RESEARCH ARTICLE

A photosynthesis operon in the chloroplast genome drives speciation in evening primroses

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One-sentence summary: Photosynthesis genes encoded on the chloroplast genome establish hybridization barriers.

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ABSTRACT
Genetic incompatibility between the cytoplasm and the nucleus is thought to be a major factor in species formation, but mechanistic understanding of this process is poor. In evening primroses (Oenothera spp.), a model plant for organelle genetics and population biology, hybrid offspring regularly display chloroplast-nuclear incompatibility. This usually manifests in bleached plants, more rarely in hybrid sterility. Hence, most of these incompatibilities affect photosynthetic capability, a trait that is under selection in changing environments. Here we show that light-dependent misregulation of the plastid psbB operon, which encodes core subunits of photosystem II and the cytochrome b6f complex, can lead to hybrid incompatibility, and this ultimately drives speciation. This misregulation causes an impaired light acclimation response in incompatible plants. Moreover, as a result of their different chloroplast genotypes, the parental lines differ in photosynthesis performance upon exposure to different light conditions. Significantly, the incompatible chloroplast genome is naturally found in xeric habitats with high light intensities, whereas the compatible one is limited to mesic habitats. Consequently, our data raise the possibility that the hybridization barrier evolved as a result of adaptation to specific climatic conditions.

INTRODUCTION
Incompatibility between the nuclear and organellar genomes represents a mechanism of reproductive isolation that is observed in a wide range of taxa (Levin, 2003; Chou and Leu, 2010; Greiner et al., 2011; Burton et al., 2013; Greiner and Bock, 2013; Sloan et al., 2017; Fishman and Sweigart, 2018; Bogdanova, 2020; Postel and Touzet, 2020). However, with the exception of cytoplasmic male sterility, a commercially important trait, little is known about the molecular and evolutionary mechanisms of cytoplasmic incompatibility (CI). Mechanistic studies are available from only a few cases (Schmitz-
Linneweber et al., 2005; Harrison and Burton, 2006; Lee et al., 2008; Burton et al., 2013; Meiklejohn et al., 2013; Fishman and Sweigart, 2018), in contrast with the great biological importance of the phenomenon. Increasing evidence is accumulating that CI arises early in the separation of two genetic lineages (Levin, 2003; Fishman and Willis, 2006; Greiner et al., 2011; Burton et al., 2013; Barnard-Kubow et al., 2016; Postel and Touzet, 2020), and thus represents an initial barrier leading to reproductive isolation. This suggests that CI is a major determinant of species formation, highlighting the need for a mechanistic understanding of its molecular basis in the context of population genetics.

The evening primrose (*Oenothera*) is a model plant that is uniquely suited to address the mechanisms of reproductive isolation via hybrid incompatibility. Crosses between *Oenothera* species usually produce viable offspring that regularly display incompatibility between the chloroplast and the nuclear genomes (plastome-genome incompatibility; PGI). These incompatibilities usually manifest in bleached plants, more rarely in hybrid sterility. They represent the only strong hybridization barrier between *Oenothera* species, which often co-occur in overlapping ecological niches within hybridization zones (Stubbe, 1964; Dietrich et al., 1997; Greiner et al., 2011) (Figure 1). Hybrid incompatibility of nuclear loci is essentially absent (Stubbe and Raven, 1979b; Greiner and Köhl, 2014), underscoring the importance of CI as the cause of incipient isolation of the hybrid. In addition, *Oenothera* is a prime example for hybrid speciation (Cleland, 1957; Hollister et al., 2019), in that permanent translocation heterozygosis, a form of cross-inducible functional asexuality, can occur. Such genotypes display a meiotic ring and breed true upon self-fertilization. In crosses, this can lead to an immediate fixation of a hybrid (Cleland, 1972; Rauwolf et al., 2008; Golczyk et al., 2014; Harte, 1994) (see Methods and below).

In evening primroses (section *Oenothera* subsection *Oenothera*), based on phenotype, phylogeography, and behavior in crossings, three genetic lineages (A, B, and C) exist that are genetically separated by PGI (Stubbe, 1964; Cleland, 1972; Greiner et al., 2011). These genetic lineages occur in either homozygous (AA, BB, or CC) or in a stable heterozygous (AB, AC, or BC) state. They can be combined with five basic chloroplast genome types (I–V), which differ in their compatibility relations to the nuclear genotypes (Figure 1B).
The presence of distinct nuclear and chloroplast genomes and the sexual separation of the species by PGI has led to the development of a genetic species concept describing 13 species for *Oenothera* (Stubbe, 1964; Raven et al., 1979; Stubbe and Raven, 1979b; Dietrich et al., 1997). This species concept (of the so-called American school) is in sharp contrast to the largely morphological species definition of the European school that recognizes about 80 species (Rostański, 1982; Rostański, 1985; Rostański et al., 2010; Woźniak-Chodacka, 2018). According to the American school, however, specific combinations of nuclear and chloroplast genomes define the species (Dietrich et al., 1997). Other genome combinations can occur in weak or inviable hybrids, thus sexually separating the species (Figure 1B). Strikingly, these chloroplast-mediated speciation barriers rely on photosynthesis, a trait that is under selection in changing environmental conditions (Arntz and Delph, 2001; Flood, 2019). This makes *Oenothera* an appealing model to understand the genetic basis of speciation. The incompatibility loci causing separation of the species are part of the species concept and, hence, relevant for speciation by definition. They may even be a result of adaptive evolution.

For example, hybridization between *Oenothera elata* (an AA-I species) and *O. grandiflora* (a BB-III species) produces the incompatible combination AB-I that displays a bleached-leaf phenotype. This genetic incompatibility creates the major hybridization barrier between AA and BB species that appears to prevent colonization of western North America by B genome species (Figure 1C) (Greiner et al., 2011). It should be emphasized that hybridization is frequent in the genus in nature. As a rule, hybridization occurs if plastome-genome combinations permit it. Importantly, all viable combinations can be confirmed in hybrids in nature (Dietrich et al., 1997). Hence, PGI appears to act as a major mechanism preventing gene flow. In addition, ecological species separation is likely to occur. For example, existence of the green and compatible hybrid AA-II can be confirmed, but it does not establish stable populations in nature (Dietrich et al., 1997) (Figures 1A and 1B).

