness is maximized by complete, but opposite, drive of the sexes (complexes) (Úbeda and Haig 2004). In addition, in different bivalent forming *Oenothera* species special phenomena such as pollen tube competition (Renner 1917a), embryo sac competition (Renner 1921a; Harte 1994) and/or balanced lethals (gametophytic and/or sporophytic lethal factors) (Renner 1914, 1946; Shull 1923; Gerhard 1929; Langendorf 1930; Krumholz 1930), influence segregation ratios of markers significantly and are thought to be widespread within the genus. Also, the segregation ratios are not constant. The frequency of crossing over is often increased in hybrids as compared to the parental lines, but tends to decrease in later generations derived from these hybrids (Renner 1942). Because of these findings, meaningful statements about homologous recombination events in *Oenothera* are difficult or even impossible at present. Providing molecular evidence of suppressed homologous recombination in permanent translocation heterozygous *Oenothera* species and the clarification of meiotic affairs in bivalent forming *Oenothera* species were therefore primary goals of this work. Therefore, a modern molecular tool, AFLP analysis, was established and performed with appropriately selected lineages of the genus *Oenothera*.

4.1.2 AFLP ANALYSES AS A MOLECULAR TOOL TO EVALUATE SEGREGATION RATIOS IN TRANSLOCATION HETEROZYGOUS HYBRIDS FROM *OENOTHERA*

AFLP analyses provide possibilities for a wide range of applications including studies of genetic diversity (Mariette *et al.* 2002; Nybom 2004; Meudt and Clarke 2007). In the genus *Oenothera*, new combinations of chromosome complexes with foreign plastomes can be generated just by simple crossing experiments which is one of several special features possible with that material. For this, a distinct complex has to be combined with appropriate complexes to result in terminal translocation heterozygous hybrids (⊙14). In this work, the haplo-complex ^{h}johansen (AA-I^{joh}) was equipped with the foreign plastome type III from *Oenothera glazioviana* strain rr-lamarckiana Sweden (III^{lam}), by combining the complex four times with the complex

^{h}johansen	**/1•2**	**14•13**	**8•9**	**4•3**	**6•5**	**7•10**	**11•12**	****
^{G}albicans	****	**2•14**	**13•8**	**9•4**	**3•6**	**5•7**	**10•11**	**12•1/**

Fig. 4-1 The configuration of a ring (⊙) of 14 chromosomes, formed by the Renner complexes ^{h}johansen and ^{G}albicans, can be predicted by the chromosome formulas of the respective complexes.

^{G}albicans (Fig. 3-1). Both complexes form a ring of all 14 chromosomes during diakinesis (Fig. 4-1). The ^{G}albicans (A) complex is the egg cell complex of the terminal translocation heterozygote *Oenothera biennis* strain suaveolens Grado [AB genotype; A = ^{G}albicans (♀); B = flavens (♀♂)]. *Oenothera biennis* strain suaveolens Grado was collected in Europe; *Oenothera elata* subsp. *hookeri* strain johansen originated in California (USA). So, there is only a limited relationship of the two complexes, which normally results in a pronounced divergence of both haplogenomes. At least an increased divergence between the two complexes ^{h}johansen and ^{G}albicans can be provided by comparison with the divergence between the complexes ^{h}cholula and ^{h}puebla (10.91%; 8.57 polymorphic bands/primer combination) which were derived from the same habitat in Mexico. AFLP analyses were used to monitor genetic diversity ("degree of contamination") between AA-I^{joh} and AA-III^{lam}, and thus to reveal the segregation ratio between the two complexes (^{h}johansen and ^{G}albicans) involved in the crossing experiments. Ten primer combinations tested (a total of 711 bands) revealed just one single band that was polymorphic between the naturally occurring AA-I^{joh} and the artificially made AA-III^{lam} (0.14%). The data corroborate and extend *Oenothera* genetics, postulating that in ring forming hybrids (⊙14) single Renner complexes, *i.e*. entire haploid genomes, behave as a single coupling group, otherwise a much higher degree of contamination from other genomes, used as crossing partners, would have been expected. Free segregation of chromosomes is suppressed by the meiotic ring and if homologous recombination occurs, it is a rare event, most probably restricted to telomeric ends (Cleland 1972). *This is the first molecular evidence corroborating postulations from classical Oenothera genetics for permanent translocation heterozygotes.*

4.1.3 FIRST AFLP LINKAGE MAPS OF *OENOTHERA,* USING NATURALLY OCCURRING BIVALENT FORMERS

AFLP markers and co-dominant markers were used to calculate linkage maps of different genotypes of *Oenothera*. Linkage analyses identified seven coupling groups in the interspecific cross BB-III^{lam} x AA-III^{lam} (^{h}tuscaloosa x ^{h}johansen) and in the intraspecific cross AA-I^{cho} x AA-I^{pue} (^{h}cholula x ^{h}puebla). This corresponds to the cytologically defined chromosome number observed in all *Oenothera* species investigated so far. Both F1 generations formed bivalents during meiosis. It is one of the striking findings of this work that a single cluster comprising ~90% of markers was detected for each chromosome. The clustered markers displayed not a single homologous recombination in more than 300 meioses investigated. A single homologous recombination event would suffice to split the bulk of markers into two. No single chromosome was found which would show more than one marker cluster, indicating that homologous recombination is completely absent, disregarding telomeric parts.

In permanent translocation heterozygous *Oenothera* lines, the lack of homologous recombination was explained by chromosome configurations (ring) present during meiosis (Stubbe 1989; Levin 2002). Such material is thought to derive from bivalent forming *Oenothera* plants after reciprocal arm translocation events. Another striking finding of this study is that the loss or reduction of homologous recombination is not exclusively restricted to ring-formation during meiosis. Thus, the strong limitation of homologous recombination is probably not a result of reciprocal translocation events, but seems to be immanent in the material and may have been evolved before. This implies new views on the evolutionary history of the genus and the evolution of permanent translocation heterozygosity in general.

Clustered markers result in small sizes of linkage maps expressed in centi Morgan (cM). Compared with the sizes detected in *Lotus japonicus* (Gifu B-129 487.3 cM; Miyakojima MG-20 481.6 cM) (Hayashi *et al.* 2001) or *Arabidopsis thaliana* (*Ler/Col* 427 cM; *Ler/Cvi* 475 cM) (Alonso-Blanco *et al.* 1998) the A genome of ^{h}johansen and B-genome of ^{h}tuscaloosa provided basic maps of 135 and 165 cM. These results do not imply that the *Oenothera* genome is approximately three times smaller than that

of *Arabidopsis.* Indeed, the *Arabidopsis thaliana* genome is at least four times smaller compared to that of evening primroses (Ingle *et al.* 1975; Sliwinska and Thiem 2007) (database: http://data.kew.org/cvalues/introduction.html). Distances in *Oenothera* differ in the sizes of single coupling groups: in AA-III^{lam} from 3 cM (coupling group 1A) to 39 cM (coupling group 4A) (Fig. 3-5a), in BB-III^{lam} from 0 cM (coupling groups 1A, 3A, 7A) to 23 cM (coupling group 2A) (Fig. 3-5b). Such small sizes reflect disturbed segregation of markers. Thus, in bivalent forming *Oenothera* hybrids, suppression of homologous recombination along with disturbed segregation of about 90% of the markers was detected. *This is the first case of sexual inheritance described with homologous recombination limited to telomeric regions described from a bivalent forming species.*

4.2 POSSIBLE REASONS FOR SUPPRESSION OF HOMOLOGOUS RECOMBINATION

Generally, homologous recombination decreases depending on the degree of polymorphism between genomes (Haber *et al.* 1991). Our analyses indicate 39.78% divergence between AA-III^{lam} and BB-III^{lam} and 10.91% between AA-I^{cho} and AA-I^{pue} (Table 3-3a). This is comparable to linkage analyses of other plant taxa (*e.g.* Alonso-Blanco *et al.* 1998). Particularly with regard to the low diversity between AA-I^{cho} and AA-I^{pue} it can be excluded that the loss of homologous recombination is caused due to high sequence divergence of the genomes investigated.

