

# Chapter 11

## Plastome Mutants of Higher Plants

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### Summary

This chapter provides an overview of higher plant plastome mutants and their application in molecular biology, cytoplasmic genetics and biotechnology. Starting from an outline on plastid inheritance, the sources of mutants, methods of their maintenance and molecular approaches to identify the underlying genetic changes are presented. Subsequently, the

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molecularly characterized plastome mutants and their impact on our current knowledge about plastids are summarized. Recent developments in genomics will likely overcome technical limitations connected with the elucidation of mutant loci in plastome mutants. The great potential of plastid mutants in future research, for example in studying plastid gene regulation, as well as suitable model plants and available genetic resources are discussed.

## I. Introduction

Plastome mutants can occur spontaneously, or can be induced by either chemical or nuclear-gene-mediated mutagenesis. Alternatively, they can be directly generated by plastid transformation (Kutzelnigg and

Stubbe 1974; Börner and Sears 1986; Hagemann 1992; Bock 2001; Sect. III). Classic plastid mutants are recognized as bleached leaf areas in variegated plants (Fig. 11.1 and Sect. II). Such material has been used as a genetic tool since the very beginning of formal genetics. Baur's and Corren's fundamental work on plastome mutants in *Mirabilis jalapa* (four o'clock flower) *Pelargonium zonale* and *Antirrhinum majus* (snapdragon) laid the foundation for cytoplasmic genetics (Baur 1909; Correns 1909; Baur 1910; Kirk and Tilney-Bassett 1978; Hagemann 2010). Since then, plastome mutants have provided compelling evidence for the existence of an independent genetic system in plastids (Baur 1909; Renner 1934; Hagemann 2010), and they still play an important role in elucidating the rules of non-Mendelian inheritance (Sect. II). The use of plastome mutants facilitated the initial development of chloroplast transformation technology in *Chlamydomonas reinhardtii* (Boynton et al. 1988). Also, plastid mutants provide selectable markers for antibiotic or herbicide resistances that are widely used in molecular biology and agriculture (Sect. VI). However, primarily due to technical limitations, the contribution of plastome mutants to illuminating gene content and gene functions in plastids was meager (Sect. V). Nonetheless, plastome mutants keep playing important roles in molecular research on plastids. Some mutants display particularly interesting developmental phenotypes (Sect. VI), and/or genetic patterns beyond the classic rules of plastid genetics (Sect. VIII). Next-generation Sequencing (NGS) technologies employed for mapping and identification of plastome mutants may herald a renaissance of this field. The aim of this chapter is to summarize the currently

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*Abbreviations:* AA – Amino acid; *accD* – Acetyl coenzyme A carboxylase subunit D gene; *aadA* – Aminoglycoside 3-adenylyltransferase gene; AtMSH1 – *Arabidopsis thaliana* MutS homolog 1; *AtMsh1* – *Arabidopsis thaliana* MutS homolog 1 gene; *atp* – ATP synthase subunit gene; *AtWhy* – *Arabidopsis thaliana* WHIRLY protein; bp – Base pair; *clpP* – Chloroplast caseinolytic protease subunit P gene; CMS – Cytoplasmic male sterility; *cpRecA* – Chloroplast RecA gene; dsDNA – Double-stranded DNA; EMS – Ethyl-methanesulfonate; IF1 – Translation initiation factor 1; indel – Insertion/deletion; InfA – Translation initiation factor A; *infA* – Translation initiation factor A gene; *matK* – Maturase K gene; MNNG – Methyl-nitro-nitrosoguanidine; N/A – not available; NGS – Next-generation Sequencing; NMU – *N*-nitroso-*N*-methyl-urea; PEG – Polyethylene glycol; *petB* – Cytochrome *b<sub>6</sub>/f* subunit B gene; PGI – Plastome-genome incompatibility; PQ – Plastoquinone; PS I – Photosystem I; PS II – Photosystem II; *Psa* – Photosystem I subunit; *psa* – Photosystem I subunit gene; *Psb* – Photosystem II subunit; *psb* – Photosystem II subunit gene; ptDNA – Plastid DNA; *rbcL* – Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit gene; RbcL – Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit; RbcS – Ribulose-1,5-bisphosphate carboxylase oxygenase small subunit; RFLP – Restriction length polymorphism; *rps12* – Ribosomal protein small subunit 12 gene; RuBisCO – Ribulose-1,5-bisphosphate carboxylase oxygenase; SAM – Shoot apical meristem; TILLING – Targeting induced local lesions in genomes; *trnE(UUC)* – tRNA-Glu (anticodon UUC); UV – Ultraviolet; *ycf* – Hypothetical chloroplast reading frame; *ZmWhy* – *Zea mays* whirly gene

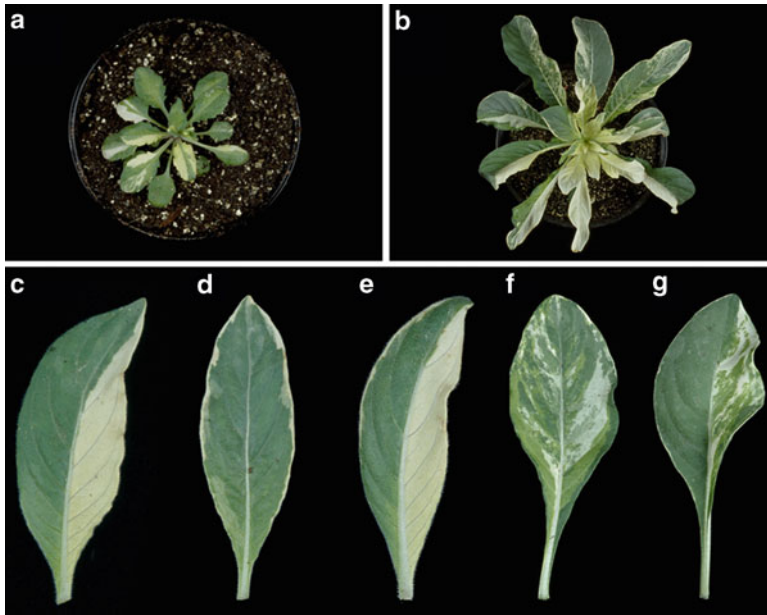


Fig. 11.1. Sorting-out of genetically distinct plastids and variegation patterns in *A. thaliana* (a) and *O. elata* (b–g). Variegation in plant rosettes produced by EMS mutagenesis (a) or sexual crosses of mutated (*pale*) and wild type (*green*) plastid genotypes (b–g). Different sorting-out patterns in leaves (c–g): Sectorial chimera (c), periclinal chimera (d), mesoclinal chimera (e), mosaic pattern (f), and combination of the former types (g). Note that *light green* tissue results from overlaying cell layers as produced by adaxial and abaxial sorting-out (f, g).

existing literature on plastid mutants, to evaluate promising applications in molecular genetics and to outline perspectives for future research.

## II. A Brief Survey of Plastid Genetics

Non-Mendelian inheritance was described already shortly after the rediscovery of Mendel's laws. It differs fundamentally from Mendelian inheritance and is characterized by a predominantly maternal inheritance recognized as reciprocal differences in sexual crosses, somatic segregation of divergent organelle genotypes and by a virtual absence of homologous recombination of the DNAs involved. Plastome mutants were the major tools in elucidating the rules for cytoplasmic inheritance. The following paragraphs briefly summarize the most relevant aspects.

### A. Transmission of Plastids

Mostly based on work with pale plastome mutants, it appeared that plastids can undergo three different modes of inheritance: maternal (initially referred to as *status albomaculatus*), biparental (*status paralbomaculatus*) or paternal. Well-known examples for maternal inheritance are *Nicotiana tabacum* (tobacco), *A. majus* and *Arabidopsis thaliana*. Biparental transmission is best studied in *Oenothera* (evening primrose) and *Pelargonium*. Paternal transmission or a strong paternal bias was observed for gymnosperms and the angiosperm *Medicago sativa* (alfalfa), respectively (Hagemann 1964, 1992, 2004; Grun 1976; Gillham 1978; Kirk and Tilney-Bassett 1978). The predominant mode of plastid inheritance in seed plants is the maternal pattern, but low-level or occasional biparental inheritance may be present in about 1/3 of all plant taxa studied

(Mogensen 1996). More and more evidence accumulates, that uniparental inheritance is often not absolute, and that paternal leakage (resulting in heteroplasmy) may be a general feature of higher plant populations (e.g., Birky 2001; Wolfe and Randle 2004; Petit and Vendramin 2007). Plastid mutants or plastids genetically modified by transformation, served in a variety of plant species as visible phenotypic or selectable markers in the analysis of sexual crosses to screen for paternal leakage events. In contrast to direct investigations of the DNA inherited, plastome mutants allow the easy, large-scale, and unambiguous identification of rare paternal transmission events of the chloroplast genome (Hagemann 1992; Azhagiri and Maliga 2007; Ruf et al. 2007; Svab and Maliga 2007; and citations therein).