It is assumed that climate changes and periods of glaciation during the Pleistocene have shaped the genetic and ecological characteristics of the basic lineages A, B and C in *Oenothera* (Cleland, 1972). This is well supported both by the estimated divergence time of the chloroplast genomes (Greiner et al., 2008b) and by nuclear genome variation.
Following this view, the three lineages originated from Middle America and reached the North American continent in several waves. The current lineages resemble the ancestral sexual and homozygous species AA-I, BB-III and CC-V, and crosses between them usually result in PGI (Stubbe, 1964; Greiner et al., 2011). However, during the Pleistocene, hybridization between the basic lineages must have happened that produced viable offspring (Cleland, 1957, 1972; Dietrich et al., 1997). Those were fixed in the structural heterozygous and functional asexual species (AB-II or BA-III, AC-IV, and BC-IV). Hence, plastomes II and IV can be seen as relict genotypes of earlier stages of plastome evolution (Stubbe, 1964; Dietrich et al., 1997). Interestingly, those relict genotypes are now quite likely maintained in adaptive niches, since they are not outcompeted by the more recent, faster multiplying and more aggressive, chloroplast genomes (Stubbe, 1963, 1964):

Biparental chloroplast inheritance, which is the rule in evening primrose, leads to selfish evolution of aggressive or more competitive (e.g. faster multiplying) chloroplast genotypes upon hybridization (Grun, 1976; Greiner et al., 2015; Sobanski et al., 2019). In *Oenothera*, the most competitive chloroplasts are fixed as current evolutionary end points in the homozygote lineages AA-I, BB-III, and CC-V. These competitive chloroplast genotypes (I, III, and V) have the potential to outcompete the weaker ones (II and IV) in hybrid genotypes. For example, the common evening primrose (*O. biennis*) harbors plastome II and III in overlapping subpopulations (Cleland, 1972; Dietrich et al., 1997). Since plastids with plastome III are more aggressive than those with plastome II, they should outcompete plastome II in this species. Observed, however, is that separation of plastome II and III in the two major subpopulations coincides with the expansion of the Wisconsin glacier (Anstett et al., 2015) (Figure 1A). This suggests that post-glaciation dispersal events and the presence of an ecological niche maintain the less aggressive chloroplast type in the northern parts of the *O. biennis* population.

Taken together, even recent plastome divergence in evening primrose appears to be a consequence of glaciation that is fixed by an interplay of adaptive and selfish evolution. The influence of genetic drift on hybrid incompatibly among the *Oenothera* populations is currently unknown.
Our aim was to understand the mechanism of the AB-I incompatibility that genetically separates the A and B lineages (Figure 1). We were further asking to what extent the underlying incompatibility might have evolved as a result of adaptive divergence in the two lineages. Based on association mapping in the chloroplast genome, the dual promoter region in the intergenic spacer between the clpP operon and the psbB operon was proposed to be involved in the incompatibility AB-I (Greiner et al., 2008a). The clpP gene encodes the proteolytic subunit of the Clp protease, the psbB operon encodes core subunits of photosystem II (PSII) and the cytochrome b$_{6f}$ complex (Cyt$b_{6f}$). However, the molecular mechanism and the physiological effects underlying the incompatibility have remained enigmatic. In addition, involvement of the ClpP protease in the phenotype of the incompatibility was still unclear.

RESULTS
AB-I plants are unable to acclimate to higher light conditions
AB-I plants display a yellow-green (lutescent) leaf chlorosis, caused by disturbed PSII activity (Greiner et al., 2008a) (Figures 2A and 2B). These photosynthetic defects occur specifically under high light intensities (Figure 2B). At 300 µE m$^{-2}$s$^{-1}$ (low light, LL), the compatible wild type AB-II and the incompatible hybrid AB-I are indistinguishable from each other, but higher light intensities cause severe photodamage in AB-I. Consistent with our previous study, already at 450 µE m$^{-2}$s$^{-1}$ (high light, HL), a substantial portion of PSII was photodamaged. Interestingly, AB-I plants are also unable to perform an efficient light acclimation response when shifted to HL conditions (Figure 2B; see Supplemental Text in the Supplemental Data pdf file containing the Supplemental Figures and Supplemental Tables): Whereas AB-II plants responded to the increased growth light intensity by strongly increasing their chlorophyll content (Supplemental Figure 1A) and the contents of all redox-active components of the electron transport chain (Figure 2B), AB-I plants were incapable of performing this light acclimation response efficiently. This behavior, inhibited in AB-I plants, is a typical reaction when plants that were previously grown under light-limited conditions are transferred to higher light intensities (Schöttler and Tóth, 2014). Finally, this leads to a relative reduction of the components of the electron transport chain, namely PSI, PSII and Cyt$b_{6f}$, but not the ATP synthase and...
plastocyanin, in AB-I plants compared to AB-II (Figures 2B and 2C; Supplemental Figure 1, Supplemental Text). In summary, AB-I plants display a light-dependent phenotype of photosynthetic acclimation that cannot be assigned to a single component of the electron transport chain. In addition, the disturbance in acclimation response is independent of ATP synthase and PC function.

The better adaptation of wild-type AA-I plants to high light is conferred by the chloroplast genotype

To examine whether the genetic differences between plastome I and II also have phenotypic effects under high light conditions in a compatible background (i.e., could be subject to selection in the parental species), we compared the light-acclimation response of green wild-type AA-I (O. elata) plants with green wild-type AB-II (O. biennis). In addition, to investigate whether potential differences are due to the chloroplast genome, we included the green chloroplast substitution line of the two genotypes (AA-II; Figure 1B). Since this experiment involved green material only, a harsher and more natural light shift from 300 µE m$^{-2}$s$^{-1}$ to 600 µE m$^{-2}$s$^{-1}$ (harsh high light, HHL) could be analyzed. The latter condition already induces severe damage in the incompatible AB-I genotype (Supplemental Text). However, it should be emphasized that 600 µE m$^{-2}$s$^{-1}$ is still a relatively moderate light intensity when compared to 1,000 to 1,500 µE m$^{-2}$s$^{-1}$ that can be reached in open land at noon.

In 300 µE m$^{-2}$s$^{-1}$, all photosynthetic parameters investigated (chlorophyll a/b ratio, chlorophyll content, $F_{v}/F_{m}$, linear electron transport capacity, and chloroplast ATP synthase activity) were very similar among the three genotypes (AA-I, AA-II, and AB-II, Table 1). However, after the shift to high light, pronounced differences were observed. While most parameters were not, or only weakly, affected in AA-I plants, the AB-II genotype showed a drastic loss of electron transport capacity. This was accompanied by marked decreases in the chlorophyll a/b ratio, chlorophyll content per leaf area, and chloroplast ATP synthase activity. Strikingly, similar changes also occurred in AA-II plants, indicating that the genetic background of the plastome, and not the nuclear genome, causes these differences in light acclimation. However, in comparison to typical light-acclimation responses of angiosperms, which (due to degradation of the chlorophyll
binding antenna proteins) result in increased electron transport capacity, chlorophyll content and chlorophyll a/b ratio (Schöttler and Tóth, 2014), the response of AA-I plants to increased light intensity is limited. It, therefore, can be concluded that plastome I is better adapted to cope with high light conditions than plastome II, although at least under the conditions tested, the johansen Standard strain of O. elata, originally isolated in California (Cleland, 1935; Dietrich et al., 1997), does not behave like a typical high light plant.

**RNA editing is not involved in the AB-I incompatibility**

Chloroplast loci that cause the described phenotypes could be related to mRNA editing sites, which often display great variability among even closely related species (Schmitz-Linneweber et al., 2005; Kahlau et al., 2006). RNA editing in chloroplasts of seed plants involves C-to-U conversions at highly specific sites (Takenaka et al., 2013). It is of particular interest, since the only previously described mechanism of PGI is based on an editing deficiency of the tobacco (*Nicotiana tabacum*) *atpA* transcript (encoding a core subunit of the plastid ATP synthase) when exposed to the nuclear genetic background of the deadly nightshade *Atropa belladonna* (Schmitz-Linneweber et al., 2005). However, as evidenced by our sequencing results of the chloroplast transcriptomes of *O. elata* (AA-I), *O. biennis* (AB-II), and *O. grandiflora* (BB-III), mRNA editing does not play a role in the AB-I incompatibility. As judged from our data all compatible wild-type genome combinations of the three species share the same 45 mRNA editing sites (Supplemental Table 2, updated GenBank records AJ271079.4, EU262889.2, and KX014625.1). This analysis also includes partially edited sites, whose biological relevance is doubtful (see Supplemental Table 2 and Methods). These results exclude the possibility that editing sites in the plastome and/or nucleus-encoded editing factors differ among the genotypes involved in the AB-I incompatibility, as this was reported for an experimentally produced cytoplasmic hybrid (cybrid) of *Atropa* and tobacco (Schmitz-Linneweber et al., 2005).