Meiotic exchanges are generally not distributed randomly along the length of eukaryotic chromosomes. Regional variation in recombination frequency has usually been observed, with certain regions having high levels of recombination (hotspots) and others having low levels (coldspots) (Koren *et al.* 2002). A dramatic repression of exchange is found near centromeres and some telomeres (Mather 1936, 1939; Alonso-Blanco *et al.* 1998; Qi *et al.* 1998). The repression of homologous recombination adjacent to the centromere is assumed to be caused by large blocks of heterochromatin present at that region (Willard 1990; Murphy and Karpen 1995). This is quite obvious for example for the *Drosophila* X chromosome, in which the centric heterochromatin, comprising half of the cytogenetic length of the chromosome, barely contributes to its genetic length (Mather 1939; Roberts 1965). A

similar centromere-associated repression of recombination was also found for autosomes of *Drosophila* (Beadle 1932; Painter 1935; Thompson 1963). Furthermore, it was described that in regions around translocation breakpoints homologous recombination is also repressed (Hayashi *et al.* 2001), most probably due to sequence divergence. Reciprocal translocations of entire chromosome arms are one of the basic genetic characteristics of the genus *Oenothera.* Due to the fact that only one single cluster of markers was detected on each chromosome, the translocation breakpoints of chromosome arms is supposed to be at heterochromatin-rich centromeric regions. However, it seems to be rather unlikely that especially the reciprocal breakpoints of chromosome arms inhibit recombination of around 90% of the markers assigned. It is also conceivable that various successive translocations on a chromosome are responsible for the loss of homologous recombination. This latter idea can also be excluded because of the relatively low degree of polymorphism detected between the plant species used. With the occurrence of successive translocations along the length of a chromosome (~90% of a chromosome), if they differ between the crossing partners, high divergence between species is expected. Furthermore, recombination is generally assumed to take place during the meiotic prophase. Therefore, cytological studies of meiotic stages were performed to investigate possible structural reasons for the suppression of homologous recombination.

4.3 AN UNEVEN CHROMOSOME CONDENSATION DURING PROPHASE I IN *OENOTHERA*

Cytological analysis revealed for both parental species and the F1 hybrid seven bivalents at diakinesis with two end-chiasmata per bivalent (Fig. 3-3) and a regular meiotic segregation at late anaphase I (Fig. 3-10d). The fourteen chromocenters in premeiotic nuclei most probably represent pericentromeric chromosome segments (Fig. 3-10a). In contrast to an expected step-wise contraction from leptotene to pachytene (Zickler and Kleckner 1998; 1999), uneven chromosome condensation takes place very early during meiosis in *Oenothera*: Chromosomes form *large* and *strongly condensed* proximal segments and two diffuse, *relaxed* distal regions already at leptotene (Fig. 3-10b, c). A similar chromosome behavior was observed for the mitotic prophase in *Oenothera* (Kurabayashi *et al.* 1962). The chromocenters,

seen at early leptotene, were larger than those in the premeiotic interphase and had chromosome-like appearance. This suggests that quite a large fraction of chromatin has an untypical, higher-order organisation as a result of precocious condensation already before zygotene pairing (formation of the meiotic bouquet). A mechanism restricting recombination may act in the form of an interplay between centromere-driven chromatin condensation and telomere-led synapsis. The dynamics of chromatin allocycly, a term referring to differences in the coiling behavior shown by chromosomal segments (pericentric heterochromatin) or whole chromosomes, in *Oenothera* can be related to the restriction of meiotic recombination. The chromosomes pair in a group at one pole in a highly polarized Rabl-configuration before zygotene (Fig. 3-10b) and remain clustered until early diplotene (Golczyk *et al.* 2008). Generally, a lack of open pericentromeric chromatin configuration was observed during whole meiosis, being a likely factor of preventing initiation of meiotic recombination throughout large chromosome portions (McKee and Handel 1993). Especially topoisomerase II is known to be involved in chromosome condensation (Hartsuiker *et al.* 1998). It is of high interest, whether this enzyme is involved in *Oenothera* cytology, because also investigations of chromosomes in different plant tissues revealed an untypical, not regular condensation of autosomes, comparable to the human barr body. In nearly all tissues investigated, except in metabolic active tapetum cells and root hair cells, large blocks of chromocenters were found (Dr. Hieronim Golczyk, personal communication). In order to clarify the particular features of *Oenothera* meiosis, dynamics and character of meiotic condensation and pairing need to be characterized more precisely at the ultrastructural level.

4.4 REPRODUCTION IN *OENOTHERA* AS PARTHENOGENESIS-LIKE FORM OF INHERITANCE

Sexual reproduction with negligible homologous recombination at telomeric regions is presumably present in all *Oenothera* species. In complete translocation heterozygous species chromosomes separate as a set without intermixing, giving rise to a progeny identical to the parental lines, whereas in bivalent forming *Oenothera* entire chromosomes freely segregate but often underly embryo sac or pollen tube competition. The production of genetically identical offspring (clones) was up to now a phenomenon just known from species reproducing by apomixis, differing only in the

form of reproduction (*Oenothera*: sexual; apomixis: unisexual). Species obeying this pattern are designated as true breeding species. Bivalent forming *Oenotheras*, in which genotype mixing is limited to free segregation of entire chromosomes, can be regarded as a preform. Parthenogenesis is defined as an unisexual reproduction, developing its offspring from unfertilized egg cells. Two basic forms of parthenogenesis are distinguishable: (1) parthenogenesis with meiosis [arrhenotoky, thelytoky (automixis)] or (2) parthenogenesis without meiosis (developing directly out of diploid germ line cells) (apomixis) (Oliver 1971, Slobodchikoff and Daly 1971). According to present knowledge, parthenogenesis is regarded as difficult or impossible in higher mammals and marsupials, in contrast for instance to plant species. The reason for this is the so-called imprinting (Kono 2006). Genomic imprinting is a principle of inheritance, independent of classical Mendelian inheritance: genes involved in the genomic imprinting are actively or inactively inherited, *i.e.* they receive a parental, genomic imprinting depending on their parental origin. This inheritance scheme does not obey Mendelian rules. Imprinting is based on additional epigenetic modifications of the DNA, which are established in addition to the genetic code in the germ cells. By this pattern, one of the two parental alleles of the imprinted genes is active and the other one inactive. Although *Oenothera* reproduction appears in a sexual way with male and female gametes, genetic imprinting is not excluded. Thus, the mechanism of inheritance in *Oenothera* (terminal translocation heterozygosity) can be handled as parthenogenesis-like (apomixis-like) inheritance, in which genomic imprinting is a possible cause.