### *B. Sorting-Out and Variegation*

Biparental transmission of genetically distinct plastids produces a zygote harboring two plastid types, a so-called mixed cell. During subsequent cell divisions, maternal and paternal plastids are randomly distributed to the daughter cells. After several division cycles, mixed cells disappear and cell lineages containing only one of the two plastid genotypes form. If one of the plastid types is marked by a mutation primarily or secondarily impairing photosynthesis, variegated plants containing mutated and wild-type plastids in distinct tissues appear (Fig. 11.1). Sorting-out takes place in each tissue and developmental stage of an individual plant. Consequently, after de novo mutation of a single plastid genome, sorting-out begins during the first subsequent cell division cycle, producing a mosaic pattern of leaf variegation with sharp tissues borders after completion of the sorting-out process (Fig. 11.1a, b, f, g). Along this border, mixed cells can be found. The concept of sorting-out is a fundamental feature of non-Mendelian inheritance, and its theoretical properties were originally worked out by Michaelis (1955) utilizing pale plastome mutants (Hagemann 1964; Kirk and Tilney-Bassett

1978; Birky 2001). In dicotyledonous plants, depending on plastid distribution in the meristem, completed sorting-out can lead to different types of variegation and (leaf) chimeras: (1) Sectorial chimeras, in which the three layers (L1–L3) of the shoot apical meristem (SAM) carry mutated and non-mutated cells. Sorting-out can lead to different genetic identities of the two sides of the organ axis. In the leaf, the genetically different tissues are arranged in lateral sectors (Fig. 11.1c). (2) Periclinal chimeras, in which sorting-out gives rise to at least one homoplasmic layer of the three meristematic cell layers [tunica (L1 and L2) and corpus (L3)]. The individual layers differ genetically and consequently epidermis (L1), subepidermal cell layer (L2; phenotypically apparent in the leaf margin) and/or corpus (L3) have different genetics identities as far as their plastids are concerned (Fig. 11.1d). (3) Mesoclinal chimeras represent a combination of sectorial and periclinal chimerism (Fig. 11.1e). (4) Mosaic patterns are observed if sorting-out is not completed within a leaf and/or in distinct domains of the meristem. This is typically the case in early plant development (Fig. 11.1f). (5) Finally, ad- and abaxial sorting patterns are possible as well as a combination of all sorting-out patterns mentioned above (Fig. 11.1f, g). For graphical presentations of variegation patterns in the SAM and its genetic and phenotypic consequences, see Kirk and Tilney-Bassett (1978). In monocotyledonous plants, where the leaf basal meristem continuously mediates proximal growth, variegation patterns are recognized as striping, since cells of different genetic identities are arranged in parallel (Kirk and Tilney-Bassett 1978).

### *C. Identification of Plastome Mutants by Means of Classic Genetics*

The challenge of identifying plastome mutants by employing classic genetic methods is not obvious at first glance. Variegation, a first indication of the presence of a plastome mutation, can also be caused by various nuclear alleles or by mitochondrial dysfunction

(Kirk and Tilney-Bassett 1978; Rodermel 2002; Yu et al. 2007). Although sorting-out of plastids results in definable intercalated patterns (Michaelis 1957, 1958a, b; cf. Fig. 11.1f), non-Mendelian inheritance must be proven to demonstrate the cytoplasmic origin of a mutation. In the case of uniparental transmission, this is evident by maternal inheritance in reciprocal crosses. In plants with biparental plastid transmission, confirming variegation, sorting-out and non-Mendelian segregation in the F1 generation represent accepted methods (Kirk and Tilney-Bassett 1978; Hagemann 1982). To rule out mitochondrial mutations, mixed cells containing both mutated and wild-type plastids need to be identified. In terms of classic genetics, together with the demonstration of sorting-out, this is a strong indication of the presence of a plastome mutation. It illustrates that the cause of the impaired plastid phenotype rests within the plastid itself. However, strictly speaking, even in this case the presence of a mitochondrial mutation cannot be excluded. The mixed cell looked at may be still heteroplasmic for a mitochondrial mutation, secondarily leading to a plastid malfunction (cf. Kirk and Tilney-Bassett 1978; Sect. III).

#### *D. Competition of Plastids with Genetically Different Plastome Types*

In *Oenothera* species, plastid genomes marked by mutation uncovered different multiplication rates in sexual crosses, depending on the plastid genotype. Based on the “variegation value” of F1 seedling populations, Schötz and co-workers measured the relative strengths of diverging plastome types to each other. For the five genetically distinguishable plastome types in *Oenothera*, three different multiplication speeds (fast, medium, and slow) could be identified. Refined analysis revealed that the competitive advantage of a given plastome is largely independent of the nucleus and can even exist if the more competitive plastid genotype is incompatible to the host plant. In extreme cases, biparental transmission can be suppressed by a

combination of a “fast” and a “slow” plastome, if incompatible plastome/genome combinations are involved. Consequently, at least in *Oenothera*, the determinants mediating plastid competition seem to be predominantly plastome encoded (Schötz 1954; Grun 1976; Gillham 1978; Kirk and Tilney-Bassett 1978; Chiu et al. 1988; Chiu and Sears 1993; Harte 1994). Although data are limited, different multiplication rates are probably an intrinsic feature of the plastid genome and a general phenomenon in nature. Comparable results were obtained in *M. sativa* using cybrids in cell culture (Fitter and Rose 1993) and some evidence also exists for *Pelargonium* (Hagemann and Scholze 1974; Hagemann 1976; Abdel-Wahab and Tilney-Bassett 1981). However, mode and control of biparental inheritance in the genus *Pelargonium* strikingly differs from that in *Oenothera* (Tilney-Bassett 1975; Kirk and Tilney-Bassett 1978; Tilney-Bassett 1994) and, clearly, the findings obtained in *Pelargonium* require further investigation (Tilney-Bassett 1994). The detection of different plastome replication rates in *Oenothera* contributed substantially to the hypothesis of selfish cytoplasmic elements (Grun 1976; cf. Hoekstra 2000; Barr et al. 2005). However, the underlying loci, most probably origins of replication (Hornung et al. 1996; Sears et al. 1996), have not yet been identified. In this regard, the identification of slower or faster multiplying plastome mutants could represent a viable approach.

#### *E. Sexual Recombination of Different Plastome Types*

Higher plant plastids seem not to undergo sexual recombination, not even in plant taxa transmitting chloroplast genomes regularly by both sexes. Chiu and Sears (1985) performed a study with *Oenothera* using two independent plastome mutants, which were crossed with 10 different other plastome mutants. In the 20 F1 generations (each heteroplasmic for a different pair of mutations), a total of about 7,500 seedlings were raised. Recombination events were expected to



result in the appearance of green leaf spots in the seedlings and would indicate a rescue of a mutant by recombination. Since such an event was not observed, recombination between different plastome types is either completely absent or present at only very low frequencies (cf. Kutzelnigg and Stubbe 1974). However, in the Chiu and Sears study some recombination events may have escaped detection due to incomplete sorting-out, the physiological state of some of the mutants (prohibiting re-greening after a genetic complementation), or close genetic linkage. The virtual absence of sexual recombination in higher plant plastids is indeed surprising, since homologous recombination between single plastid genomes within a plastid is quite frequent (e.g., Palmer 1983; Day and Madesis 2007). At least occasionally, it also can be induced in somatic cybrids generated by protoplast fusion. For instance, the *Nicotiana plumbaginifolia* line SR1-A15 carries two plastome mutations, one mediating streptomycin resistance and the other greening deficiency. A second line (LR400) is resistant to lincomycin, also due to a plastome mutation, but is normally green. Protoplasts of the cell lines were mixed, fused in the presence of polyethylene glycol (PEG), and subsequently selected for green calli on streptomycin-containing media. Regenerated green lines were supposed to carry a recombinant plastome from the streptomycin-resistant (white) and lincomycin-resistant (green) plastids. Double selection of the cybrid plants on medium containing both streptomycin and lincomycin as well as RFLP analysis confirmed this assumption (Medgyesy et al. 1985). Comparable data were independently obtained in similar experiments, including interspecific protoplast fusions (e.g., Thanh and Medgyesy 1989; Trabelsi et al. 2005; Bidani et al. 2007; but also see Petit and Vendramin 2007). These results show that higher plant plastids can, in principle, undergo recombination of different genotypes. At least, this is possible under strong selection and in a tissue culture system including PEG, which might have induced artificial plastid fusion. That plastid fusion is the critical prerequisite for sexual recombination of

plastid DNA (ptDNA) is evident from work on the isogamous green alga *Chlamydomonas*. In this organism, the two chloroplasts of the crossing mates ( $mt^+$  and  $mt^-$ ) fuse in the zygote. Typically the  $mt^-$  ptDNA is degraded (>90%), but UV irradiation of the  $mt^+$  parent can greatly increase the frequency of heteroplasmic zygotes. This strategy, together with various antibiotic-resistant plastome mutants, was particularly useful for constructing recombination maps of the plastid genomes of *Chlamydomonas* species (Gillham 1978; Gillham et al. 1991; Boynton et al. 1992). Therefore, the explanation for the apparent absence of homologous recombination between plastomes of different genotypes in sexual crosses of higher plants may lie in the lack of plastid fusion in the germline (Meyer and Stubbe 1974; Kirk and Tilney-Bassett 1978; Sears 1980; Kuroiwa 2010; Nagata 2010). There is also only limited evidence for plastid fusion in somatic tissue (Esau 1972; Sears 1980; Vaughn 1981). In contrast, some theoretical and circumstantial phylogenetic evidence indicates the presence of sexual recombination of plastid genomes (cf. Hagemann 1992; Birky 1995; Wolfe and Randle 2004; Barr et al. 2005; Petit and Vendramin 2007; Greiner et al. 2011). The true situation in natural populations of higher plant species remains unclear and needs further investigation.