**Association mapping to identify plastid loci causing the AB-I incompatibility**

Having ruled out the involvement of mRNA editing, we performed an association mapping in the chloroplast genome of *Oenothera* to pinpoint the causative loci for the AB-I
incompatibility. In contrast to the green alga *Chlamydomonas*, chloroplast genomes of higher plants are not amenable to linkage mapping (Chiu and Sears, 1985; Greiner et al., 2015). Hence, identification of functionally relevant loci is usually based on correlation of a polymorphism to a phenotype (e.g. Greiner et al., 2008a; Simon et al., 2016; Sobanski et al., 2019). In the case of the AB-I incompatibility, this can be achieved by manual inspection of an alignment of fully sequenced chloroplast genomes and a search for specific polymorphisms in plastome I vs. II, III and IV. Those polymorphisms are considered candidates for causing the AB-I incompatibility because only plastome I confers the bleached *lutescent* phenotype in the AB nuclear genetic background, whereas plastomes II, III and IV are all green when combined with the same nuclear genome (Stubbe, 1964; Greiner et al., 2008a). Our original analyses of the AB-I phenotype had included only four chloroplast genomes and yielded 16 candidate regions (Greiner et al., 2008a).

Using the power of next-generation sequencing technologies, we were able to base the association mapping on 46 full chloroplast genomes whose genetic behavior had been determined by extensive crossing studies (Stubbe, 1959, 1960, 1963; Wasmund, 1980) (Methods; Supplemental Table 1). The chosen strains represent the material used for generalization of the genetic species concept in *Oenothera* that is based on the basic A, B and C nuclear and I to V chloroplast genotypes (Cleland, 1972; Dietrich et al., 1997) (see Introduction). The strains reflect the full natural distribution range of *Oenothera* and cover all species accepted at the time the crossing data were gathered. Altogether, the mapping panel included 18 chloroplast genomes representing plastome type I (Methods; Supplemental Table 1). Although the individual plastomes differ by various single-nucleotide polymorphisms and insertions/deletions (indels) (cf. Greiner et al., 2008a; Greiner et al., 2008b), only four polymorphisms were absolutely linked with the AB-I phenotype, i.e. they were specific to plastome I and could potentially be involved in the AB-I incompatibility: (i) a 144-bp deletion in the *clpP* - *psbB* operon spacer region, (ii) a combined 5-bp deletion/21-bp insertion in the *psbM* - *petN* spacer (genes encoding a PSII and a Cytb6f subunit, respectively), (iii) a 194 bp deletion in the *ndhG* - *ndhi* spacer (two genes encoding subunits of NADH dehydrogenase complex), and (iv) a 21-bp insertion in the *trnL-UAA* - *trnT-UGU* spacer (Supplemental Files 1-3).
Due to the lack of measurable sexual recombination frequencies in chloroplast genomes of seed plants (Greiner et al., 2015) (see above), genetic methods cannot be employed to further narrow down the causative loci for the AB-I incompatibility in plastome I. We, therefore evaluated the remaining candidate polymorphisms with respect to their potential for causing the incompatible phenotype. Based on the deletions in the ndhG - ndhI and trnL-UAA - trnT-UGU spacers it was difficult to explain the observed light-dependent reduction of specific photosynthetic complexes in AB-I incompatible material (Figure 2). The neighboring genes do not encode components of the electron transport chain, and, moreover, knockouts of NDH complex subunits lack a discernible phenotype (Burrows et al., 1998). From possible effects on the expression of trnL-UAA and/or trnT-UGU, two essential tRNAs, one might expect a more pleiotropic phenotype that does not depend on the light intensity. As judged from the functions of the genes affected, a contribution of the latter two polymorphisms to the AB-I phenotype is hence unlikely, although strictly speaking we cannot fully exclude this. By contrast, the polymorphisms affecting the psbB operon and the psbM/petN spacer are more likely candidates because they can potentially affect both PSII and Cytb6f, in agreement with the physiological data (Figure 1B).

The psbN-petN spacer region makes a minor contribution to the AB-I incompatibility
To examine the contribution of the combined 5-bp/21-bp indel in the psbM - petN spacer, transcript and protein analyses were performed in incompatible AB-I plants and compatible controls under LL and HL conditions (Figure 3). The indel is located in the 3' UTR of both genes (Figure 3A) and, therefore, could potentially affect the stability of their transcripts.

RNA blot analyses revealed that both genes are affected by the indel. For psbM, reduction of the 0.35-kb monocistronic transcript was observed for AA-II and AB-I under LL, and for AB-II and AB-I under HL conditions. Although there is no obvious explanation for this light-dependent effect, it is independent of the AB-I incompatibility because the levels of psbM mRNA cannot be linked to the AB-I phenotype (Figure 3B and below). Moreover, as judged from knockout mutants in tobacco, even complete loss of the PsbM
protein does not lead to a strong phenotype that would be comparable to the phenotype of AB-I plants (Umate et al., 2007). By contrast, petN encodes an essential subunit of the Cytb<sub>f</sub> (Hager et al., 1999; Schwenkert et al., 2007), and reduced petN transcript stability, therefore, could affect Cytb<sub>f</sub> accumulation. RNA gel blot analysis of petN mRNA accumulation detected a mature transcript of 0.3 kb (Figure 3A). Under LL conditions, petN transcript accumulation is unaltered in the incompatible hybrid, whereas under HL, the petN mRNA is significantly reduced in AB-I material (Figure 3C). Immunoblot analyses showed that this leads to a reduction of the protein level to approximately 80% (Figure 3D), an estimate that is well supported by our spectroscopic quantification of Cytb<sub>f</sub> (Figure 1B).

Taken together, these data do not exclude the possibility that the psbM/petN region influences the incompatibility phenotype, and they suggest a minor contribution. However, involvement of psbM is unlikely, because down regulation of its mature transcript is observed also in compatible AA-II plants under LL conditions (Figure 3B). Nonetheless, a role of petN is unlikely as well, since reduction of about 20% of the Cytb<sub>f</sub> content (Figure 2B, Figure 3D) does not affect accumulation of the photosystems (Anderson et al., 1997; Hager et al., 1999; Schöttler et al., 2007b; Schwenkert et al., 2007). Hence, another chloroplast locus is responsible for the AB-I incompatibility.