4.5 THE SENSE OF SEX AND HOMOLOGOUS RECOMBINATION

The sense of sex and homologous recombination is an unresolved question. There are a number of reasons why sex might be disadvantageous. Females pay the full cost of reproduction in contrast to males, but provide only half of the genes to sexually produced offspring. In asexually reproducing females a complete set of their genes is transmitted at the same energetic cost per offspring. This fact is called ´two-fold´ cost of sex (*e.g.* Maynard-Smith 1978; Rice 2002; Nielsen 2006; Agrawal 2006). The success of asexual lineages is shown by the frequently outnumbering of their sexual progenitors, the persistence for thousands of generations and the geographical distributions that frequently far exceed those of their sexual progenitors

(Stebbins 1950; Maynard-Smith 1978; Rice 2002). Therefore, it is obvious that recombination frequently provides a long-term, rather than an immediate, advantage (Rice 2002; Agrawal 2006). Furthermore, sex may be expensive because of the costs of searching for mates and of engaging in mating (Agrawal 2006). Also, while selection is expected to make appropriate allele combinations disproportionately common, genetic mixing tends to break down this excess of favorable allele combinations, generating unfavorable combinations in the process (negative epistasis) (Agrawal 2006). But also the opposite case can be assumed that sexual recombination speeds up the rate of adaptive evolution, because it could allow beneficial mutations to be combined in the same individual (positive epistasis) (Agrawal 2006). Many species, in particular plant species, are capable of both, sexual and asexual reproduction (Stebbins 1950; White 1978; Agrawal 2006). Obviously, sexual processes are not essential for reproduction. Nevertheless, the trend in evolution goes clearly in the direction to sexual inheritance, a phenomenon which because of its disadvantages and lack of proven advantages compared to asexual inheritance is often described as the paradox of sex.

It is highly relevant to mention that even within some obligately sexual species, the extent of sex is reduced by suppression of recombination in some individuals, *e.g.* in male *Drosophila melanogaster* (Wurglics and Becker 1993; Agrawal 2006), demonstrating the possibility of sexual reproduction without genetic mixing. From a genetics perspective, sex is synonymous with homologous recombination (Lamb *et al.* 2007). The best known advantage of sexual recombination is faster adaptation to changing environment. The evolutionary driving force and ´source´ of evolution and adaptation are mutations (Dobzhansky 1937; Stebbins 1950; Cavalier-Smith 2002). To adapt to changed evolutionary pressure beneficial mutations have to evolve and to be fixed in one individual. Homologous recombination could on the one hand be advantageous in bringing the beneficial mutations together. On the other hand, it can be disadvantageous as coupled beneficial mutations can also be separated again by crossing-over events. The chance for the latter increases with the number of beneficial mutations needed to adapt. It was also reported that new alleles are only beneficial if they accumulate in a prescribed order (Kondrashov 1984). This may also be relevant for adaptation. Only asexual or sexual reproducing organisms which do

not mix their genetic information allow some overlap of successive allele replacements.

The general consequence from the ability of homologous recombination is a high genetic variability within a population. The maintenance of this system can be based on at least three facts: (1) maintenance of the flexibility for adaptation with environmental changes, (2) distribution of accumulated harmful (primarily deleterious) mutations within the entire population for increasing individual fitness (Muller´s ratchet, mutational load), or (3) protection, for instance, against pathogens (pathogen ratchet theory). All three aspects are important for the survival of organisms. Different organisms can differ in managing these points, as it has to be the case in the genus *Oenothera.*

4.6 THE *OENOTHERA* SYSTEM AND ITS CONSEQUENCES FOR ADAPTATION TO CHANGING ENVIRONMENT

Oenothera is a highly successful, cosmopolit plant (Dietrich *et al.* 1997, Punt *et al.* 2003). It originated in the North American continent and invaded Europe after 1500 A.D. (Harte 1994). This implied the ability of fast adaptation to changed environment. *Oenothera* adapted and ´conquered´ almost all continents and is even able to adapt to environments with extreme conditions, as it is, for instance, the case in Chernobyl (Boubriak *et al.* 2008). The ability to adapt to such areas is an indication for the fact that the plant developed special adaptive survival mechanisms, which may include an effective DNA repair system.

Bioinformatical analysis of plastome sequences dates the evolutionary distances for the five distinct *Oenothera* plastomes to approximately one million years ago (Greiner *et al.* 2008b). During this time, distinct changes in one of the organellar genome lead inevitably to a high pressure in the others. This form of fast adaptation is visible in the genus, represented in incompatible artificial plastome-genome combinations (Fig. 1-4). Incompatibilities are result of missing co-evolved (co-adapted) cellular subgenomes that occurred in natural parental combinations.

Lack of exchange of genetic material between two homologous DNA fragments stabilizes genetic information encoded on individual chromosomes. Nevertheless, genetic information foreign to a species can be established in a different way in the *Oenothera* system, due to interfertility of its species. More or less entire chromosomes can be replaced by another one in bivalent forming *Oenothera* species, whereas in permanent translocation heterozygotes even entire haploid chromosome sets can be exchanged. Manifestation of a beneficial chromosome foreign to a species in a bivalent forming *Oenothera* hybrid can be supported by embryo sac or pollen tube competition that significantly increases the chances of