#### F. Plastid Restitution

Plastid restitution is defined as the re-greening of a mutated plastid by an additional stable mutation. With this strict definition, plastid restitution does not necessarily require a reversion or suppressor mutation within the plastome itself. Classic genetic problems associated with the identification of restitution events, such as uncompleted sorting-out, have been discussed previously (Tilney-Bassett 1975; Kirk and Tilney-Bassett 1978). Additional examples have come from *P. zonale* (spontaneous restitution), *Oenothera* (mutated by a nuclear plastome mutator allele), *Helianthus annuus* (second-site chemical mutagenesis and

spontaneous mutants), and presumably also *Hordeum vulgare* (barley), caused by a plastome mutator (Abdel-Wahab and Tilney-Bassett 1981; Johnson et al. 1991; Prina 1992; Usatov et al. 2004). Work on *Epilobium hirsutum* suggests that restitution events can lead to changes in plant or plastid morphology compared to the original wild type (Michaelis 1969). Michaelis could further demonstrate the overcoming of hybrid incompatibility and sterility by a restituted plastid (Michaelis 1969; Kirk and Tilney-Bassett 1978). However, it is worth mentioning that the material Michaelis used in his restitution experiments was generated under the influence of the nuclear plastome mutator allele *mpl*, for which circumstantial evidence suggests an additional cytoplasmic (likely mitochondrial) mutator activity (Sect. III). Unfortunately, none of the published restitution events was characterized at the molecular level, although the phenomenon clearly deserves systematic studies. In line with a high plastome copy number and small genome size (Bock 2007b) and depending on the original mutation, restitution can be rather frequent. Occasionally, restitution depends on environmental factors, indicating no “full” rescue to the original wild-type plastome (Michaelis 1969; Tilney-Bassett 1975; Kirk and Tilney-Bassett 1978). Elucidating suppressor relations between partially plastid-encoded supramolecular machineries, such as the thylakoid membrane system or the organellar ribosomes, and/or between chloroplast and mitochondrial genomes provides fascinating opportunities for further research.

A special case of plastid restitution is the rescue of plastome-genome incompatibility (PGI). These speciation-relevant barriers are the result of a co-evolution between the plastid and the nuclear genome, often leading to bleached chloroplasts in an alien nuclear background (reviewed in Levin 2003; Greiner et al. 2011). Mutagenesis of incompatible tissue and screens for restitution events can potentially become a general method to pinpoint plastome-encoded determinants for PGI. Utilizing chemical mutagenesis, the

albinotic phenotype of a plastome-genome incompatible cybrid between the nuclear genome of *Atropa belladonna* (deadly nightshade) and the plastome of *N. tabacum* was cured. Analysis of relevant plastid loci revealed a single base pair exchange within the *atpA* gene, mimicking a species-specific editing site for the *N. tabacum* plastome (Schmitz-Linneweber et al. 2005).

### III. Sources of Plastome Mutants

As outlined above, plastid mutants were used as an important tool for the analysis of non-Mendelian genetics. What sources of such mutants are available to researchers? The following section provides an overview of the three different origins of mutants: spontaneous occurrence, chemical mutagenesis and nuclear-gene induced mutations by plastome mutator alleles (Kutzelnigg and Stubbe 1974; Kirk and Tilney-Bassett 1978; Börner and Sears 1986; Hagemann 1992).

#### A. Spontaneous Occurrence

Spontaneous plastome mutations are known from several plant species (Sect. VI; Table 11.1 and citations therein). Published values for rates of spontaneously arising chlorophyll deficiency observed by variegation or striping vary between 0.006% and 0.3% in *A. majus*, *A. thaliana*, *Epilobium*, *H. vulgare* and *Oenothera* (Maly 1958a; Röbbelen 1962; Michaelis 1964; Kutzelnigg and Stubbe 1974; Kirk and Tilney-Bassett 1978; Prina 1992). Inconsistent values between species and experiments are presumably caused by different experimental setups and/or selection criteria, such as plant size and age. It is important to note that the approach systematically misinterprets plastome mutation rates, since only mutants with chlorotic phenotypes and completed sorting-out are usually recognized (cf. Michaelis 1958a; Kutzelnigg and Stubbe 1974; Kirk and Tilney-Bassett 1978). In general, plastome mutation rates are about two-fold lower than those of the nuclear genome (Wolfe

Table 11.1. Plastome mutants with impaired plastid gene function<sup>a</sup>

Plastid function	Organism/variety	Mutant line	Gene/locus	Mutation	Phenotype	Origin	Reference
Photosynthetic light reaction	<i>Antirrhinum majus</i> Sippe 50	en:alba-1	<i>psaB</i>	Transversion; premature stop codon	Yellowish white	Spontaneous	Schaffner et al. (1995)
Photosynthetic light reaction	<i>A. majus</i> Sippe 50	en:alba-4	<i>psbD</i>	Transition; single AA-exchange	Yellowish	NMU	Schaffner (1995)
Photosynthetic light reaction	" <i>Oenothera suaveolens</i> " <sup>b,c</sup>	II-gamma	<i>psbE</i>	5 bp duplication, premature stop codon	Light green to yellowish white	Spontaneous	Hupfer (2002)
Photosynthetic light reaction	" <i>O. suaveolens</i> " <sup>b,c</sup>	II-theta	<i>petB</i> intron	2 bp deletion, splicing deficiency	Light green to white	Spontaneous	Hupfer (2002)
Carbon fixation	" <i>O. hookeri</i> " var. hookeri de Vries <sup>d</sup>	I-sigma	<i>rbcL</i>	5 bp deletion, premature stop codon	Light green	Spontaneous	Gordon et al. (1980); Winter and Herrmann (1987)
Carbon fixation	<i>Oenothera</i> <sup>e</sup>	IV-beta	<i>rbcL</i>	Transversion; single AA-exchange	Pale to green depending on light conditions	Spontaneous	Dauborn and Brüggemann (1998)
Carbon fixation	<i>Nicotiana tabacum</i> var. Techné with <i>debneyi</i> cytoplasm <sup>f</sup>	Sp25	<i>rbcL</i>	Transition, single AA-exchange	Light green ( <i>viridis</i> );	EMS	Shikanai et al. (1996)
Carbon fixation	<i>N. tabacum</i> var. Xanthi	XV1	<i>rbcL</i>	Transition, single AA-exchange	Light green	NMU	Avni et al. (1989)
Translation	<i>Hordeum vulgare</i> <sup>e</sup>	Cytoplasmic line 2 (CL2)	<i>infA</i>	Transition, single AA-exchange	Gradually greening	Plastome mutator	Landau et al. (2007); Colombo et al. (2008)
Translation	<i>H. vulgare</i> <sup>e</sup>	Cytoplasmic line 2-like (CL2-like)	<i>infA</i>	Transition, single AA-exchange	Gradually greening	Plastome mutator	Landau et al. (2007)
PS I assembly	<i>H. vulgare</i> <sup>e</sup>	cytoplasmic line 3 (CL3)	<i>ycf3</i> intron 1	Two point mutations (transition and single bp insertion), splicing deficiency	Light green ( <i>viridis</i> ); temperature sensitive	Plastome mutator	Landau et al. (2009)
mRNA maturation	<i>Cryptomeria japonica</i> var. <i>Wogon-Sugi</i>	<i>Wogon-Sugi</i> (likely)	<i>matK</i>	19 bp insertion, premature stop codon	Periodically pale ( <i>virescens</i> )	Spontaneous	Hirao et al. (2009)

<sup>a</sup>The list excludes plastome mutations conferring herbicide resistances and deletion mutants from cereal tissue culture (for review and references, see Oettmeier 1999; Day and Madesis 2007; Powles and Yu 2010). Antibiotic-resistant mutants are summarized in Table 11.2

<sup>b</sup>The correct taxonomic designation of *O. suaveolens* is now *O. biennis*. For details, see Dietrich et al. (1997)

<sup>c</sup>Identity of mutated and reference strain supposable, but not fully established in the cited references

<sup>d</sup>The correct taxonomic designation of *O. hookeri* is now *O. elata* ssp. *hookeri*. For details, see Dietrich et al. (1997)



et al. 1987). All types of mutations, including indels and point mutations, can arise spontaneously (for references, see Table 11.1).

### B. Spontaneously Induced Large Deletions of ptDNA in Cereal Tissue Culture

Within the Poaceae, for which plastid translation is not an essential growth requirement, plastome mutants lacking a big portion of the plastid genome can be isolated. Such mutants arise spontaneously from regenerated anthers or in long-term cell culture or can be induced by the plastid translational inhibitor streptomycin. Typically, relatively short linear DNA fragments and sometimes circular forms of deleted plastomes, are observed, often at high abundance. Mapping of such fragments regularly revealed the presence of ptDNA regions around the *trnE(UUC)* gene. Interestingly, this region is not identical to any of the origins of replication mapped in somatic tissue by independent methods (for summary and references, see Day and Madesis 2007). It is noteworthy in this respect that *trnE(UUC)* is not only involved in translation, but also required for tetrapyrrole biosynthesis (Schön et al. 1986), which likely explains the retention of this gene in all deletion lines.

### C. Nuclear Plastome Mutator Alleles Causing Multiple Plastid Mutations

Several nuclear alleles have been documented, which recessively induce various kinds of mutated plastids at frequencies much higher than spontaneous mutations. Such plastome mutator alleles were described for *A. thaliana* (*chloroplast mutator*), *E. hirsutum* (*mp<sub>1</sub>*, *mp<sub>2</sub>*), *H. vulgare* (*chloroplast mutator*), *Nepeta cataria* (*mutation-allowing*), *O. elata* ssp. *hookeri* (Syn: *O. hookeri*) (*plastome mutator*), *Petunia hybrida* (*a-*) and *Solanum nigrum* (*cpm*) (Grun 1976; Kirk and Tilney-Bassett 1978; Arntzen and Duesing 1983; Börner and Sears 1986; Hagemann 1986; Prina 1992, 1996; Prina et al. 2009). However, at least for the *E. hirsutum* allele *mp<sub>1</sub>* and for the *N. cataria*

line, formal genetic evidence suggests the mitochondrial genome as (a second) site for mutagenesis (cf. Kirk and Tilney-Bassett 1978; Sect. II). This also holds true for the *A. thaliana chloroplast mutator* (*chm*), although this allele has been a classic example for inducing mutated plastids. Its mutator activity was even confirmed by maternal inheritance, sorting-out and mixed cells (Rédei 1973). Molecular analysis revealed that impaired gene regulation from rearranged mitochondrial loci could explain the variegation phenotype. The corresponding allele was cloned, re-designated *AtMsh1* and found to be a homologue of the *Escherichia coli* MutS gene, a factor for mismatch repair and DNA recombination (reviewed in Rodermeil 2002; Yu et al. 2007). AtMSH1 is involved in mitochondrial substoichiometric DNA shifting and mitochondrial DNA recombination (Abdelnoor et al. 2003; Arrieta-Montiel et al. 2009).