**The promotor region of the psbB operon is a major determinant of the AB-I incompatibility**

Next, we analyzed the transcript patterns of the clpP and psbB operons that flank the 144-bp deletion in the spacer region (Supplemental Figure 2A). RNA gel blot analyses revealed that accumulation of both the clpP precursor transcript and the mature clpP mRNA did not differ in control plants vs. incompatible plants under HL conditions. All clpP transcripts accumulated to similar levels as in the compatible lines. Similarly, no difference in transcript accumulation of the remaining operon genes residing upstream of clpP (rpl20 and 5'-rps12) was observed (Supplemental Figure 2B). In addition, analyses of ClpP protein accumulation and the integrity of the plastid ribosomes revealed no difference between compatible and incompatible material (Zupok, 2015). Based on these
findings, a contribution of the *clpP* operon to the incompatibility phenotype can be excluded.

By contrast, transcript accumulation of all *psbB* operon genes (*psbB*, *psbT*, *psbH*, *petB* and *petD*) was found to be reduced in AB-I plants under HL, but not LL conditions (Figures 4B and 4F; Supplemental Figure 2). Run-on transcription analyses revealed that this effect was due to impaired transcription rather than being an effect of altered transcript stability. Transcription of the *psbB* operon was specifically reduced under HL conditions in the incompatible hybrids (Figure 4D). Consequently, in contrast to the green AA-I, AA-II and AB-II plants, the deletion in plastome I in the AB background affected regulation of the *psbB* operon promoter in a light-dependent manner (Figure 4D). Importantly, the same promoter was used in all genetic backgrounds, as evidenced by mapping of the transcription start sites (Figure 4C), which are also highly conserved among species (Figure 4A). The deletion does not affect the TATA box of the *psbB* operon promoter, but resides 7 bp upstream of the -35 box. This may suggest that polymerase binding is not affected, but instead that binding of auxiliary proteins such as sigma factors is impaired by the deletion in the incompatible hybrids (see Discussion).

Interestingly, *pbf1* (*photosystem biogenesis factor 1*, previously designated *psbN*), a gene involved in PSI and PSII assembly, is down-regulated in AB-I incompatible plants under HL conditions (Figure 4E). *pbf1* is transcribed from the opposite strand by its own promoter, which lacks any polymorphism in all *Oenothera* plastomes sequenced so far (Supplemental Files 1-3). Therefore, the reduction in *pbf1* transcript accumulation might result from the sense-antisense interaction with the *psbT* mRNA, as previously described for *Arabidopsis* (Zghidi-Abouzid et al., 2011; Chevalier et al., 2015). Alternatively, it could be the result of a type of unknown feedback regulation. In any case, the interaction results in a strong reduction of Pbf1 accumulation (Figure 4F) that is a result of a decreased translation activity of the reduced *pbf1* transcript and its antisense counterpart *psbT* (Figure 4H). Since *pbf1* knockouts are extremely light-sensitive and show severe defects in PSII and, to a lesser extent, also in PSI accumulation (Krech et al., 2013; Torabi et al.,
2014), it appears likely that the effect on the \textit{pbf1} mRNA also contributes to the incompatibility phenotype.

**DISCUSSION**

PGI was reported from a large number of plant species and is an important phenomenon in plant speciation (Greiner et al., 2011). Our work reported here shows that light-dependent misregulation of a core photosynthesis operon leads to hybrid incompatibility, thus causing reproductive isolation and ultimately, speciation. Interestingly, the underlying genetic architecture was shaped during the last ice age by periods of glaciation, as noted in the Introduction. The mechanism we have discovered is different from that of the two other case of PGI mechanistically studied so far. RNA editing of the \textit{atpA} transcript was identified as the cause of chloroplast-nuclear incompatibility in an \textit{Atropa}/tobacco synthetic cybrid (Schmitz-Linneweber et al., 2005) (see above). Variation in the coding regions of \textit{accD} (the plastid-encoded subunit of the acetyl-CoA carboxylase, catalyzing the first step of fatty acid biosynthesis) was suggested as a genetic determinant of PGI in pea (Bogdanova et al., 2015). However, the \textit{Atropa}/tobacco case represents an unnatural, artificial combination of the plastid and the nuclear genomes of two sexually incompatible species, and, unfortunately, the evidence for possible causative loci for the incompatibility in pea are currently not strong enough to judge their impact on natural populations (Nováková et al., 2019). Moreover, in both cases, the ecological relevance of the suggested PGI loci is unclear and cannot be deduced from the identified polymorphisms.

By contrast, our data indicate that the AB-I incompatibility might have evolved as a result of ecological selection, representing a genotype x genotype x environment interaction (GxGxE). Although cytoplasmic GxE integrations were reported earlier in bread wheat (\textit{Triticum aestivum}) (Ekiz et al., 1998), especially for the chloroplast, cases in plants are hardly described. Recent work in animals, however, indicates that GxGxE interactions may be relatively common (Hoekstra et al., 2018; Hill et al., 2019; Rand and Mossman, 2020). That cytoplasmic incompatibly, in turn, can result from ecological selection is obvious from work in sunflower (using a cross between \textit{Helianthus annuus} and \textit{H. petiolaris}), in which common garden experiments in xeric and mesic habitats
demonstrated maintenance of cytoplasmic incompatibility by positive selection (Sambatti et al., 2008). The underlying genes and physiology, however, have remained enigmatic.

Our study demonstrates that photosynthesis-related genes encoded in the chloroplast genome can establish hybridization barriers. The incompatible phenotype is only visible under HL condition—AB-I plants cannot perform the necessary acclimation response as described above. Strikingly, plastome I in the native AA nuclear genetic background of *O. elata* (a species adapted to the western United States and Mexico) copes better with high light conditions than does the AB-II genotype of *O. biennis*, which is native to the North American woodlands (Dietrich et al., 1997). This effect is plastome-dependent, since the green AA-II chloroplast substitution line behaves photosynthetically very similar to *O. biennis*, but fully resembles the *O. elata* morphotype. However, to what extent the identified incompatibility locus is involved in a light acclimation response of AA-I species in their natural habitats remains to be addressed in further investigations.

Within their natural distribution ranges, species carrying plastome I colonized central and southwest North American (xeric habitats with high light irradiation), whereas species carrying plastomes II, III, IV or V are limited to the mesic sites of eastern North America (Figure 1C) (Cleland, 1972; Dietrich et al., 1997). Hence, plastome I seems to be required for colonization of habitats exposed to higher irradiation. This assumption is further supported by the fact that *O. biennis* (AB-II or BA-III), a species that spread west of the Great Plains after 1970, is only rarely found in the southern parts of the USA and is still absent from Mexico (Cleland, 1972; Dietrich et al., 1997).

Consequently, the loci underlying the AB-I incompatibility seem to prevent colonization of the south western parts of North America by the B genome by creating an asymmetric hybridization barrier between AA-I and AB-II, BA-III and BB-III species. At the same time, the compatible nuclear-chloroplast genome combination AA-I may have facilitated physiological adaptation of the corresponding species by nuclear-cytoplasmic coevolution. Therefore, it seems reasonable to assume that, as a result of higher light intensities (and/or light quality differences) in xeric habitats, the deletion upstream of the *psbB* operon promoter coevolved with nucleus-encoded proteins that interact with the (bacterial-type) plastid-encoded RNA polymerase (PEP).
Strong candidates for these interacting proteins are the PEP sigma factors, which were shown to regulate polymerase binding in response to both light quality and light quantity (Noordally et al., 2013; Chi et al., 2015). Moreover, regulation by sigma factors (e.g., through redox-induced phosphorylation) influences the stoichiometry of the protein complexes of the photosynthetic electron transport chain (Shimizu et al., 2010). Thus, the failure of AB-I plants to acclimate to high light intensities could be a direct consequence of disturbed transcriptional regulation by sigma factors. Coevolution and coordinated rates of molecular evolution of PEP core subunits and sigma factors could be a common principle in plant evolution, as suggested by recent findings in Geraniaceae, a family in which PGI is also widespread (Zhang et al., 2015).