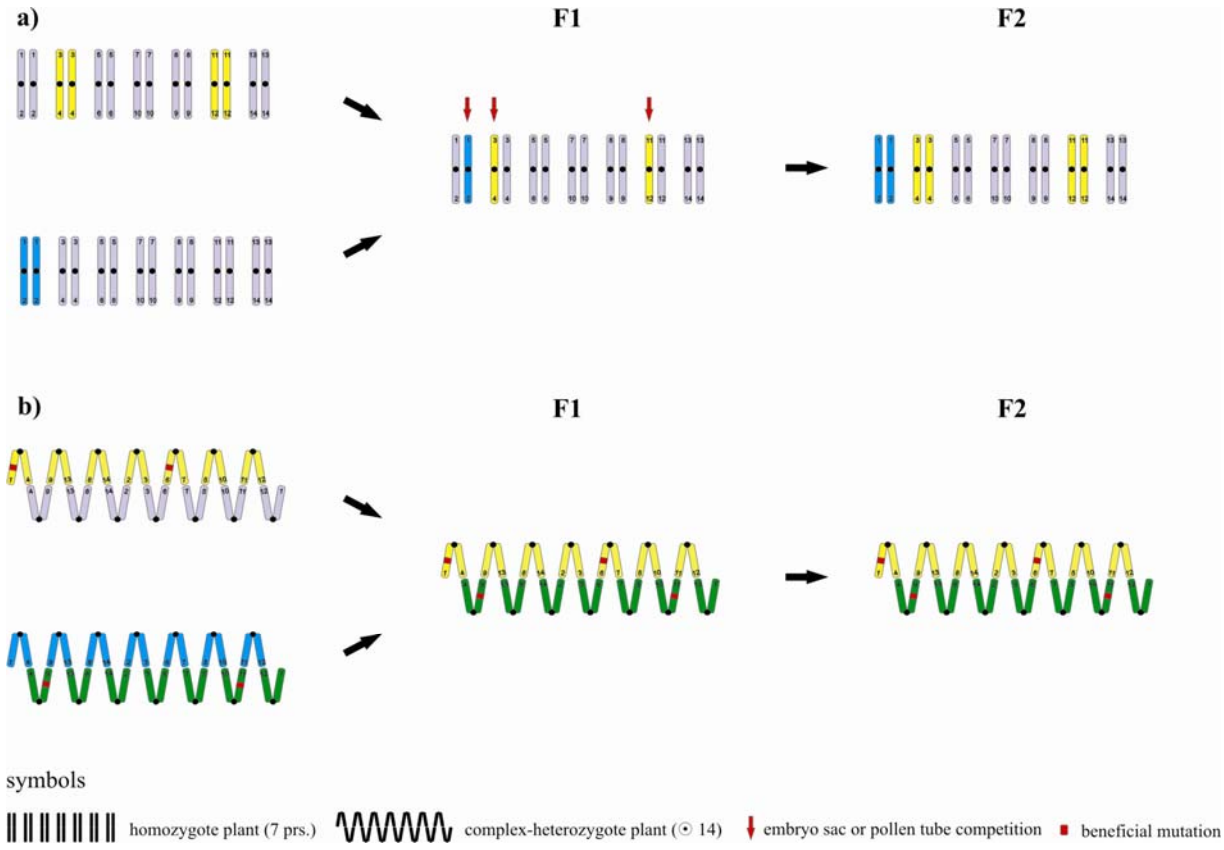


Fig. 4-2: Schemes of adaptation by manifestation of beneficial chromosomes in **(a)** bivalent forming and **(b)** permanent translocation heterozygote *Oenothera*. **(a)** Chromosomes carrying beneficial mutations are in colour (yellow or blue). Beneficial chromosomes in F1 (red arrow) underly theoretically an advantage in fitness while developing in the embryo sac (embryo sac competition) and/or pollen tube (pollen tube competition). This leads to a non-Mendelian segregation of the beneficial chromosomes concerned in F2, in which the better adapted chromosomes arise with higher frequency. **(b)** Beneficial mutations on chromosomes are symbolized in red. In F1 the egg cell complex (yellow) from the female parental plant and the pollen complex (green) from the male parental plant are combined. Each of the Renner complexes carries beneficial mutations (red), which, because of the *Oenothera* genetics (gametophytic and/or sporophytic lethal factors), become already fixed in the following generations. This way, an entire beneficial haploid chromosome set will be fixed in permanent translocation heterozygote and the co-evolved, “harmonious” complex is fixed as a whole.

such a manifestation because of its advantage in fitness. This form of new combination of chromosomes by introducing one or more foreign beneficial chromosomes into a stable (not recombining) genetic system leads to a “steady improvement of the chromosome set” and hence also to an effective form of adaptation (Fig. 4-2a). In permanent translocation heterozygous *Oenothera* species even an entire Renner complex carrying beneficial mutations can be inherited. The co-evolved genes, encoded on the Renner complex, thus will be fixed as a whole and the “harmonious gene pool” of one complex will not be separated (Fig. 4-2b).

The genetic behavior of permanent translocation heterozygosity occurs with variations in many plant families, *e.g.* in Campanulaceae, Commelinaceae, Clusiaceae, Iridaceae, Paeoniaceae, Papaveraceae (Holsinger and Ellstrand 1984; Raven 1979), Asteraceae (Hunziker *et al.* 2002), Euphorbiaceae (Szweykowski 1965), Fabaceae (Ashraf and Bassett 1986), Poaceae (Castellarin *et al.* 1993), Alliaceae, Liliaceae, Salicaceae, Hypericaceae, Rosaceae, Solanaceae and Alismataceae (Darlington 1937; Stebbins 1950). Furthermore, translocation heterozygosity was described from various animal taxa, too (*e.g.* Henricson and Bäckström 1964; Grützner *et al.* 2004), demonstrating that this phenomenon is neither rare nor an exception.

Finally, *Oenothera* is able to adapt relatively fast to different environmental conditions even without essential homologous recombination. In addition, it is assumed that *Oenothera* possesses a specific, effective DNA repair system because of its ability to grow on strongly mutagenic territories (Boubriak *et al.* 2008), which possibly can compensate the accumulation of deleterious mutations (mutational load).

As mentioned above, a high genetic variability can also be of advantage in an increased protection against pathogens. In this respect, no explicit investigations were performed with *Oenothera,* but neither in field-grown nor in greenhouse-grown material an increased infestation of parasits was observed. In nature, *Oenothera* occurs regularly, but only rarely cumulated at one location. Thus, a spatial barrier is given for pathogens to attack an entire population. A general statement about

resistence against pathogens is difficult to make, but all observations available did not show a conspicuous liability against pathogens.

4.7 THE PLASTOME-GENOME INCOMPATIBLE (PGI) AA-III HYBRID AS A POSSIBLE EXAMPLE FOR A DOBZHANSKY-MULLER INCOMPATIBILITY (DMI)

Oenothera genetics allows the exchange of plastids and nuclei by simple crossings. Distinct plastome-genome combinations lead to incompatible phenotypes (Fig. 1-4), which reflect a special case of the DMI. Disturbances caused by interspecific organelle exchanges usually affect a multitude of ontogenetic processes. Most conspicuously are lesions of the photosynthetic apparatus (hybrid bleaching, hybrid variegation) and of the generative phase that can be impaired at various gametophytic and/or sporophytic stages, such as pollen and/or ovule development (summarized in Harte 1994). These serious developmental disturbances are caused by co-evolution of cellular subgenomes (see Chapter 1.2.3). The PGIs described for the *Oenothera* system are based on one (or more) gene pair(s), of which one element is located in the nucleus ("nucleus-encoded factor"), the other one in the plastid ("plastid-encoded factor"). Such a Dobzhansky-Muller gene pair has not yet been described. This is quite surprising since in the order of 25 - 30% of the nuclear coding capacity is required for the management of the organelles (Herrmann 1997). Therefore, such a plastome-genome interacting DMI gene pair is very likely because of the co-evolution of the organellar genomes. By combining plastome sequence information and bioinformatic approaches with formal genetic data and molecular, biochemical and biophysical analysis, regions on the plastid chromosomes can be unveiled that are potential candidates for plastome-encoded determinants of interspecific PGI (Greiner *et al.* 2008c). Mapping approaches can in principle be used to identify the nuclear determinants, but not in non-recombining species, as *Oenothera*. In evening primroses, mapping approaches can be used for assigning the nuclear determinants to respective chromosomes or to determine the number of coupling groups involved. We have started to characterize and narrow down potential candidates that are responsible for the plastome-genome incompatibility AA-III.

The lack of homologous recombination allows a fast determination of the coupling groups involved. A small number of F2 plants, around 20, is already sufficient to determine the chromosome(s) the sought gene is located on. An accurate mapping of the gene is not possible at present, unless a mutant line can be found or generated, allowing homologous recombination. If it is true that the irregular condensation of the chromosomes causes suppression of homologous recombination during meiosis, a possible approach would be to generate RNAi lines to inactivate a gene that is necessary for condensation. Such a mutant was already described, *e.g.* for *Arabidopsis*. Chromatin assembly factor 1 (CAF-1), a heterotrimeric complex consisting of three subunits (p150/p60/p48), is known to play a role for reconstitution of S-phase chromatin. A *fas1-4* mutant (defective in p150 subunit of the CAF-1 complex) of *Arabidopsis* showed, in addition to reduced heterochromatin, a more open confirmation of euchromatin, a reason why homologous recombination is dramatically increased in that line (Kirik *et al.* 2006). Another classical approach is to perform a mutagenesis approach followed by a screen for a recombining *Oenothera* individual, to establish a T-DNA insertion line or to perform ´happy mapping´ (Dear and Cook 1992).