A clear case established by molecular analysis of plastome mutations induced by a nuclear allele was described for *O. elata*. Individuals homozygotic for the *plastome mutator* allele *pm* show a 200–1,000 times higher mutation frequency than the wild type. Deletions up to 600 bp, small insertions, point mutations and additional nucleotides in poly-A/T stretches were detected in such lines (Epp 1973; Sears et al. 1996; Stoike and Sears 1998). Mutation frequency, at least for deletions, is biased to five hot-spots and directed to tandem repeats (Chiu et al. 1990; Chang et al. 1996). These regions may overlap with the hot-spots in sequence divergence identified in comparative analyses of *Oenothera* chloroplast genomes (Gordon et al. 1982; Chiu et al. 1990; Greiner et al. 2008b). Mechanistically, template slipping due to the absence of a DNA helicase or another DNA-binding protein was proposed (Stoike and Sears 1998). The locus corresponding to the mutator remains to be identified. Likewise, the possible influence of the *pm* locus on the mitochondrial genome needs to be investigated.

Characterization of plastome mutations induced by the *chloroplast mutator* (*cpm*) in

*H. vulgare* so far resulted in the exclusive detection of point mutations (single base pair transitions or insertions; Prina et al. 2009). The mutator does not seem to induce major structural changes in the ptDNA (Colombo et al. 2006), as judged from studies of various mutant lines derived from this material. These lines include mutations in *infA*, *ycf3* and *psbA*. The latter is atrazine-tolerant (Rios et al. 2003; Prina et al. 2009; Sect. VI). However, the specificity for ptDNA still needs to be verified.

Recently reported double knockouts of the *A. thaliana* *whirly1* (*AtWhy1*) and *whirly3* (*AtWhy3*) genes can induce different types of variegation in about 4.6% of the progeny. Due to illegitimate recombination between short direct repeats, ptDNA rearrangements resulting in head-to-tail concatemers and/or monomeric circles were observed in independent mutants. The rearranged regions are 10–25 times more abundant than the wild-type ptDNA. Illegitimate recombination was also shown in single knockout mutants of *AtWhy1*, *AtWhy3*, and in the corresponding ortholog in *Zea mays* (*ZmWhy1*; Maréchal et al. 2009). In general, the Whirly protein family is known as single-strand DNA binding proteins involved in DNA metabolism, including transcriptional regulation and telomere homeostasis (Desveaux et al. 2005; Cappadocia et al. 2010). Multiple functions also were proposed for the family members discussed here. *ZmWHY1* binds to several plastid transcripts and to DNA throughout the plastid genome. It further mediates splicing of the *atpF* intron. Strong *ZmWhy1* mutant alleles are deficient in plastid ribosomes (Prikryl et al. 2008). For *AtWHY1* and *AtWHY3*, circumstantial evidence suggests a role as transcription factors in the nucleus (Xiong et al. 2009). Finally, it was shown that they bind to single-stranded DNA and are involved in repairing double-strand DNA (dsDNA) breaks (Cappadocia et al. 2010).

Reverse genetic and proteomics approaches may elucidate further factors responsible for plastid/organelle DNA maintenance and sta-

bility. For example, *A. thaliana* lines homozygous for a T-DNA insertion in *cpRecA* (*RecA1*) displayed variegated seedlings with a frequency of 1.1% and 4.2% in the fourth and later generations, respectively. This *E. coli* *RecA* homolog is targeted to the chloroplast, but its functional homology still needs to be proven (Rowan et al. 2010; cf. Chap. 8). Virus mediated post-transcriptional genes silencing of gyrases A and B, both dually targeted to plastids and mitochondria, can induce leaf variegation in *Nicotiana benthamiana*. Both organelle morphology and functionality are altered in these plants. Interestingly, the affected organelles display a significantly higher DNA content. Disturbed plastid nucleoids as well as alterations in size and structure of ptDNA were observed (Cho et al. 2004). Whether these approaches can be utilized as general tools for plastome mutagenesis, remains to be proven and so does their specificity for the plastid genome (see below).

#### D. Nuclear Mutator Alleles Secondarily Affecting the Plastid

A second class of plastome mutator lines, which produces variegated plants but only a single type of mutated plastids, was reported for *A. thaliana* (*albomaculans*), *Capsella bursa-pastoris* (*albovariabilis*), *Capsium annuum* (one line), *H. vulgare* (*albostrians*, Okina-mugi, Okina-mugi tricolor, Saskatoon, *striata-4*, *white*, *white-streak-3*), *Orzya sativa* (two lines), and *Z. mays* (*iojap*, *chloroplast mutator*). The chlorophyll deficiencies are transmitted maternally and, for some lines, the presence of mixed cells could be confirmed. Appropriate summaries of genetic evidence are provided elsewhere (Grun 1976; Kirk and Tilney-Bassett 1978; Hagemann 1986; Rodermeil 2002; Yu et al. 2007). However, molecular analyses question this class of plastome mutators. In none of these lines, a mutation in the plastome was verified by sequencing analysis. For the classic examples – the *iojap* allele in *Z. mays*, and the two *H. vulgare* mutants *albostrians* and Saskatoon – it turned out that ptDNA is

not, or not obviously, affected (Börner and Sears 1986). Comparable to the *ZmWhy1* knock-out lines (see above), loss of plastid ribosomes is induced in these lines (Börner and Sears 1986; Hagemann 1986; Rodermel 2002; Yu et al. 2007). In addition, CMS phenotypes and changes in mitochondrial DNA were observed in the *iojap* background, segregating independently from the “chloroplast mutation” in these lines (Hagemann 1986; Lemke et al. 1988). The *iojap* gene was cloned and it was shown that its product is associated with the plastid ribosomal 50S subunit, but the gene function of this locus still remains unclear (Han et al. 1992; Han and Martienssen 1995). It has been postulated that the striped *iojap* phenotype and maternal inheritance of its white plastids are caused by an irreversible loss of plastid ribosomes, and hence, that the Iojap-protein is involved in plastid ribosome assembly and/or stability. However, the protein has no similarity to any characterized RNA-binding protein family or other known proteins. It was further speculated that the *iojap* phenotype may also result from irreversibly impaired mitochondrial function (cf. Börner and Sears 1986; Lemke et al. 1988; Rodermel 2002; Yu et al. 2007). Some evidence of altered mitochondrial function was also given for the *striata-4* allele in *H. vulgare* (von Wettstein and Eriksson 1965).

Taken together, it is advisable to treat plastome mutators with some caution. Various phenotypes seem to reflect secondary effects of mitochondrial disturbance, and classic genetics does not allow to clearly distinguish between the two DNA-containing organelles (Sect. II). Also cases proven by molecular approaches in *O. elata* (*pm*), *H. vulgare* (*cpm*) and *A. thaliana* (*AtWhy1*, *AtWhy3*) need further investigation to evaluate their possible influence on the mitochondrial genome. The putative targets of plastome mutator alleles, the plastid DNA stability and replication machinery, are barely understood (for review see Day and Madesis 2007). Some components seem to be organelle specific, such as AtWHY1 and AtWHY3 which are localized in the plastid. Their homolog

AtWHY2 is targeted to mitochondria (Krause et al. 2005). However, further factors, such as the DNA polymerases, one of the three RecA homologues identified in *A. thaliana* (RecA2) or the gyrase subunits A and B in both *A. thaliana* and *N. benthamiana* are dually targeted (Day and Madesis 2007; Shedge et al. 2007). Hence, it seems reasonable to postulate the existence of machineries for organelle DNA replication and maintenance that are at least partially overlapping between plastids and mitochondria.

### E. Induction of Plastome Mutations by Chemicals

Interestingly, an abundant classic genetic literature reports resistance of plastids to mutagenic agents, such as radiation or chemicals (cf. Kutzelnigg and Stubbe 1974; Grun 1976; Kirk and Tilney-Bassett 1978; Hagemann 1982). The first unequivocal work describing a successful induction of plastome mutations by chemical treatment was published by Beletskii et al. (1969). The chemical compound *N*-nitroso-*N*-methyl-urea (NMU) was subsequently successfully applied to various higher plants species and has become a standard chemical agent to induce plastome mutations (e.g., Hagemann 1982; Schmitz-Linneweber et al. 2005; Azhagiri and Maliga 2007). NMU is a DNA alkylation agent with major effects on the guanidine N7 and O6 residues, inducing point mutations and chromosomal damage (Hagemann 1982; Beranek 1990; Doak et al. 2007). Its efficiency could be increased by heat shock in *H. annuus* (Guskov et al. 2001). Applied in appropriate concentrations, it is relatively specific to ptDNA, probably due to the lack of methyltransferases in plastids (Sears 1998). Screening the first generation following the mutagenesis treatment (M1 generation) for variegation is an effective approach to identify plastome mutants (Hagemann 1982). Since the discovery of the mutagenic action of NMU, several other chemicals, including nucleic acid base analogues and antibiotics, were described to induce mutations in the plastome. Successful reports exist for

*N*-nitroso-*N*-ethyl-urea, methyl-nitro-nitrosoguanidine (MNNG), ethyl-methane-sulfonate (EMS), or 5-bromo-2'-deoxyuridine (Kirk and Tilney-Bassett 1978; Hagemann 1982), more recently also for 9-aminoacridine hydrochloride causing single base pair deletions or small inversions (GuhaMajumdar et al. 2004), and ciprofloxacin as a gyrase inhibitor inducing double strand breaks in organelle DNA (Wall et al. 2004).