Finally, it should be emphasized that, although the psbB operon does not encode PSI-related genes, its transcriptional misregulation also explains the observed effect on PSI, due either to the antisense interaction with the pbf1 mRNA or an unknown mechanism of feedback regulation (Figure 4). Interestingly, while the psbB operon (including the pbf1 gene on the opposite strand) displays extremely high structural conservation from cyanobacteria to higher plants, light regulation of pbf1 transcript abundance was shown to be highly variable among species (Kuroda and Sugiura, 2014).

METHODS
Plant material
Throughout this work, the terms Oenothera and evening primrose refer to subsection Oenothera (genus Oenothera, section Oenothera, Onagraceae; 2n = 2x = 14) (Dietrich et al., 1997). Plant material used here is derived from the Oenothera germplasm resource harbored at the Max Planck Institute of Molecular Plant Physiology (Potsdam-Golm, Germany). This includes the living taxonomic reference collection of subsection Oenothera (Greiner and Köhl, 2014). Part of this reference collection is the Renner Assortment, a medium-sized collection of European lines that were thoroughly characterized by members of the genetic school of Otto Renner (Cleland, 1972; Harte, 1994). In addition, it includes the Cleland collection, a large set of North American strains of subsection Oenothera that was extensively studied by Ralph E. Cleland and colleagues (Cleland, 1972). Also present are North American accessions analyzed by Wilfried
Stubbe and coworkers, and these represent species of this subsection that were recognized later than the 1960s (Stubbe and Raven, 1979a; Steiner and Stubbe, 1984, 1986; Wasmund and Stubbe, 1986; Wasmund, 1990; Schumacher et al., 1992; Schumacher and Steiner, 1993; Stubbe and Steiner, 1999). The availability of this material allowed us to employ the original source of lines on which the genetic species concept of subsection Oenothera was based (cf. Cleland, 1962) The lines employed for association mapping of the plastidic AB-I locus were extensively analyzed by classical genetics for the compatibility relations of their nuclear and chloroplast genomes (Supplemental Table 3 for details).

RNA editing analyses were performed with the wild type strains of Oenothera elata subsp. hookeri strain johansen Standard (AA-I), O. grandiflora strain Tuscaloosa (BB-III) and O. biennis strain suaveolens Grado (AB-II). Supplemental Table 3 contains a summary of all wild-type strains used in this work.

For most other genetic or physiological work presented here, the wild type strains johansen Standard (AA-I) and suaveolens Grado (AB-II) or chloroplast substitution lines between them (AA-II and AB-I) were used. Here, AA-I refers to the wild type situation, i.e. strain johansen Standard with its native nuclear and chloroplast genomes. AA-II refers to the nuclear genome of johansen Standard combined with the chloroplast genome of suaveolens Grado. AB-II designates nuclear and chloroplast genomes of the wild type strain suaveolens Grado, and AB-I the nuclear genome of suaveolens Grado equipped with the chloroplast genome of johansen Standard. Generation of AA-II and AB-I from the wild types AB-II and AA-I is detailed below (see the summary in Supplemental Table 4).

For ribosomal profiling and RNA-seq analyses incompatible (AB-I) and compatible (AB-II) plants were obtained from F1 seeds of crosses between the wild type or mentioned chloroplast substitution lines of johansen Standard (AA-I or AA-II) as the seed parent and grandiflora Tuscaloosa (BB-III) as the pollen parent. Here, AA-I x BB-III yielded AB-I plants, and AA-II x BB-III produced AB-II plants. Selection against the paternally transmitted plastome III was done using appropriate markers (Rauwolf et al. 2008). The material produced largely recreates the AB-I genotype that was characterized by Greiner et al. (2008a). For the tobacco (Nicotiana tabacum) wild type, the cultivar Petit Havana was used. The tobacco ΔpetN mutant was obtained from Hager et al., 1999.
Generation of chloroplast substitution lines

In *Oenothera*, the genetics of permanent translocation heterozygosity, combined with a biparental transmission of plastids, offer an elegant opportunity to substitute chloroplasts between species in only two generations while leaving the nuclear genome constitution unaltered (Stubbe, 1959, 1960, 1989). For the interested reader, general principles, including a detailed discussion of crossing examples, are presented in Rauwolf et al. (2008). The chloroplast substitution between the strains suaveolens Grado and grandiflora Tuscaloosa [described in Figure 6 of Rauwolf et al. (2008)] resembles the chloroplast substitution between suaveolens Grado and johansen Standard used in this work.

In brief, due to reciprocal chromosomal translocations, many species of *Oenothera* form permanent multichromosomal meiotic rings. If all members of a given chromosome complement are involved in a single ring, they establish two regularly segregating sets of genetically linked chromosomes. This leads to formation of two superlinkage groups, each involving one complete parental haploid chromosome set (α and β). Suppression of homologous recombination avoids genetic reshuffling between the two haploid sets. Additional genetic properties, especially presence of gametophytic lethal factors that lead to sex-linked inheritance of a given haploid set, eliminate homozygous segregants (α·α or β·β). This results in permanent heterozygous progeny (α·β) that is identical to the parental plant. The phenomenon of structural heterozygosity is a form of functional asexuality. However, truly sexual species also exist in *Oenothera*, i.e., species that display bivalent-pairing and regular meiotic segregation. In contrast to the structurally heterozygous species, they lack lethal factors and are homozygous for their haploid sets (haplo-haplo vs. α·β from above) (Cleland, 1972; Harte, 1994).