4.7.1 DETERMINATION OF COUPLING GROUPS INVOLVED IN THE PGI AA-III BY A MAPPING APPROACH

In a mapping approach a cross of compatible BB-III and incompatible AA-III (*virescent*) plants was used. In F2 different incompatible phenotypes were observed (Fig. 3-4), indicating that the co-evolution reflected is complex and that at least two nuclear loci are involved. Exact segregation ratios of incompatible plants in F2 could not be determined because of varying patterns of incompatible plants during the first days of development (Schötz 1958). In addition, the development of the incompatible phenotypes depends on environmental factors. As a consequence of lacking homologous recombination, entire chromosomes of each single plant can be assigned to its genotype, AA, AB or BB. Comparison of genotypes between compatible and incompatible F2 plants revealed two coupling groups, chromosome 4 and 7, to be unequivocally involved in causing the AA-III incompatibility. Furthermore, the analysis revealed that these two loci interact. A BB genotype on one of the two chromosomes involved is able to reverse the incompatible phenotype. This can have

two reasons, (1) a gene duplication in the *Oenothera* history or (2) a further gene compensates the effect of the other.

Furthermore, an additional nuclear factor, or a special combination of chromosomal genotypes has to be involved in causing the AA-III incompatibility, because in four incompatible plants the AA genotype was detected neither on chromosome 4 nor on chromosome 7. The multiplicity of phenotypes could not be reconciled with special combinations of chromosomal genotypes, probably due to a phenotypical dependency on environmental conditions.

4.7.2 NARROWING DOWN THE IDENTITY OF THE PLASTID-ENCODED FACTOR OF THE PGI AA-III

The five basic plastome sequences (I – V) (Greiner *et al.* 2008b) can be used to decipher the potential plastid-encoded determinant of AA-III plants by bioinformatical sequence comparisons. Since the nuclear background AA is incompatible with plastome type III, showing a *virescent* phenotype, plastome type III must differ in at least one region in the sequence from the other four. Plastome V was excluded in analysis because AA-V (as well as AB-V and BB-V), in which fertility and morphogenesis are severly affected (Stubbe 1963; Stubbe *et al.* 1978), may be caused by (1) multiple Dobzhansky-Muller plastid-nuclear gene pairs, or (2) different PGIs. Both would result in a severe phenotype and thus should not be considered in the comparison of plastome sequences. The weak incompatibility caused by plastome IV in an AA background differs from that caused by plastome III. In addition, a special combination of complexes with the genotype A is known for plastome IV (^{G}albicans • undans combined with IV^{atro}; Stubbe 1960), which can reverse the incompatibility with plastome IV but not that of AA-III, indicating that most probable a single locus within this plastome, different from that of AA-III, causes the *chlorina* phenotype. The revertant observed in AA-III (Fig. 3-12) indicates a single plastid-encoded factor to be responsible for incompatibility in AA-III plants. An influence of more than one region within the plastome can thus be regarded as improbable. Furthermore, our mapping approaches revealed more than one nuclear locus to be involved in the AA-III phenotype (see in Chapter 3.1.4, Fig.3-6). That makes parallel “backmutations” in nuclear genes unlikely and indicates the potential

plastid-encoded factor to be “backmutated” in the revertant. In *Oenothera*, a nuclear gene, the so-called *pm*-factor (plastome mutator), was described that causes a higher mutation frequency in plastomes (Epp 1973; *Epp et al.* 1987; Sears and Sokalski 1991; Stoike and Sears 1998). This factor could be used, for instance, in further crossing experiments to generate AA-III plants, containing this factor and search for another AA-III revertant.

The genic differences, leading to an exchange in amino acid sequence, were compared as well as large deletions or insertions in non-coding sequences. Plastome III specific genic differences were found in the genes *atp*A, *clp*P1, *ndh*A, *ndh*E, *rps*3, *rps*8, *rps*18, *ycf*1, *ycf*2, *ycf*4, *ycf*5, *rpo*C2 and *acc*D (Table 3-10a, Fig. 3-13), large intergenic differences between *trn*G_{UUC}/trnS_{GCU}, *trn*Q_{UUG}/accD, *psa*J/*rpl*33, *rrn*16/*trn*I_{GAU}, and directly before *atp*B/E operon (Table 3-10b). All differences involving the NADPH complex (*ndh*A and *ndh*E) can be disregarded, because knock-out mutants of individual NDH subunits in tobacco lack a conspicuous phenotype (Burrows *et al.* 1998; Kofer *et al.* 1998). In addition, sequence polymorphisms between other plastomes, *e.g.* of types I and II, can be disregarded too. A contribution of these regions to PGI is unlikely, since plastomes I and II remain fully compatible in an AA background (Fig. 1-4). This excludes the genes *acc*D, *ycf*1 and *ycf*2 and the intergenic region *trn*Q_{UUG}/*acc*D. In *rpo*C2, a threonin-to-serine amino acid exchange is found in the plastome III sequence. The two amino acids share attributes (small, polar, neutral). An effect of such an amino acid exchange is quite unlikely, and supported by former macroarray analysis of AA-III plants (Mráček *et al.* 2006), which did not indicate a general malfunction of transcript profiles.

Frameshifts in genes are also possible candidates causing incompatibility. A point mutation causes an alternative stop codon in *atp*A, leading to a length polymorphism of two amino acids in plastome III (Fig. 3-13) that can be detected by Western analysis (Herrmann *et al.* 1980b). Multiple base pair indels lead to a different stop codon in *rps*18. Plastome IV has most of the same indels in *rps*18, but differs by an unchanged stop-codon. In contrast to plastome III the last ten amino acids were unchanged in plastome IV. This part is highly conserved in other *Oenothera* plastomes but it is polymorphic between the different organisms used for alignment

comparison (Fig. 3-13). Although an involvement of *rps* genes in the AA-III incompatibility is not very likely, *rps* genes could not be rigorously excluded as potential candidates causing PGI. The *virescent* phenotype disappears after 10 - 12 weeks indicating a development-dependent malfunction. In the first weeks, plants elongate fastly and significantly increase number and size of their leaves. During this developmental stage many proteins need to be synthesized, which are required for structures, *e.g.* of the thylakoid membrane system. It is conceivable that the temporally affected development is due to an impaired functionality of the ribosomal complex in AA-III plants compared to the others. To verify this, it would be useful to compare polysomes of incompatible AA-III with compatible AA-I plants.

All differences detected in the cytochrome complex (*ycf*5 and *pet*D intron) or the ATP synthase (*atp*A, 13 base pair deletion directly upstream of *atp*B/E operon) are essential and can not be excluded, because PAM measurements revealed on one hand high non-photochemical quenching in incompatible AA-III, possibly due to a high proton gradient across the thylakoid membrane that can be due to an ATP synthase malfunction. On the other hand, problems could arise as well in the "downstream electron cascade" to reoxidize Q_B that would influence assembling of functional complexes following photosystem II, the cytochrome complex and photosystem I. The photosystem II phenotype observed could also be caused by metabolic defects. In photosystem I, PAM measurements indicate no significant differences between compatible AA-I and incompatible AA-III plants. Furthermore, Western analyses revealed an increased ClpP1 protein level in AA-III plants. ClpP1 encodes a plastid determinant of the 350 kDa Clp complex (plastid protease), consisting of a multitude of subunits (different types of ClpP, ClpP-like (ClpR), ClpS, ClpD) (*e.g.* Sokolenko *et al.* 1998; Sokolenko *et al.* 2002; Koussevitzky *et al.* 2006; Adam *et al.* 2006, Sjögren *et al.* 2006). At present, information about specific targets of the Clp complex is not available. Western analyses disclose an additional PsaF band that was already observed in incompatible AB-I plants, in which a 148 bp deletion of the divergent promoter containing (NEP and PEP promoters) intergenic region between *psb*B operon and *clp*P1 in plastome type I is considered to cause the incompatibility (Greiner *et al.* 2008c). So, it is conceivable that PsaF is a potential target of the Clp complex. This suggests that ClpP1 is also a probable factor

responsible for the incompatibility in AA-III plants. This suggestion is supported by an observed *virescent* phenotype in the *clpR1* mutant line in *Arabidopsis thaliana*, which is very similar to the phenotype observed in AA-III plants (Koussevitzky *et al.* 2006). To summarize, bioinformatical analyses could narrow down potential candidates responsible for the incompatibility in AA-III plants. Further biochemical and biophysical approaches are necessary to settle the molecular basis of AA-III incompatibility.