#### *F. Effects of Radiation on ptDNA*

Studies on the influence of radiation on ptDNA are still inconsistent. Although it was reported that UV light induces pyrimidine dimers in ptDNA, stable mutations presumably cannot be isolated. UV treatment may reduce the effective copy number of plastid chromosomes, indicating an efficient degradation mutated ptDNA molecules (cf. Sears and Sokalski 1991; Draper and Hays 2000; and references therein). Furthermore, the presence of a cyclobutane pyrimidine dimer photolyase, targeted to all three genetic compartments was recently reported for *Oryza* (Takahashi et al. 2011). Remarkably, X-ray treatment also seems not to induce recoverable mutations in ptDNA, or at least does so only at extremely low frequencies. The classic genetic literature is rich in reports about unsuccessful induction of cytoplasmic mutations by X-ray irradiation. Probably successful cases, many of them supported by mixed cells, sorting-out and maternal inheritance, were reported from *A. thaliana* (0.07% increase after X-ray treatment against a similar background; Röbbelen 1962) and *Epilobium* (between 0.06% and 0.15% induced with X-ray, <sup>35</sup>S or <sup>32</sup>P, which, however, is not much more than two times higher than the spontaneous frequency observed for these experiments; Michaelis 1958a, b, 1967). Data on some putative X-ray induced plastome mutations described for Pteridophyta are vague (cf. Maly 1958b; Kirk and Tilney-Bassett 1978). An explanation for the “resistance” of ptDNA to X-ray irradiation may lie in the expected induction of dsDNA breaks in ptDNA. Plastid chromosomes are probably

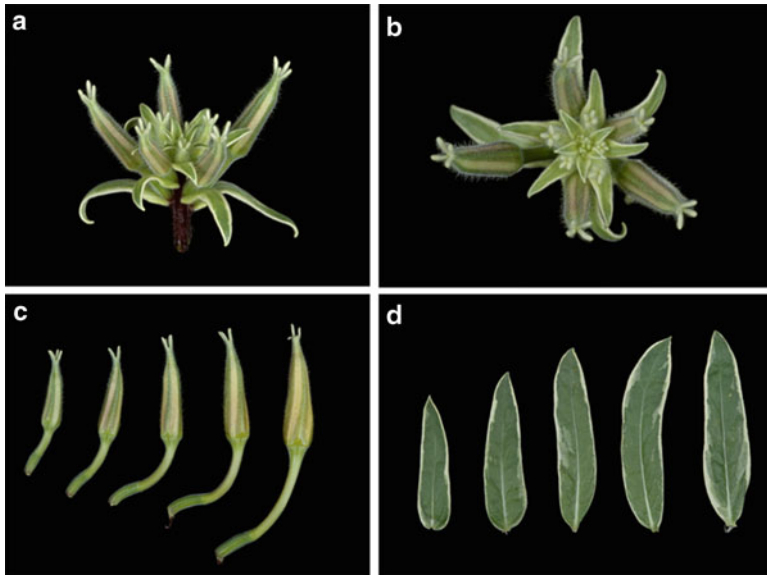
unable to repair such breaks by non-homologous end joining (Kohl and Bock 2009). Recent analyses of the ptDNA repair machinery indicate repair mechanisms by homologous recombination and/or microhomology-mediated break-induced replication (Cappadocia et al. 2010).

## IV. Maintenance of Plastome Mutants

As mentioned above, plastome mutants are usually recognized in the form of green–white (or pale, yellow) variegated plants (Fig. 11.1). Variegation is a result of random sorting-out of mutated and wild-type plastids (Sect. II). White, yellow or pale green sectors of these plants harbor only mutant plastids. In a chimeric plant, however, the survival of the impaired tissue is facilitated by the adjacent green tissue, which supplies the mutant plastids and cells with components they are unable to synthesize. Such plant material can be maintained in several ways, depending on the species (and sometimes even the variety) and its mode of chloroplast inheritance (Kutzelnigg and Stubbe 1974; Kirk and Tilney-Bassett 1978; Stubbe and Herrmann 1982; and references cited in Table 11.1).

### *A. Recovery of Homoplasmic Plastome Mutants*

Preferably, plants are maintained in a homoplasmic state in soil or, if plastome mutants cannot grow autotrophically, in sterile culture on sugar-containing media. Material homoplasmic for a mutation can be obtained by either regeneration of mutated leaf sectors or by selfing flowers containing solely the mutated plastome in the germ line. Such flowers can be recognized on white or pale stems with completed sorting-out for the mutation. In many dicotyledonous plants, however, stems whose leaves display periclinally chimeric phenotypes (Sect. II) are equally suited or even preferred. They typically show higher vigor in growth, seed and pollen development.



*Fig. 11.2.* Periclinal chimeric plant organs indicating the presence of a homoplasmic plastome mutation in the germline of evening primroses. **(a)** Inflorescence – lateral view. **(b)** Inflorescence – top view (note that the bracts represent periclinally chimeric leaves). **(c)** Striped buds from a periclinally chimeric inflorescence. **(d)** Successive leaves from a periclinally chimeric stem.

As mentioned above, periclinal chimeras occur after a completed sorting-out of plastids in the L1, L2, and L3 histogenetic layers of the SAM, resulting in different genetic identities of these layers. In the developed leaf, the L1 and L2 layers give rise to the tunica (epidermis and subepidermal tissue, respectively). The L3 layer forms the corpus (Sect. II). At leaf margins, the L2 layer is significantly enriched. Plants with white leaf margins are therefore homoplasmic for a plastome mutation in the L2 layer of the SAM. Since the germ cells originate from the L2 layer, flowers exclusively inheriting a mutated plastome can be recognized on shoots whose bracts show a pale leaf margin (Fig. 11.2). Selfing of such flowers leads to homoplasmic offspring (Kutzelnigg and Stubbe 1974; Kirk and Tilney-Bassett 1978; Stubbe and Herrmann 1982).

### *B. Propagation of Variegated Plants*

If a homoplasmic plastome mutant is not viable on soil, or tissue culture approaches

are not available, maintenance of plastome mutants is difficult, at least for plants with uniparental inheritance of chloroplasts. In such cases, plants must be kept heteroplasmic during propagation – the wild-type plastome is needed to nourish the mutant tissue (see above) – but heteroplasmy cannot be induced sexually. Propagation by seeds from variegated branches is possible but not reliable. Variegated offspring can only be obtained from flowers, in which sorting-out was not completed in the zygote. Sectorial chimaeras or mosaic patterns (Sect. II) in the inflorescence may indicate the potential that such zygotes can form, but neither the yields of heteroplasmic offspring are predictable nor the degree of variegation in the progeny (cf. Kirk and Tilney-Bassett 1978). In addition, backcrossing of such plastome mutants obtained from mutagenesis approaches in order to “clean” the nuclear genome from background mutations is advisable, but challenging for the same reasons. Hence, for juvenescence such mutants are often maintained by cutting variegated stems, a generally



difficult approach and not feasible for many higher plant taxa.

A solution to this severe problem is the use of model organisms displaying biparental plastid inheritance, like *Pelargonium* or *Oenothera*. In these genera, plastome mutants can be propagated by seeds and kept as variegated plants in soil. Biparental transmission allows a directed generation of variegated plants by crossing, using individuals with mutated and wild-type plastomes as crossing mates. Although the frequencies of variegated offspring differ depending on species and strain, this strategy is reliable enough to maintain large collections of mutants (Kutzelnigg and Stubbe 1974; Stubbe and Herrmann 1982; Tilney-Basset 1994). For *Oenothera*, a particularly elegant genetics for isolation and seed propagation of plastome mutants is available (Sect. VII).

## V. Identification of Plastome Mutants

Since higher plant plastomes are not amenable to linkage mapping approaches (Sect. II), direct methods for the identification of a plastome mutant are based on sequence analysis, preferably of the entire mutated plastid chromosome (Hirao et al. 2009). RFLP analysis of ptDNA, frequently performed in the early time of plastid molecular genetics, detects only larger rearrangements and rarely point mutations or small indels (e.g., Day and Ellis 1984; Lee et al. 1989; Chiu et al. 1990; To et al. 1992). The standard approach used so far relies on physiological and biochemical analyses of the mutant, which allow to make predictions about the mutated gene. This strategy was applied to most plastome mutations identified by molecular approaches (for references, see Tables 11.1, 11.2), and is still successfully used in screens for the molecular causes of plastid-encoded herbicide resistances in plant populations (Thiel et al. 2010; cf. Sect. VI). As long as Sanger sequencing was the limiting step in this analysis, single nucleotide polymorphism detection (e.g., using gel-shift assays) was performed (To et al. 1993; Schaffner et al. 1995). However,

the approach of combining physiological characterization with local sequence analysis suffers from three serious limitations: (1) The mutated gene needs to be identified and characterized first (e.g., Fromm et al. 1987; Winter and Herrmann 1987), which ironically prevented the elucidation of the plastid gene content by mutant analysis. (2) It is very difficult to identify unknown gene functions and/or unknown target sites for herbicides or antibiotics. (3) The presence of a second site mutation cannot be ruled out. Obviously, NGS technologies will overcome these technical constraints. If applied to organisms with established, high-quality plastid isolation protocols and a reference plastome sequence available, identification of plastome mutants should become routine. Highly purified ptDNA may even not be required – taking advantage of the high abundance of ptDNA in total DNA preparations, complete plastomes were re-mapped to a reference using Illumina deep sequencing (Nock et al. 2011). In a similar approach, starting from enriched ptDNA obtained by a rapid chloroplast isolation protocol, the plastome of *Corynocarpus laevigatus* (karak nut) was assembled de novo, using the related plastome of *Cucumis sativus* (cucumber) as guiding sequence (Atherton et al. 2010). However, the purity requirements of the ptDNA preparations subjected to NGS analysis and the general accuracy of the resulting genome assemblies remain to be carefully examined. It is widely known that, as the result of plastid-nuclear gene transfers, pieces or even whole plastome sequences are located in the nucleus of many plant species (e.g., Bock and Timmis 2008). Hence, if not removed, these promiscuous sequences may lower assembly quality and potentially interfere with mutation mapping in such approaches.