As a consequence of this genetic behavior, entire haploid chromosome sets in evening primrose can behave as alleles of a single Mendelian locus. These so-called Renner complexes are designated using (Latin) names; e.g. *G. albicans-G. flavens* (α·β) for the structurally heterozygous strain suaveolens Grado or *hjohansen Standard-hjohansen Standard* (haplo-haplo) for the homozygous line johansen Standard. A cross between them (suaveolens Grado x johansen Stanard = *G. albicans-G. flavens x hjohansen Standard-hjohansen Standard*) yields in the F1 the offspring *G. albicans-hjohansen...*
To equip johansen Standard (AA-I) with the chloroplast of suaveolens Grado (AB-II), the F1 hybrid \textit{G. albicans} \textit{\textsuperscript{h}johansen Standard} was used. Due to biparental inheritance of chloroplasts in evening primroses, it carries the chloroplasts of both johansen Standard (I-johSt) and suaveolens Grado (II-suavG). (The other hybrid \textit{G. flavens} \textit{\textsuperscript{h}johansen Standard} is not of interest and therefore discarded.) Since \textit{G. albicans} \textit{\textsuperscript{h}johansen Standard} I-johSt/II-suavG displays a full meiotic ring, this leads to suppression of homologous recombination as well as elimination of random chromosome assortment in meiosis (see above). Therefore, as a result of Mendelian segregation of the \textit{\textsuperscript{h}johansen Standard} complex, the johansen Standard strain (\textit{\textsuperscript{h}johansen Standard-I} johansen Standard) can be recreated from \textit{G. albicans} \textit{\textsuperscript{h}johansen Standard} upon selfing. (\textit{G. albicans} \textit{\textsuperscript{h}johansen Standard} x s = \textit{G. albicans-G. albicans}, \textit{G. albicans\textsuperscript{h}johansen Standard} and \textit{\textsuperscript{h}johansen Standard\textsuperscript{h}johansen Standard}; the sergeant \textit{G. albicans-G. albicans} is not realized due to a male gametophytic lethal factor in \textit{G. albicans}). When a \textit{G. albicans} \textit{\textsuperscript{h}johansen Standard} plant homoplasmic for II-suavG is used for selfing, the johansen Standard plant in F2 now carries plastome II-suavG (AA-II). If AB-I plants are desired, the \textit{G. albicans} \textit{\textsuperscript{h}johansen Standard} I-johSt/II-suavG hybrid in F1 is selected for I-johSt and backcrossed with suaveolens Grado (\textit{G. albicans-G. flavens} II-suavG). BC1 then reassembles \textit{G. albicans-G. flavens} I-johSt/II-suavG which, due to the maternal dominance of biparental transmission in evening primrose (Schötz, 1954; Chiu et al., 1988; Sobanski et al., 2019), contains a major proportion of \textit{G. albicans-G. flavens} I-johSt, i.e., AB-I plants.

**Plant cultivation, growth conditions, and tissue harvest**

For crossing studies, plastome sequencing and analysis of RNA editing, \textit{Oenothera} plants were cultivated in a glasshouse as previously described (Greiner and Köhl, 2014). AA-I, AA-II, AB-I and AB-II plants for molecular genetic and physiological analyses were cultivated in soil in growth chambers using a 16-h light/8 h-darkness cycle and 24°C at low light intensities (\~150 \text{µE m}^{-2}\text{s}^{-1}). At the beginning of the early rosette stage, about 21 days after germination (cf. Greiner and Köhl, 2014), plants were transferred to higher light intensities, i.e., 300 \text{µE m}^{-2}\text{s}^{-1} (low light, LL), 450 \text{µE m}^{-2}\text{s}^{-1} (high light, HL) or 600 \text{µE m}^{-2}\text{s}^{-1} (very high light, VHL).
s\(^{-1}\) (harsh high light, HHL), and then kept under the same growth regime. Hence, in all subsequent experiments the material was analyzed during the early rosette stage, which is defined as the young rosette 21-30 days after germination (see Greiner and Köhl (2014) for details and Figure 1A). 600 \(\mu\text{E m}\(^{-2}\text{s}\(^{-1}\)\) was used only for a single experiment, because it resulted in severe photodamage of the incompatible combination AB-I (see Supplemental Text). To avoid pleiotropic effects, the yellowish material of the bleached leaf tip, a typical characteristic of the \textit{lutescent} AB-I incompatible phenotype (Figure 1A), was excluded from all experiments. The tobacco \textit{ΔpetN} mutant and its corresponding wild type were cultivated as reported earlier (Hager et al., 1999).

**Thylakoid membrane isolation from \textit{Oenothera} leaves**

For spectroscopic measurements and blue-native PAGE, an improved thylakoid membrane isolation protocol was developed for \textit{Oenothera} leaf tissue that contains high amounts of mucilage and starch. All steps were performed at 4°C. Solutions were pre-chilled, leaves shortly placed in ice-cold water and dried with a salad spinner. Approximately 10 g of mature leaf tissue that was dark adapted for 1 h was homogenized in a blender adding 200 mL of isolation buffer [330 mM sorbitol, 50 mM HEPES, 25 mM boric acid, 10 mM EGTA, 1 mM MgCl\(_2\), 10 mM NaF (optional, if protein degradation is apprehend), pH 7.6 with KOH, and 5 mM freshly added sodium ascorbate]. 100-mL aliquots of the homogenate were then filtered through a double layer of cheese cloth (Hartmann), followed by filtering through a single layer of Miracloth (Merck). After that, the following procedure was applied twice: After adjustment of the solution to 200 ml with isolation buffer, it was centrifuged for 5 min at 5,000 \(g\) and the pellet was resuspended in 40 mL of isolation buffer using a 30-cm\(^3\) Potter homogenizer (VWR, mill chamber tolerance: 0.15 to 0.25 mm). Following the second homogenization step, the solution was adjusted to 200 mL with washing buffer (50 mM HEPES/KOH pH 7.6, 5 mM sorbitol, and optionally 10 mM NaF) followed by a filtering step through one layer of Miracloth. Subsequently, the thylakoid homogenate was centrifuged for 5 min at 5,000 \(g\), the pellet resuspended with a 30-cm\(^3\) Potter homogenizer in 30 mL washing buffer and centrifuged for 5 min at 5,000 \(g\). Then, after resuspending the thylakoids in 5 mL of washing buffer, the homogenate was placed on a 85% Percoll cushion [Percoll stock solution: 3% (w/v)
polyethylene glycol 6000, 1% (w/v) BSA, 1% (w/v) Ficoll 400, dissolved in Percoll; 85% Percoll: 85% PBF-Percoll stock solution, 330 mM sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM MgCl$_2$; pH 7.6 with KOH] in a 30 mL Corex tube and centrifuged for 5 min at 5,000 g. This step effectively removes starch from the isolation. Finally, thylakoids, which do not enter the Percoll cushion, are collected, washed in a total of 25 mL of washing buffer, centrifuged for 5 min at 5,000 g and resuspended in the desired buffer and volume.

**Spectroscopic methods**

For quantification of isolated thylakoids, chlorophyll amounts were determined in 80% (v/v) acetone (Porra et al., 1989). The contents of PSII, PSI, Cyt$b_6f$ and PC were determined in thylakoids as described previously (Schöttler et al., 2004). PSI was quantified from P700 difference absorption signals at 830 to 870 nm in solubilized thylakoids using the Dual-PAM-100 instrument (Walz) (Schöttler et al., 2007a, b). Contents of PSII and Cyt$b_6f$ were determined from difference absorption measurements of cytochrome b$_{559}$ and Cyt$b_6f$, respectively. Measurement procedures and data deconvolution methods have been described previously in detail (Kirchhoff et al., 2002; Schöttler et al., 2007a). Maximum F$_{v}$/F$_{m}$ values were measured in leaves adapted to darkness for one hour. Chlorophyll fluorescence was recorded with a pulse amplitude-modulated fluorimeter (Dual-PAM-100) on intact plants at room temperature. A F-6500 fluorometer (Jasco) was used to measure 77 K chlorophyll-a fluorescence emission spectra using an amount of freshly isolated thylakoid membranes equivalent to 10 μg chlorophyll mL$^{-1}$. The sample was excited at 430 nm (bandwidth of 10 nm), and the emission spectrum was recorded between 655 and 800 nm in 0.5 nm intervals (bandwidth of 1 nm). Dark-interval relaxation kinetics of the electrochromic shift, a measure of the proton motive force across the thylakoid membrane, were used to determine the thylakoid conductivity for protons (gH$^+$), which is a proxy for ATP synthase activity. Electrochromic shift signals were measured and deconvoluted using a KLAS-100 spectrophotometer (Walz) as previously described (Rott et al., 2011).
Antibody sources and anti-PetN serum production