4.8 BENEFIT AND APPLICATION OF CO-DOMINANT MARKERS IN *OENOTHERA* BREEDING

Oenothera plants contain two genome complexes, which can be haplo-complexes or Renner complexes. In *Oenothera* breeding strategies, *e.g* for assembling plastome-genome incompatible plants, genome restructuring or for commercial *Oenothera* breeding (Fieldsend 2007), complexes were newly combined and progenies often have to be selected for desired plastome-genome combination. Using the few phenotypic markers, a trained eye and experience for the most of the selections is required (Dietrich *et al.* 1997). The characteristics for different genotypes were described in Dietrich *et al.* (1997). Heterozygotes (AB, AC and BC) in most cases show a phenotype, which is intermediate between the characterstics of both genomes. In most cases, these are not clearly distinguishable during the first weeks of development and become visible only in adult stage. Thus, molecular co-dominant markers are of high interest in *Oenothera* breeding but were not available up to now (Mráček *et al.* 2006; Larson *et al.* 2008).

In order to solve this problem and to make the Onagracean material accessible for straightforward breeding and mapping approaches, a PCR marker system was developed for genomes and plastomes, to distinguish the most important complexes (marker allele M40; Table 3-8; see Chapter 3.3.1) and the most important basic plastomes and their subplastomes (marker allele *rrn*16-*trn*I_{GAU}; Table 3-9; see Chapter 3.3.2) (Rauwolf *et al.* 2008b; Greiner 2008). This way, immediate genetic access to a variety of lineages was provided. Up to now, basic and subplastome types could only be distinguished by time-consuming RFLP analyses (Herrmann *et al.* 1980b; Gordon 1981, 1982; Chapman *et al.* 1999) or the time-consuming usage of

bleached plastome mutants (Stubbe 1959; Stubbe 1989). The number of crosses can now be significantly reduced using the plastome marker allele, which allows monitoring of plastome types by a single PCR.

It is likely that most of the co-dominant molecular markers designed can be applied to the majority of complexes (Rauwolf *et al.* 2008b; Mráček *et al.* 2006; Greiner 2008). Therefore, it will be possible to merge the classical map and the molecular map presented in this work by segregation analyses of an appropriate cross. In a pilot study with the nuclear marker M58 it was possible to correlate chromosome 9·8 with linkage group 7 (Rauwolf *et al.* 2008b; Greiner 2008). Furthermore, with appropriate lines co-dominant markers can be used to define individual chromosome arms which is a crucial step in *Oenothera* breeding. The highly diverging M40 marker, in turn, can be applied to distinguish between individual complexes and can be used to monitor basic genotypes in ring forming hybrids and a large number of distinct haplo-complexes. Screening of splitting generations with different complexes at the seedling stage by a single PCR is the major benefit of this marker. This is a significant relief in working with annual herbs in general. In summary, the markers described represent a significant progress in *Oenothera* genetics. They allow a precise and easy molecular identification of plastomes, Renner complexes, single chromosomes and chromosome arms by PCR in crossing programs. They will also have an impact on commercial *Oenothera* breeding.

4.9 COMMERCIAL INTEREST IN *OENOTHERA*

Oenothera has become an interesting material for industry with the discovery of essential unsaturated fatty acids in this genus, used as food supplies and in medical application (Horrobin 1990; Morse and Clough 2006). Consequently, commercial cultivars as well as attempts for genetic manipulation and breeding do exist (de Gyves *et al.* 2004; Fieldsend 2007). In human health, a balance between Omega-6 and Omega-3 fatty acids is vital (1:1 to 4:1). Gamma-linoleic acid (GLA) belongs to the Omega-6 family and appears in relatively high amounts in seeds of *Oenothera* species (“evening primrose oil”). The human body needs GLA to produce prostaglandins, a type of hormone-like substance (Horrobin *et al.* 1984a). Prostaglandins are believed to be involved in many processes, including in the

regulation of the immune system. An imbalance between Omega-6 and Omega-3 fatty acids contributes to the development of long-term diseases. Various research data suggest that GLA may be useful for diabetes (Keen *et al.* 1993), eye disease (Brown *et al.* 1998), chronical fatigue syndrome (CFS) (Behan *et al.* 1990; Theander *et al.* 2002), osteoporosis (Kruger and Horrobin 1997; Kruger *et al.* 1998), alcoholism (Horrobin 1984b; Horrobin 1987; Corbett *et al.* 1991), menopausal symptoms (Chenoy *et al.* 1994), premenstrual symptoms (Horrobin 1983; Horrobin and Brush 1983; Khoo *et al.* 1990; Budeiri *et al.* 1996; Bendich 2000), (atopic) eczema (Schalin-Karrila *et al.* 1987; Morse *et al.* 1989; Fiocchi *et al.* 1994; Worm and Henz 2000; Yoon *et al.* 2002), allergies (Wakai *et al.* 2001), rheumatoid arthritis (Joe and Hart 1993; Belch and Hill 2000; Leventhal *et al.* 1993; Calder and Zurier 2001, Darlington and Stone 2001), attention deficit/hyperactivity disorder (Arnold *et al.* 1989), cancer (Davies *et al.* 1999, Kenny *et al.* 2000, Menendez *et al.* 2001), migraine (Wagner and Nootbaar-Wagner 1997), weight loss (Garcia *et al.* 1986), high blood pressure and heart disease (Frenoux *et al.* 2001), ulcers (al-Sabanah 1997), Raynaud´s syndrome (Belch *et al.* 1985), epilepsy (Puri 2007), multiple sclerosis (Cunnane *et al.* 1989) and HIV infections (Mpanju *et al.* 1997). Because of the possible broad field of applications, research in the genus *Oenothera* is important and marker systems will provide important progress in its breeding.

4.10 FISH AS TOOL TO DETECT CHROMOSOME ARM COMBINATIONS PRESENT IN *OENOTHERA*

Chromosomes in *Oenothera* lineages differ in their chromosome arm combinations which are the result of frequently observed reciprocal translocation events. Thus, the genetic behavior of unanalyzed *Oenothera* lineages in plant breeding or simple crossing experiments is generally unknown. In the past, all complexes used, egg cell complexes and pollen complexes, were analyzed separately by means of crossing experiments, based on combination with complexes with known chromosome configurations (listed in Cleland 1972) and following cytological analyses of chromosome patterns during meiosis (Cleland 1972). The relatively long generation time of *Oenothera* (annual herb) already implies that this approach is time-consuming and requires experienced personnel.