## VI. Types of Plastome Mutants

Over several decades, a substantial amount of plastome mutants have been described from various higher plant species and were characterized cytologically (e.g., by electron microscopy), physiologically and biochemically.

Table 11.2. Mapped antibiotic-resistant plastome mutants in higher plants

Resistance	Organism/strain	Mutant line	Gene/locus	Mutation	Origin	Reference
Spectinomycin	<i>Nicotiana tabacum</i> var. Xanthi	X/ <i>spe</i> <sup>R</sup> 40	16S rRNA	G to A (1012)	NMU	Fluhr et al. (1985); Fromm et al. (1987)
Spectinomycin	<i>N. tabacum</i> var. Petit Havana mut. SR1	SPC23 <sup>a</sup>	16S rRNA	G to A (1333)	Spontaneous (spectinomycin selection)	Svab and Maliga (1991)
Spectinomycin	<i>N. tabacum</i> var. Petit Havana	SPC1	16S rRNA	A to C (1138)	Spontaneous (spectinomycin selection)	Svab and Maliga (1991)
Spectinomycin	<i>N. tabacum</i> var. Petit Havana mut. SR1	SPC2 <sup>a</sup>	16S rRNA	C to T (1139) <sup>b</sup>	Spontaneous (spectinomycin selection)	Svab and Maliga (1991)
Spectinomycin	Nicotiana line 92 <sup>c</sup>	92/ <i>spe</i> <sup>R</sup> 4	16S rRNA	G to A (1140)	NMU	Fluhr et al. (1985); Fromm et al. (1987)
Spectinomycin	<i>Solanum nigrum</i> var. SN or SNR	StSp1 <sup>a</sup>	16S rRNA	C to T (1140) <sup>d</sup>	NMU	Kavanagh et al. (1994)
Spectinomycin	Nicotiana line 92 <sup>c</sup> mut. <i>str</i> <sup>R</sup> 7/ <i>spe</i> <sup>R</sup> 1 <sup>a</sup>	92/ <i>str</i> <sup>R</sup> 7/ <i>spe</i> <sup>R</sup> 1 <sup>a</sup>	16S rRNA	G to A (1140) <sup>e</sup>	NMU	Fluhr et al. (1985); Fromm et al. (1987)
Spectinomycin	<i>Arabidopsis thaliana</i> var. RDL	RLD-Spc1	16S rRNA	G to A (1141) <sup>f</sup>	N/A	M. Skarjinska, Z. Svab and P. Maliga in Azhagiri and Maliga (2007)
Streptomycin	Nicotiana line 92 <sup>c</sup>	92/ <i>str</i> <sup>R</sup> 6	16S rRNA	C to T (472) <sup>b</sup>	NMU	Fluhr et al. (1985); Fromm et al. (1989)
Streptomycin	<i>Nicotiana plumbaginifolia</i> var. Viviania	SR1007	16S rRNA	C to T (473) <sup>g</sup>	MNNG	To et al. (1989); Yeh et al. (1994)
Streptomycin	<i>N. plumbaginifolia</i> var. Viviania	SR1021	16S rRNA	G to A (833) <sup>h</sup>	MNNG	To et al. (1989); Yeh et al. (1994)
Streptomycin	<i>N. tabacum</i> var. Petit Havana	SR1	16S rRNA	C to A (860)	Spontaneous (streptomycin selection)	Maliga et al. (1973); Maliga et al. (1975); Eitzold et al. (1987)
Streptomycin	Nicotiana line 92 <sup>c</sup>	92/ <i>str</i> <sup>R</sup> 7	16S rRNA	C to T (860) <sup>b,i</sup>	NMU	Fluhr et al. (1985); Fromm et al. (1989)
Streptomycin	<i>N. plumbaginifolia</i> var. Viviania	SR1009, SR1012, SR1019, SR1020, SR1025, SR1036, SR1037, SR1043, SR1046	16S rRNA	C to T (860) <sup>j,k</sup>	MNNG	To et al. (1989); Yeh et al. (1994)

(continued)

Table 11.2. (continued)

Resistance	Organism/strain	Mutant line	Gene/locus	Mutation	Origin	Reference
Streptomycin	<i>N. plumbaginifolia</i> var. <i>Viviania</i>	SR1018	<i>rps12</i>	A to G (codon 88: K to R)	MNNG	Hsu et al. (1993)
Streptomycin	<i>N. tabacum</i> var. Xanthi	X/ <i>sp</i> <sup>a</sup> 6	<i>rps12</i>	C to T (codon 90: P to S)	NMU	Fluhr et al. (1985); Gatili et al. (1989)
Streptomycin	<i>S. nigrum</i> var. SN or SNR	StSp1 <sup>a</sup>	<i>rps12</i>	A to C (codon 87: K to Q)	NMU	Kavanagh et al. (1994)
Lincomycin <sup>1</sup>	<i>N. plumbaginifolia</i>	LR400	23S rRNA	G to A (2032) <sup>m</sup>	NMU	Cseplö et al. (1988)
Lincomycin <sup>1</sup>	<i>N. plumbaginifolia</i>	LR415	23S rRNA	A to G (2058) <sup>m</sup>	NMU	Cseplö et al. (1988)
Lincomycin <sup>1</sup>	<i>N. plumbaginifolia</i>	LR421, LR446	23S rRNA	A to G (2059) <sup>m</sup>	NMU	Cseplö et al. (1988)
Lincomycin	<i>S. nigrum</i> var. SNR	L17A1	23S rRNA	T to C (2073) <sup>n</sup>	NMU	Kavanagh et al. (1994)

<sup>a</sup>The mutants SPC2, SPC23, 92/*str*<sup>a</sup>7/*spe*<sup>a</sup>1 and StSp1 are streptomycin/spectinomycin double resistance mutants. For SPC2 and SPC23, the spectinomycin resistance arose from a second-site mutation in the already streptomycin resistant mutant SR1 (Svab and Maltga 1991). The molecular cause of the SR1 mutant is listed above. In the case of 92/*str*<sup>a</sup>7/*spe*<sup>a</sup>1, the streptomycin-resistant mutant 92/*str*<sup>a</sup>7 served as source material for subsequent second-site mutagenesis (Fluhr et al. 1985; Fromm et al. 1987). For the underlying mutation of *str*<sup>a</sup>7, see this table. The mutant StSp1 was obtained from separate mutagenesis and selection steps, initially on streptomycin, subsequently on spectinomycin (Kavanagh et al. 1994)

<sup>b</sup>Mutated site originally published as RNA sequence

<sup>c</sup>*Nicotiana* line 92 is a hybrid harbouring *Nicotiana undulata* plastids and the nuclear genome *N. tabacum* var. Xanthi. For details, see Aviv et al. (1980), Fluhr et al. (1984, 1985), and Fromm et al. (1987)

<sup>d</sup>Identical position as 92/*spe*<sup>a</sup>4 and 92/*spr*R<sup>7</sup>/*spe*<sup>a</sup>1 (*E. coli* coordinate 1193)

<sup>e</sup>Identical to 92/*spe*<sup>a</sup>4 (*E. coli* coordinate 1193)

<sup>f</sup>Original published sequence (C to T) complemented

<sup>g</sup>Original position published as *E. coli* coordinate 526

<sup>h</sup>Original position published as *E. coli* coordinate 885

<sup>i</sup>Identical position as SR1 (*E. coli* coordinate 912)

<sup>j</sup>Identical to 92/*str*<sup>a</sup>7 (*E. coli* coordinate 912)

<sup>k</sup>Original position published as *E. coli* coordinate 912

<sup>l</sup>Confirmed clindamycin cross-resistance

<sup>m</sup>*E. coli* coordinate

<sup>n</sup>Identical position as LR415 (*E. coli* coordinate 2058)

Frequently observed phenotypes are impaired plastid gene function and resistances to herbicides or antibiotics (for reviews and references, see Kirk and Tilney-Bassett 1978; Börner and Sears 1986; Somerville 1986; Hagemann 1992; Tables 11.1, 11.2; and citations therein). Some plastome mutants display mild chlorotic effects, are developmentally impaired or sensitive to environmental factors, show mottled phenotypes and/or bleach reversibly (e.g., Kirk and Tilney-Bassett 1978; Stubbe and Herrmann 1982; Colombo et al. 2008; Hirao et al. 2009). A drought and temperature tolerant plastome mutant was described from *H. annuus* (Usatov et al. 2004; Mashkina et al. 2010). Mutant lines with unexplained genetic behavior were published as well (Sect. VIII). Mutations lacking large parts of the plastid genome, spontaneously occurring in tissue cultures of cereals, were discussed in Sect. II. Unfortunately, due to technical difficulties (see above) molecular characterization of plastome mutants at the sequence level in general is not yet well developed. Excluding mutants resistant to herbicides, Tables 11.1 and 11.2 give an overview of higher plant plastome mutants of which the molecular basis is known. In the following paragraph characterized mutants are reviewed.

#### A. Mutants with Impaired Plastid Gene Function

Disregarding mutants resistant to herbicides or antibiotics (see below) so far only 12 mutations in chloroplast genes have been identified, four of them within the *rbcL* gene (Table 11.1). The spontaneous *A. majus* plastome mutant en:alba-1 was shown to be deficient in photosystem I (PS I) activity. Sequence analysis of PS I genes led to identification of a transversion in codon 136 of the *psaB* gene encoding the P700 apoprotein A2 of the PS I reaction centre changing a tyrosine (TAT) into a stop codon (TAG) to cause a premature stop of polypeptide synthesis. The truncation of the PsaB protein prevents the formation of a functional PS I complex (Schaffner et al. 1995).