The anti-Pbf1 (PsbN) antibody used in this work was described in Torabi et al. (2014). The serum was diluted 1:1,000. The anti-AtpA (AS08 304, dilution 1:5,000) antibody and the secondary antibody [Goat anti-Rabbit IgG (H&L), HRP conjugated, AS09 602, dilution 1:40,000] were obtained from Agrisera. To prepare an antibody against the PetN protein, rabbits were injected with PEG2-FTFSLSLVWGRSGL-PEG2-C-Amid (BioGenes GmbH), a highly hydrophobic peptide comprising about half of the small protein PetN. The peptide was coated with PEG2 (8-amino-3,6-dioxoactanoic acid) to ensure better solubility. Active serum was obtained after four immunizations and used in a dilution of 1:500.

Protein analyses

Blue-native PAGE was performed as previously reported (Ossenbühl et al., 2004; Schwenkert et al., 2006). To avoid protein degradation, 10 mM of NaF was optionally added to all solutions. Thylakoid membranes were solubilized with dodecyl-β-D-maltoside (DDM) at a final concentration of 1% and separated in 4-12% polyacrylamide gradient gels. Protein equivalents of 30 μg chlorophyll were loaded in each lane.

For immunoblot analyses, thylakoids were mixed with sample buffer [50 mM Tris/HCl, pH 6.8, 30% (v/v) glycerol, 100 mM DTT, 4% (w/v) SDS, 10% (w/v) Coomassie Brilliant Blue G-250] and were denatured for 5 min at 95°C under continuous agitation. Then, samples were analyzed by Tricine-SDS-PAGE (16% T separation gel and 4% T stacking gel) followed by gel blotting onto a PVDF membrane (0.2 µm) using the semi-dry PEQLAB transfer system (PEQLAB Biotechnologie GmbH). After incubation with the secondary antibody, immunochemical detection was performed with the help of the ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the supplier’s recommendations. In the relevant figures, 100% loading represents a 3 μg chlorophyll equivalent.

Isolation and purification of nucleic acids

DNA and RNA isolations from evening primroses were performed employing protocols specially developed for their mucilage and phenolic compound rich tissue, as previously
described in Massouh et al. (2016). For RNA-seq analyses, total RNA was purified from residual DNA contamination by digestion employing the Ambion® Turbo DNA-freeTM Kit.

**Association mapping of the plastid AB-I locus**

For association mapping in the chloroplast genome, we used 46 full plastome sequences of *Oenothera* for which precise genetic information is available (Supplemental Table 1 and Plant Material section). To this end, we newly determined the sequences of 30 plastomes, now available from GenBank under the accession numbers KT881175.1, KX014625.1, MN807266.1, MN807267.1, and MN812468.1 to MN812493.1. The new chloroplast genomes were annotated and submitted by GeSeq v1.43 (Tillich et al., 2017) and GB2sequin v1.3 (Lehwark and Greiner, 2019), respectively. The remaining 16 plastomes were previously published (see Supplemental Table 1 for details). Chloroplast genome sequencing from *Oenothera* total DNA was done as reported earlier (Massouh et al., 2016; Sobanski et al., 2019), but a higher version of the SeqMan NGen assembly software was used (v14.1.0; DNASTAR). Also, in contrast to earlier work, 250-bp Illumina paired-end reads (instead of 100-bp or 150-bp) were generated, with the exception of KX014625.1 (100-bp paired-end) and MN807266.1 and MN807267.1 (both 150-bp paired-end). Subsequently, for association mapping, the redundant inverted repeat A (IR_A) was removed, sequences were aligned with ClustalW (Thompson et al. 1994) and the alignments manually curated in Mesquite v3.40 (Maddison and Maddison, 2018). Polymorphisms specific to plastome I (i.e., polymorphisms that were present in all 18 plastome I genotypes but absent from all 28 plastome II, III and IV genotypes) were identified by visual inspection in SeqMan Pro v15.2.0 (DNASTAR) (cf. Greiner et al., 2008a). For the original data file, see Supplemental File 2. For readers without access to the commercial SeqMan Pro software, Supplemental File 1 and 3 are provided. Those contain the multiple sequence alignment of the 46 plastomes in standard FASTA, as well as the annotation of its consensus in the widely used GenBank format, respectively. Both files together reassemble the information included in Supplemental File 2, but can be read by any (free) sequence analyses software.
RNA editing analyses

To determine the RNA editotype of the *Oenothera* chloroplast, RNA-seq samples of the 1kp project (Hollister et al., 2015; Leebens-Mack et al., 2019) of johansen Standard (AA-I), suaveolens Grado (AB-II), and grandiflora Tuscaloosa (BB-III; NCBI SRA Accession Numbers ERS631151, ERS631122, and ERS631139; also see Plant Material section) were mapped against their respective chloroplast genomes (AJ271079.4, KX014625.1, and EU262889.2) from which the IR_A had been removed. For this we employed the reference-guided assembly - special workflows pipeline of SeqMan NGen v15.2.0. single-nucleotide polymorphisms were called in SeqMan Pro v15.2.0. To deal with the heterogeneity of the mRNA population, partial editing and sequencing errors, sites showing C-to-T (U) conversion of at least 30% were originally considered as mRNA editing sites. If editing could not be detected above this threshold at a given site in all three species, the sites were subjected to manual inspection of the original mapping data. In most cases, this procedure revealed mapping errors, however, in a few cases also partial editing below 30% in at least one of the strains was uncovered.

Gel blot detection of RNA

RNA blot analyses were performed as previously described (Massouh et al., 2016). Gene-specific PCR products used as probes were obtained by using the primers listed in Supplemental Table 5. Total *Oenothera* DNA was used as template in standard PCR reactions.

Chloroplast run-on analyses

For slot-blot preparation of DNA probes, PCR-amplified DNA probes (Supplemental Table 5) were immobilized to a Hybond-N+ nylon membrane (Amersham) through a slot-blot manifold. For this, 1.5 μg of DNA was denatured in 0.5 M NaOH and heated for 10 min at 95°C. Then, the volume of the denatured DNA probes was adjusted with water to 100 μL per spot. After heating, the probes were cooled on ice for 2 min to prevent DNA renaturation, and briefly centrifuged to collect the condensate. To each sample, 20 μL of cold 0.5 M NaOH and 0.5 μL of cold 10x DNA loading-dye [50% (v/v) glycerol, 100 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol] were added.
Subsequently, the samples were spotted to nylon membranes pre-hydrated with double-distilled H$_2$O, and then 100 μL 0.5 M NaOH was applied to each spot. After drying the membrane at room temperature for 5 min, the DNA was cross-linked to the membrane with 0.12 J/cm$^2$ using the UV crosslinker BLX-254 (BIO-LINK).