Fluorescent *in situ* hybridization (FISH) using chromosome arm specific probes solves and fastens this procedure and represents a complementary approach to arm-specific marker technology. Because of this significant relief, the implementation of this method to *Oenothera* research could again be a substantiated improvement in the history of this genus. The first data achieved were promising since chromosome arm specific signals could be obtained (Figs. 3-21 and 3-22). Still, the results were limited to a small number of metaphase chromosomes. In order to fully exclude the possibility of artifacts improvement of this method needs to be established to reproduce the signals with a higher frequency. As soon as this will have been managed, it is an easy to assign the probe to the appropriate chromosome arm by means of simple mapping. In addition, *Oenothera* chromosomes could be characterized by C-banding or karyotyping that both were also began in cooperation with Dr. Hieronim Golczyk (Krakow, Poland). C-banding, a selective chromosome stain with Giemsa that presents heterochromatic regions, is of especial interest in view of the lack of homologous recombination and chromosome condensation in evening primroses.

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6. Summary

Research on the genus *Oenothera* (evening primroses) exists since more than a century. A large variety of species and lineages were collected and analyzed during this period which differed substantially in their genetical behavior due to terminal or partial translocation heterozygosity; bivalent forming strains exist as well. Translocation heterozygosity, biparental transmission of plastids, fertility of hybrids and a general interfertility of species allow sexual exchanges of plastids, of individual or more chromosomes and of entire genomes. The exchange of plastids or of entire genomes requires lines with a combination of two chromosome complexes which form a ring (⊙14) of alternating parental chromosomes in meiosis. Such heterozygous plants are considered to inherit entire haploid chromosome sets without intermixing, except of a negligible degree at telomeres. Interpecific plastome-genome combinations, in turn, result in incompatible phenotypes which reflect a lack of co-evolution between newly combined organelles, presumably due to gene-pairs, as described by the Dobzhansky-Muller model. Up to date, research on evening primroses, notably homologous recombination and organelle exchanges between species was performed exclusively with cytological methods and/or phenotypical markers, but quite often segregation analyses resulted in only altered, atypical Mendelian segregation ratios.

The aim of this thesis was to develop molecular strategies for the classical genetic model *Oenothera*, notably to establish molecular marker technologies for genome and plastome and to investigate homologous recombination events in bivalent forming and translocation heterozygous species (AFLP technology combined with co-dominant markers). The AFLP technology was employed to (i) generate first linkage maps for the A and B genomes in subsection *Oenothera* using bivalent forming species and (ii) to verify a lack of homologous recombination in translocation heterozygotes. Remarkably, homologous recombination events were strictly limited to telomeric regions, not only in translocation heterozygous, but also in bivalent forming species. (iii) Cytological investigations of meiotic prophases uncovered an untypical, higher-order organization, predominantely condensation, of all chromosomes during meiosis as a most probable and general reason for the lack of homologous

recombination in *Oenothera*. (iv) Although species without homologous recombination are generally seen as evolutionary dead-ends, the role of the cosmopolit *Oenothera* as an exception in this case is being discussed referring to the sense of sex that is generally considered as synonymous to homologous recombination from a genetic perspective. (v) In parallel, marker systems were generated for both, nuclear genomes (marker allele M40) and (sub-)plastomes (marker allele *rrn*16-*trn*lGAU). (vi) General application of nuclear markers requires knowledge of chromosomal formulas of complexes. Up to now, determinations of floating chromosomal formulas in *Oenothera* species, reflecting diverse reciprocal translocations of entire chromosome arms, were time-consuming. To speed up this procedure significantly, a fluorescent *in situ* hybridization (FISH) technique with chromosome arm-specific probes was developed with promising perspective. (vii) Potential candidate genes in the plastome, causing the *virescent* phenotype of AA-III, were narrowed down by comparative molecular biological, biochemical and bioinformatical analyses of all five basic distinguishable plastome sequences (I – V). (viii) An AFLP mapping approach, in turn, revealed two coupling groups, 4 and 7, that are involved in the AA-III incompatibility. These two coupling groups must interact since a BB genotype in one of the groups is able to reverse the incompatible phenotype.

7. APPENDIX:

7.1 DETAIL INFORMATION ABOUT AFLP MARKERS ASSIGNED IN INTERGRATED GENETIC MAP

Coupling Group 1 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm281_453.6	0.000	27	126	88	31.4	*******	[a:h:b]	54.85
sm299_103.7	7.331	27	128	86	29.8	*******	[a:h:b]	87.76
sm299_179.8	8.155	27	124	93	35.8	*******	[a:h:b]	96.74
sm261_118.4	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_328.9	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm280_378.9	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_395.7	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm281_116.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm290_52.2	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm285_416.7	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm285_216.3	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm276_394.3	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
am280_377.9	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm276_90.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm281_193.2	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm280_441.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm279_120.0	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm276_216.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm280_221.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm279_412.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm267_372.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_43.9	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_367.1	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm279_341.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm299_345.6	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm299_271.4	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm281_312.3	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_397.0	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_321.7	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm285_375.7	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm276_334.2	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm285_73.4	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm290_129.0	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm285_220.9	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm267_344.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14

continued

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm261_134.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm276_426.2	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm279_137.9	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm290_391.7	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm280_116.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm279_170.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm290_390.5	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm276_89.8	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
sm263_493.7	8.358	27	125	92	34.8	*******	[a:h:b]	99.14
am261_246.8	9.182	27	125	92	34.8	*******	[a:h:b]	90.50
sm263_67.5	9.593	27	125	92	34.8	*******	[a:h:b]	87.25

Coupling Group 2 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm263_92.7	0.000	50	118	75	5.3	*	[a:h:b]	47.75
sm261_91.5	6.270	52	118	73	3.8	-	[a:h:b]	42.87
sm276_104.5	8.736	51	117	75	5.1	*	[a:h:b]	95.02
sm263_233.5	10.121	51	119	74	4.5	-	[a:h:b]	108.17
sm261_325.3	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm263_283.7	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm267_267.5	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm263_130.4	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm299_212.8	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm281_235.2	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm279_54.3	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm261_431.2	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm263_279.4	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm261_70.0	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm267_57.2	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm267_171.8	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm267_122.7	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm279_167.5	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm280_140.2	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm290_432.7	10.322	51	119	74	4.5	*	[a:h:b]	108.37
sm280_53.5	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm279_199.1	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm279_102.0	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm280_141.6	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm276_461.8	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm279_79.1	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm280_125.1	10.322	51	118	75	5.0	*	[a:h:b]	108.37

continued

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm276_415.2	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm280_115.2	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm281_282.1	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm299_255.3	10.322	51	118	75	5.0	*	[a:h:b]	108.37
sm280_78.7	11.553	51	118	75	5.0	*	[a:h:b]	97.35
sm261_89.5	12.836	54	113	74	4.3	-	[a:h:b]	89.51
sm267_73.5	19.776	51	120	70	3.0	-	[a:h:b]	30.99

Coupling Group 3 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm261_143.0	0.000	71	157	16	44.9	*******	[a:h:b]	65.38
sm280_304.2	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm280_372.8	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm290_285.9	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm276_212.3	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_77.0	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm280_42.1	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_385.5	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm281_150.4	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm290_346.1	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm261_315.1	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm281_60.8	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_163.9	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_369.0	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm280_390.9	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm281_174.7	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm267_251.4	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_383.6	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_175.4	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm279_151.6	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm276_452.1	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm267_58.5	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm267_146.6	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm267_101.0	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm290_179.4	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm299_96.4	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm261_389.1	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm285_377.7	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm276_174.6	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm263_239.4	6.802	71	124	49	4.0	-	[a:h:b]	106.73
sm267_39.5	7.606	71	128	45	6.1	**	[a:h:b]	99.00

continued

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm276_272.8	13.640	71	157	16	44.9	*******	[a:h:b]	65.38