The plastome mutant en:alba-4 is deficient in photosystem II (PS II) activity. This mutation was induced by treatment of *A. majus* seeds with NMU, which caused a transition (C to T) at position 1027 of the *psbD* gene changing the codon 343 for proline into serine thus effecting an amino acid exchange near the C-terminus of the D2 protein. Together with D1, the D2 protein forms the heterodimer of the PS II reaction centre, which binds the cofactors essential for charge separation. The C-terminus of D2 is highly conserved and obviously plays an important functional role as its mutational change in en:alba-4 severely impairs the function of PS II. The genes *psbD* and *psbC* in the *psbD-psbC* operon overlap within a short region. The above mutation hence simultaneously affects position -19 of *psbC* three base pairs upstream of its Shine-Dalgarno sequence. The mutation in this position, however, may have no effect on *psbC* translation since the interaction of the 16S rRNA with the Shine-Dalgarno sequence should not be disturbed (Schaffner 1995).

Immunological analysis of the *O. biennis* (Syn: *O. suaveolens*) mutant II-gamma pointed to a deficiency in cytochrome  $b_{559}$  subunits associated with PS II. Sequence analysis of the corresponding genes *psbF* and *psbE* uncovered a 5 bp duplication at position +42 in *psbE*, resulting in a frameshift and a premature stop codon 83 bases later. The important histidine residue at PsbE codon 23 co-ordinating the essential heme in cytochrome  $b_{559}$  is missing from the truncated protein version (Hupfer 2002), presumably explaining the severe photosynthetic deficiency in the mutant.

Several mutations in the *rbcL* gene encoding RuBisCO are known to impair function of the enzyme. The *N. tabacum* EMS mutant Sp25 displays a transition (G 964 A) resulting in an exchange of glycine to serine. The mutant synthesizes both RbcL and RbcS, but is defective in holozyme assembly (Shikanai et al. 1996). In the mutant XV1 of *N. tabacum*, NMU mutagenesis led to a C to T transition at position 335 in the *rbcL* reading frame (changing serine 112 to

phenylalanine). Functional RuBisCO could not be identified, but a precursor, the so-called B-complex, accumulates in the mutant (Avni et al. 1989). Two spontaneous *rbcL* mutants were described in *Oenothera*: The mutant IV-beta was identified as a single point mutation (G 337 C) resulting in an exchange from valine to leucine at position 113. RuBisCO assembly is impaired to 90%, again the B-complex accumulates. However, the remaining functional holoenzyme displays wild-type-like kinetic properties (Dauborn and Brüggemann 1998). In the mutant I-sigma, a TTAAC deletion (position 808–812) causes a frameshift at codons 270/271 and leads to a premature stop seven triplets later (Winter and Herrmann 1987). The mutant is deficient for the RuBisCO enzyme (Hildebrandt et al. 1984), and was the first plastome mutant, whose defect was characterized on the DNA level. It was also used to show the possibility of plastome mutant complementation by gene expression from the nuclear genome (allotropic transformation). A full-length *rbcL* cDNA equipped with the RbcS transit peptide and promoter sequences from *Pisum sativum* (pea) was transformed into the *Oenothera* nuclear genome, rescuing the I-sigma phenotype (Winter 1986; Nagley and Devenish 1989; Kanevski and Maliga 1994).

Particularly interesting point mutants were reported for the *infA* coding region in the CL2 and CL2-like lines of *H. vulgare* (Landau et al. 2007). The gene is a homologue of the bacterial translation initiator factor 1 (IF1; Sijben-Müller et al. 1986). However, *infA* gene function in higher plants could not be studied by transplastomic approaches so far. Model species amenable for plastid transformation like *N. tabacum* belong to the Rosid clade (e.g., Bock 2007a), of which most species have lost *infA* by gene transfer to the nucleus (Millen et al. 2001). Characterization of the *H. vulgare infA* mutants revealed a time-dependent reversible bleaching of the upper part of the primary leaf blade, probably due to a translational defect already during embryogenesis. The bleached leaf areas are plastid ribosome deficient,

show a delay of plastid development and probably an altered plastid retrograde signaling. The mutant phenotype is temperature dependent. Plants grown under high temperature during seed formation produce offspring with lower pigment content, whereas high temperatures during vegetative growth of CL2 mutants lead to enriched pigment levels in these plants (Prina et al. 2003; Colombo et al. 2008). Reversible bleaching was also reported for mutants with a frame-shift in the *matK* gene in *Cryptomeria japonica* (Cupressaceae; Hirao et al. 2009). *MatK* is thought to be an RNA maturation factor involved in the splicing of group II introns, although direct molecular evidence for this is still lacking (Schmitz-Linneweber and Barkan 2007), mainly because *matK* knock-out-lines in *Nicotiana* appear to be lethal (R. Maier in Schmitz-Linneweber and Barkan 2007).

#### *B. Plastome Mutants Impaired in Plastid Gene Regulation*

Of particular interest are those plastome mutants that display sensitivity to abiotic environmental factors and mutants showing periodical bleaching or mottled phenotypes. These features are characteristic of several plastome mutants and some of them are even viable in soil (e.g., Stubbe and Herrmann 1982; Chia et al. 1986; Archer and Bonnett 1987, and examples discussed below). Mildly chlorotic and developmentally dependent phenotypes promise interesting insights into regulatory mechanisms of chloroplast gene expression and gene function. For example, characterization of the plastome mutant II-theta in *O. biennis* indicated a splicing deficiency of the *petB* transcript due to deletion of two conserved nucleotides in the *petB* intron (Hupfer 2002). Two point mutations in the *ycf3* intron 1 of the *H. vulgare* CL3 line exhibit a similar, but temperature-dependent splicing defect (Landau et al. 2009). The variegated phenotype of the I-iota mutant in *O. elata* may be caused by a mutation causing translational fusion of the overlapping and co-transcribed genes for the  $\beta$ - and



$\epsilon$ -subunits of the plastid ATP synthase, as judged from western analysis. However, in vitro translation of isolated mRNAs from the I-iota mutant in a heterologous system results in non fused wild-type proteins. To resolve this discrepancy, a disturbed translation signal or post-transcriptional event has been proposed. However, the exact molecular defect in this mutant remains to be identified (Sears and Herrmann 1985).

### C. Plastome Mutants Exhibiting Resistance to Antibiotics

The generation of plastome mutants resistant to antibiotics was first reported in *Chlamydomonas*, and subsequently also in higher plants (Gillham 1978; Börner and Sears 1986; Hagemann 1992). Induction of plastome-borne spectinomycin, streptomycin or lincomycin (clindamycin) resistances was successful for various plant species, notably in *A. belladonna*, *A. thaliana*, *Caspicum*, *Nicotiana*, *Onobrychis viciifolia*, *Petunia hybrid*, and *Solanum* (Syn: *Lycopersicon*) (Jansen et al. 1990; Babiychuk et al. 1995; Venkataiah et al. 2005; Azhagiri and Maliga 2007; see also references in Table 11.2 and citations therein). A somewhat unclear case of chloramphenicol resistance was reported for *Nicotiana* (Fluhr et al. 1985). Typically, following mutagenesis using agents inducing point mutations, resistant plants are regenerated under antibiotic selection. Spectinomycin resistance also frequently occurs spontaneously on selective media containing this antibiotic. Most of these antibiotic resistance mutations have been localized to the plastid genes for the 16S rRNA (spectinomycin and streptomycin) or the 23S rRNA (lincomycin). In addition, three mutations causing streptomycin resistance are due to mutations in the plastid gene *rps12* for the ribosomal protein S12. Most of the mapped mutation sites are located in the corresponding regions of functional homology in *E. coli*. For a summary, see Table 11.2 and references cited therein.

Studies of genetic recombination and paternal leakage are examples, in which such

mutants provided useful experimental tools (Sect. II). The selectable markers of such mutants were furthermore employed to detect the interspecific transfer of chloroplasts in cybrid cultures by somatic fusion of protoplasts or protoplasts and microplastids. In these approaches, cells harboring antibiotic or herbicide resistant-donor chloroplasts are typically lethally irradiated and fused to a sensitive receptor line and plant regeneration is then performed on selective media. This technique can be extended to other selection markers, like bleached plastome mutants in the recipient (then selecting for green lines containing the donor plastid) or species specific regeneration media for the recipient (e.g., Medgyesy et al. 1985; Kushnir et al. 1987; Thanh et al. 1988; Kushnir et al. 1991; Eigel and Koop 1992; Babiychuk et al. 1995; and citations therein). The interspecific cybrids obtained can be utilized as targets to transform plastid genomes of species that are originally not transformable, by introducing foreign plastids into a host that is easy to manipulate (Kuchuk et al. 2006). A further application of antibiotic-resistant plastome mutants is a transformation of the chloroplast genome, avoiding bacterial selection markers such as the *aadA* gene. However, transformation efficiency is significantly lower (Svab and Maliga 1993). Using transformation vectors with point mutations in an endogenous 16S rRNA gene mediating resistance to spectinomycin and/or streptomycin, successful manipulation of the *N. plumbaginifolia* and *S. nigrum* plastids, respectively, was reported (O'Neill et al. 1993; Nugent et al. 2005).

### D. Herbicide Resistance Induced by Amino-Acid Substitutions in *psbA*

Of commercial importance are plastome mutants, or naturally evolved alleles, that confer resistance to herbicides. As a consequence of the global use of herbicides in agriculture, numerous spontaneous resistance mutants have arisen and are documented continuously (Heap 2011). In addition to resistant alleles encoded in the nucleus and

conferring tolerance against several herbicide classes, some resistance traits, especially against herbicides of the triazine type, were proven to be plastome encoded. All of them are associated with the *psbA* gene, encoding the D1 protein, one of the two reaction center subunits of the PS II complex. Similar or identical mutations are known from photoautotrophic bacteria and algae. The literature on this topic was comprehensively reviewed (e.g., Oettmeier 1999; Powles and Yu 2010; and reviews cited therein).

Briefly, a frequently found mutation is the substitution of amino acid residue 264 of the D1 protein, which is serine in wild-type (herbicide-sensitive) plants. The serine codon was changed in different herbicide-resistant plastome mutants by single substitutions into codons for glycine, threonine, asparagine or alanine. The most prominent substitution conferring triazine resistance is the serine 264 glycine exchange. Exchange to one of the other amino acids may additionally mediate resistance to other herbicide classes, such as urea derivatives. Selection for non-triazine herbicides affecting D1 has uncovered further resistance-conferring amino acid substitutions, such as valine 219 isoleucine, known already from *Chlamydomonas* or photosynthetic bacteria. These mutations also confer triazine resistance. Within *psbA*, also more complex mutations leading to herbicide resistance have been described, including double and triple mutations as well as indels.

Analysis of herbicide-resistant mutants has greatly contributed to our understanding of the molecular structure and function of the D1 protein in PS II. It allowed the identification of a region of 57 amino acids within D1 involved in herbicide binding, which defines the binding niche for the second plastoquinone (PQ) acceptor  $Q_B$  of PS II. The herbicides compete with  $Q_B$  and their binding inhibits PS II electron flow (Rochaix and Erickson 1988; Giardi et al. 2006; Powles and Yu 2010). Many, if not all, herbicide resistance mutations reduce the PQ binding affinity and, consequently, are associated with fitness costs (Gressel 2009; Vila-Aiub et al. 2009).

It is worth mentioning that, with the Ely accession of *A. thaliana*, a natural *psbA* allele for atrazine resistance has been discovered (El-Lithy et al. 2005).

## VII. Plastome Mutants of *Oenothera*

The genus *Oenothera* contributed significantly to the understanding of cytoplasmic genetics and, for many decades, played a dominant role in this research field (cf. Sect. II). For this genus, approximately 50, predominately spontaneously arisen, plastome mutants are available, which were systematically collected by *Oenothera* geneticists in Germany during the past century. This material shows various kinds of chlorophyll deficiency, and is barely characterized in terms of the underlying mutations. Only four mutants of this collection were subjected to molecular analysis (Sect. VI and Table 11.1). A physiological characterization involving most of the mutants revealed eleven mutants deficient for PS I and six for PS II. Four mutations affect the cytochrome  $b_6/f$  complex, one the plastid ATP synthase and six influence RuBisCO activity. One mutant displays translational errors (Sears and Herrmann 1985). However, no specific defect could be assigned so far to 22 other mutants (for review and references, see Kutzelnigg and Stubbe 1974; Stubbe and Herrmann 1982). In particular the unassigned ones, together with classes of mutants showing mottled areas of green/yellowish or white cells and/or conditional pigment deficiency associated with external or developmental factors, promise interesting insights into unknown features of plastid gene regulation. For example, the mutant I-tau displays reversible bleaching (*virescent* phenotype). It can grow heterotrophically and forms bleached and green leaves in alternating order. Maintenance of the mutant in the rosette stage in soil is possible for years, as long as enough green leaves are formed to nourish the bleached ones. This finding indicates periodic alterations in plastid physiology, the genetic determinants of which reside in the

plastome (W. Stubbe unpublished). For a systematic investigation of this valuable mutant material, reference plastomes for mutant identification by deep sequencing approaches (Greiner et al. 2008a, b) as well as high-quality plastid isolation protocols are now available (Herrmann 1982; Wolfson and Sears 1989; S. Greiner unpublished).

The availability of this rich collection of plastome mutants from *Oenothera* rests on a particularly elegant genetics of their maintenance, originally elaborated by Wilfried Stubbe. The genetics of permanent translocation heterozygosity and biparental plastid transmission in *Oenothera* facilitates a fast and easy substitution of cytoplasm between lines and species (Stubbe 1960, 1989; Kutzelnigg and Stubbe 1974; Stubbe and Herrmann 1982; Rauwolf et al. 2008). This allows propagation of mutant plastids of different *Oenothera* species and strains in defined genetic backgrounds. In *Oenothera*, this is of particular interest, since PGI within the genus is frequent and a genetic background compatible with most of the five basic plastome types (I–V) is highly desirable (Stubbe 1959, 1989; Greiner et al. 2011). This prerequisite is given with the johansen Standard strain (*O. elata* ssp. *hookeri*; Syn: *O. johansen*; Cleland 1935), which is most suitable as a maintainer. It is compatible with the basic plastome types I, II and IV and only weakly incompatible with plastome III. The strain prospers with relatively short generation times, flowers reliably, is easy to cross-pollinate, produces high seed yields, is resistant to most *Oenothera* pests and diseases, and amenable to tissue culture approaches and nuclear transformation. Once transferred in this genetic background, maintenance of plastome mutants by sexual propagation utilizing biparental transmission and variegated plants to nourish the mutated plastome by wild-type plastids is feasible (cf. Sect. IV). In the genus *Oenothera*, biparental transmission of plastids is of maternal dominance; that is, F1 generation offspring is either homoplasmic for the maternal plastome or heteroplasmic for the paternal and maternal plastomes. To increase the frequency

of variegated (heteroplasmic) offspring, a johansen Standard line was equipped with the slowly multiplying plastome IV (Sect. I) as wild-type plastome and used as seed parent in crosses with mutants of all faster multiplying plastomes (I–III). Resulting F1 generations display up to 100% variegation for wild-type and mutated plastomes in the progeny. In contrast, if a mutant of the slow plastome IV is to be maintained, the mutant plastome line should be used as maternal crossing mate and crossed to a wild type carrying the basic plastome type I, a fast multiplying plastome (Kutzelnigg and Stubbe 1974; Stubbe and Herrmann 1982). A comparable genetics is available for plastome V, which is severely incompatible with the johansen Standard strain. Maintenance of mutants of this plastome is achieved in its native background. To increase variegation in F1, a maintainer line with the slowest basic plastome IV is available. For both maintainer lines, high yields of variegated plants (mutant and nursery plastome) can be obtained and kept under greenhouse conditions. Since rosette diameters of *Oenothera* plants are up to 60 cm, quite large amounts of leaf material homoplasmic for the mutations can be obtained after completed sorting-out (Fig. 11.1b), thus facilitating the detailed physiological and genetic characterization of the mutant phenotype.

## VIII. Perspectives

Although research on plastome mutants suffered from limitations in identifying mutations on DNA level, the novel NGS technologies should overcome these technical limitations (Sect. V). A relatively fast and reliable identification of plastome mutations now offers the possibility of a systematic investigation of available plastome mutant collections and systematic screens for particular phenotypes employing mutagenesis approaches. The small genome size of plastomes should even allow saturating mutagenesis.

As obvious already from the few examples of plastome mutants that were characterized

molecularly, plastome mutants can fill a gap left by chloroplast transformation approaches for several reasons. First, many of the mutants with chlorotic defects characterized so far (Table 11.1) were identified from species not (yet) amenable to plastid transformation. This offers the possibility of molecular studies on plastid genomes also in non-standard organisms. A case in point is provided by the isolation of *infA* mutants in *H. vulgare*. The *infA* gene is not present in plastomes of higher plant species currently amenable to plastid transformation (Sect. VI). Second, induction of point mutations or indels by plastid transformation is technically challenging. Beyond simple knock-out analysis, point mutations can be highly valuable in elucidating functions of chloroplast genes. They are especially valuable to determine the functions of essential chloroplast genes, such as *matK* (R. Maier in Schmitz-Linneweber and Barkan 2007), *clpP* (Shikanai et al. 2001), *accD* (Kode et al. 2005) or the open reading frames *ycf1* and *ycf2* with still unidentified functions (Drescher et al. 2000). Third, possible dual functions of plastid genes may be uncovered by isolating mutated alleles. Fourth, data on promoter motifs are limited and cis-acting target sequences of plastid RNA metabolism are still largely unknown (Bollenbach et al. 2007; Liere and Börner 2007). Mutations for these elements are not or not readily obtainable by reverse genetics. Therefore, a systematic investigation of (mild) chlorotic and/or developmentally impaired plastome mutants would be highly desirable, since these phenotypes should include mutations in these elements (Sect. VI). Fifth, most intergenic regions in plastomes are small, presumably tightly packed with regulatory elements and probably as important as the coding sequences (Herrmann et al. 1992). Systematic mutagenesis approaches on these regions may uncover novel mechanisms in plastid gene regulation. Sixth, various plastid regulatory determinants, such as the loci underlying the different plastome multiplication rates (Sect. II), have remained elusive and hence are not amenable to knock-out approaches. Finally, the study of plastid restitution events

could help elucidating the genetic interactions between chloroplast and mitochondrial loci (Sect. II). Addressing these questions will require systematic plastome mutagenesis approaches comparable to TILLING for nuclear genes (cf. Prina et al. 2009). The technological basis for such systematic studies is now available and offers rich opportunities for future research.

It is important to note that not all observed phenotypes of plastid mutants are explainable with our current knowledge. For example, a plastome mutant identified from *Epilobium* apparently induces degradation of wild-type plastids present in the same cell (Michaelis 1957). Long-range interactions of plastids or intercellular movement of plastid signals were even observed between adaxial and abaxial cell layers, as revealed by studies with some plastome mutations in *Oenothera* (Stubbe 1958; Kutzelnigg and Stubbe 1974). In conclusion, several cases have been reported, in which plastome mutants undergo a so far not understood genetic behavior. According to Michaelis (1955), sorting-out of organelles is a rather quick and random process (Sect. II; but also see Birky 2001). However, based on circumstantial evidence, Michaelis also suggested the possibility of non-random (or one-sided) sorting-out for some plastome mutations in *Epilobium* (cf. Kirk and Tilney-Bassett 1978, pp. 366–386). The significance and possible general relevance of all reports on unusual sorting-out phenomena still needs to be determined and plastid mutants will undoubtedly play a prominent role therein.

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