Coupling Group 4 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm263_192.6	0.000	56	134	54	2.4	-	[a:h:b]	73.01
sm263_117.9	4.754	57	126	61	0.4	-	[a:h:b]	98.71
sm261_223.4	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm299_121.2	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm267_240.0	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm263_196.2	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm281_133.1	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm276_388.8	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm261_478.7	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm267_225.7	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm281_166.9	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm285_370.0	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm285_371.1	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm299_61.0	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm299_478.1	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm299_206.2	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm299_220.8	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm261_349.6	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm280_478.9	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm261_49.1	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm299_481.9	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm263_88.0	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm290_341.2	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm263_351.6	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm276_422.3	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm276_196.0	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm290_108.5	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm281_88.1	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm263_200.2	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm281_441.0	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm285_474.6	5.525	57	126	61	0.4	-	[a:h:b]	106.93
sm290_105.5	5.698	57	126	60	0.4	-	[a:h:b]	106.93
sm279_143.7	6.723	57	126	61	0.4	-	[a:h:b]	95.58
sm279_211.1	6.955	57	121	64	0.4	-	[a:h:b]	98.01
sm280_89.9	11.794	57	125	60	0.3	-	[a:h:b]	73.10

continued

Coupling Group 5 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm267_184.9	0.000	65	125	53	1.4	-	[a:h:b]	65.19
sm263_97.4	7.113	69	121	54	1.9	-	[a:h:b]	89.88
sm281_433.6	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm267_42.6	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm263_263.2	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm290_214.9	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm280_47.2	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm263_332.8	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm290_246.2	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm290_301.1	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm267_103.3	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm267_174.3	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm279_390.5	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm280_43.1	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm281_131.0	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm285_447.9	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm267_159.3	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm276_424.5	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm285_170.0	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm290_166.4	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm290_210.0	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm280_154.0	9.244	66	124	54	1.3	-	[a:h:b]	107.35
sm280_262.3	12.512	65	117	60	0.5	-	[a:h:b]	85.10
sm267_286.0	17.906	65	128	51	2.2	-	[a:h:b]	63.82

Coupling Group 6 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm280_193.2	0.000	26	122	96	40.2	*******	[a:h:b]	90.28
sm263_55.5	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm280_224.3	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm299_228.7	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm280_317.0	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm279_328.0	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm263_60.6	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm299_71.5	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm263_184.6	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm263_185.6	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm261_458.7	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm261_128.8	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm261_176.1	1.866	35	113	96	31.8	*******	[a:h:b]	104.23

continued

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm276_289.1	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm261_184.7	1.866	35	113	96	31.8	*******	[a:h:b]	104.23
sm285_79.8	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm267_61.4	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm299_70.5	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm279_156.6	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm267_341.7	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm267_230.0	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm299_277.6	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm263_99.8	1.866	35	112	96	32.1	*******	[a:h:b]	104.23
sm281_432.2	7.002	35	117	88	23.6	*******	[a:h:b]	70.00

Coupling Group 7 (integrated genetic map)

Locus	Position	a	h	b	X2	Signif.	Classes	LOD
sm285_106.9	0.000	52	135	56	3.1	-	[a:h:b]	93.00
sm276_40.8	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm279_274.9	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm267_79.2	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm299_197.8	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm285_74.3	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm285_336.1	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm299_164.4	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm299_311.3	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm263_337.9	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm280_439.7	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm281_355.6	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm267_97.8	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm276_262.0	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm276_84.6	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm280_252.8	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm280_59.5	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm285_334.0	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm290_270.4	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm280_129.8	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm290_66.6	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm279_290.9	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm263_296.1	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm263_288.0	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm290_394.7	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm281_220.1	1.207	52	132	60	2.2	-	[a:h:b]	104.73
sm285_365.2	2.211	52	136	54	3.8	-	[a:h:b]	94.55

7.2 LIST OF PUBLICATIONS

Parts of this work have already been published or are in preparation to be published:

Mráček J, Greiner S, Cho WK, **Rauwolf U**, Braun M, Umate P, Altstätter J, Stoppel R, Mlčochová L, Silber MV, Volz SM, White S, Selmeier R, Rudd S, Herrmann RG, and Meurer J **(2006)** Construction, database integration, and application of an *Oenothera* EST library. *Genomics*. 88: 372-380.

Greiner S, Wang X, **Rauwolf U**, Silber M, Maier K, Haberer G, Meurer J, and Herrmann RG **(2008)** The complete nucleotide sequences of the five genetically distinct plastid genomes of *Oenothera*, subsection *Oenothera*: I. Sequence evaluation and plastome evolution. *Nucleic Acids Research*. 36: 2366-2378.

Greiner S, Herrmann RG, Wang X, **Rauwolf U**, Mayer K, Haberer G, and Meurer J (2008) The complete nucleotide sequences of the five genetically distinct plastid genomes of *Oenothera*, subsection *Oenothera*: II. A view on microevolution. Submitted.

Golczyk H, Musiał K, **Rauwolf U**, Meurer J, Joachimiak AJ, Herrmann RG, and Greiner S (2008) Meiotic events in *Oenothera* – a non-standard pattern of chromosome behaviour. Submitted.

Rauwolf U, Golczyk H, Meurer J, Herrmann RG, and Greiner S **(2008)** Molecular marker systems for *Oenothera* genetics. Submitted.

Rauwolf U, Greiner S, Braun M, Golczyk H, Mracek J, Mohler V, Herrmann RG, and Meurer J **(2008)** Sexual reproduction with homologous recombination limited to telomeric regions of chromosomes. In preparation.

Greiner S, **Rauwolf U**, Meurer J, and Herrmann RG (2008) The impact of plastids on speciation. In preparation.

Further publication by the author of this thesis:

Rauwolf U, Golczyk H, Greiner S, and Herrmann RG (2008) Variable amounts of DNA related to the size of chloroplasts: II. Biochemical determination of DNA amounts per organelle and cell. In preparation.

7.3 EHRENWÖRTLICHE VERSICHERUNG

Ehrenwörtliche Versicherung

Hiermit versichere ich, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe.

Zudem erkläre ich, dass ich keine früheren Promotionsversuche unternommen habe.

München, 20.06.2008

(Uwe Rauwolf)

7.4 *CURRICULUM VITAE*

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1989 – 1998	***Gisela-Gymnasium*** in München Abschluss: Allgemeine Hochschulreife

Beruflicher Werdegang

1998 – 1999	Zivildienst (Caritas in Unterschleißheim)
08/2002 – 07/2003	Labortätigkeit bei IMGM (Labor für medizinische Genetik; Dr. Klein, Dr. Wölpl)
07/2003 – 04/2005	studentische Hilfskraft der Ludwig-Maximilians-Universität in München (physiologische Botanik, Prof. Dr. Herrmann)
seit 10/1999	***Ludwig-Maximilian-Universität München*** *Studium der Biologie*
04/2002 – 03/2005	• Hauptstudium der Fächer Physiologische Botanik im Hauptfach, sowie Systematische Botanik, Humangenetik und Pharmakologie/Toxikologie im Nebenfach
04/2004 – 04/2005	• Erstellung des experimentellen Teils der Diplomarbeit
04/2005	• Erwerb des Diploms
	• Diplomarbeit: Analyse von *Oenothera*-Genomen; Kartierung mit AFLP-Markern und Expressionsanalyse (Etablierung von EST-Banken und Macro-„arrays“)
seit 04/2005	• Dissertation am Lehrstuhl Botanik I, Department Biologie I bei Prof. Dr. Reinhold G. Herrmann

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