To analyze strand-specific gene expression of \textit{pbf1}, single-stranded \textit{pbf1} RNA probes were generated using the Ambion® Maxiscript® T7 Kit (Invitrogen) according to the manufacturer’s instructions and immobilized through a slot-blot manifold to a Hybond-N+ nylon membrane (Amersham). The \textit{pbf1} gene of johansen Standard was amplified with the primer pair psbNRO\_F 5’-AGCATTGGGGAGGCTCATTAC-3’ and psbNRO\_R 5’-GGAAACAGCAACCCTAGTCG-3’ and cloned into to pCRTM2.1-TOPO® (Invitrogen). The vector was linearized with \textit{Hind}III and in \textit{vitro} transcription was performed according to the suppliers’ protocol. 1.5 μg of RNA was adjusted with nuclease-free water to a volume of 50 μL prior to incubation with 30 μL of 20x SSC (0.3 M sodium citrate and 3.0 M sodium chloride) and 20 μL 37% formaldehyde at 60°C for 30 min. Samples were maintained on ice and spotted to nylon membranes pre-hydrated with double-distilled H$_2$O and 10x SSC. Next, 100 μL 10x SSC was applied per slot. After drying the membrane at room temperature for 5 min the RNA was cross-linked with an UV crosslinker as described above.

For \textit{in vitro} transcription and hybridization to slot-blot membranes, chloroplasts from \textit{Oenothera} leaves harvested 8-10 weeks after germination were isolated and counted according to a previously published protocol, applying the same minor modifications as described in Sobanski et al. (2019). Then, a chloroplast suspension containing 4.9 x 10$^7$ chloroplasts was transferred to a fresh tube, centrifuged at 5,000 g for 1 min and the supernatant was removed. To start the \textit{in vitro} transcription, 20 units of RNase Inhibitor (Promega GmbH), 50 μCi of [α-32P] UTP, and 94 μL transcription buffer (50 mM Tris–HCl pH 8.0, 10 mM MgCl$_2$, 0.2 mM CTP, GTP, and ATP, 0.01 mM UTP, 10 mM 2-mercaptoethanol) were added, mixed and incubated for 10 min at 25°C. Next, the reaction was stopped by adding 10 μL of stop buffer [5% (w/v) Na-lauroylsarcosine, 50 mM Tris–HCl, pH 8.0, 25 mM EDTA] followed by a RNA isolation protocol, where 100 μL of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the reaction, vortexed, incubated for 10 min at room temperature, and centrifuged at 18,000 g for 10 min at 4°C.
Afterwards, the upper phase was collected and nucleic acids were precipitated overnight at -20°C using 3 volumes of 100% (v/v) ethanol, 0.3 M sodium acetate and 1 μL GlycoBlue™ (Invitrogen). On the next day, the sample was centrifuged at 20,000 g for 1 h at 4°C. After centrifugation, the pellet was washed in 75% (v/v) ethanol and dissolved in 50 μL of RNase-free water. Next, the RNA was denatured at 75°C for 15 min and cooled for 2 min on ice. Before hybridizing the slot blots with the isolated RNA, the membrane was prehybridized with 20 mL of Church buffer [1 mM EDTA, 7% (w/v) SDS, 0.5 M NaHPO₄ pH 7.2] in hybridization tubes at 65°C for 1 h. Hybridization was performed at 65°C overnight. Subsequently, the membrane was washed once with 1 x SSC and 0.2% (w/v) SDS for 10 min, and once with 0.5 x SSC and 0.2% (w/v) SDS for 10 min. After washing, the membrane was wrapped in a transparent foil and exposed to a storage phosphor screen for 5 days. The signals were detected using an Amersham Typhoon IP scanner.

5’-RACE experiments to map transcription start sites
TAP transcript 5’-end mapping in Oenothera chloroplasts was performed as previously described (Kühn et al., 2005). In brief, primary transcripts of bacteria and cell organelles have triphosphates at their 5’ ends, while processed transcripts possess monophosphates at the 5’ end. The TAP enzyme (tobacco acid pyrophosphatase) removes the additional phosphates from the 5’-end of primary transcripts. After this treatment, both primary and processed transcripts can serve as substrate for RNA ligase. This allows us to distinguish between primary and processed transcripts when +TAP and -TAP treated samples are compared. In -TAP samples, ligation products originating form primary transcripts are absent. Hence, to map the transcription start sites of the psbB operon and to distinguish them from processing sites in close proximity, a 5’-RACE from +TAP (Epicenter) and -TAP RNA samples was performed. For this, RNA samples of both treatments were ligated to an RNA linker (5’-GUGAUCCAAACCGACGCGACAAGCUAAUGCAAGANNN-3’). After cDNA synthesis with a psbB gene-specific primer (psbB_cDNA_jn 5’-GCTGGGCTGTCCATATAATGCATACAGC-3’), two PCRs were performed: The first PCR used the linker-specific primer RUMSH1 (5’-TGATCCAACCGACGCGAC-3’) and the
psbB-specific primer psbB_cDNA_jn. The second PCR used the linker-specific nested primer RUMSH2 (5’-ACCGACCGACAAGCTAATGC-3’) and the primer psbB_5prime_jn (5’-GGAAAGGGATTATTAGGCATACCAATCG-3’). PCR products (30 μL of PCR solution) were run on 1% agarose gels (w/v) and, prior to sequencing, cloned into pCR®2.1-TOPO® (Invitrogen).

**Ribosomal profiling**

*Monosome and polysome isolation:* Polysome isolation from *Oenothera* leaves was performed based on a protocol modified from Ingolia et al. (2009) and Zoschke et al. (2013): 300 mg of leaf tissue were ground in liquid nitrogen and 3 mL of extraction buffer [40 mM Tris-aceate (pH 8.0), 0.2 M KCl, 10 mM MgCl₂, 1% (v/v) Triton X-100, 2% (v/v) polyoxyethylene (10) tridecyl ether, 100 μg mL⁻¹ chloramphenicol, 100 μg mL⁻¹ cycloheximide, 0.2 M sucrose, 10 mM 2-mercaptoethanol, 15 mM boric acid and 5 mM EGTA]. The suspension was filtered through glass wool, centrifuged at 15,000 g for 10 min, and the supernatant was collected. For nuclease digestion, 300 U/mL RNaseI nuclease (Thermo Fisher Scientific) was added. The samples were incubated at room temperature for 1 h in a rotator. Subsequently, in the prepurification step, the suspension was transferred onto a 2 mL sucrose cushion [40 mM Tris-aceate (pH 8.0), 100 mM KCl, 15 mM MgCl₂, 100 μg mL⁻¹ chloramphenicol, 100 μg mL⁻¹ cycloheximide, 25% (w/v) sucrose, 5 mM 2-mercaptoethanol, 15 mM boric acid and 5 mM EGTA]. After ultracentrifugation for 1.5 h at 303,800 g at 4°C in a SW55 Ti rotor, the supernatant was discarded and the pellet, which contained the nuclease digested monosomes were stored at −80°C.

*Ribosome footprint isolation:* After nuclease digestion, the monosome pellet was suspended in 500 μL ribosome footprint isolation buffer [10 mM Tris (pH 8.0), 1 mM EDTA, (pH 8.0), 100 mM NaCl, 1% (w/v) SDS, 0.1 M EGTA, pH 8.0] and transferred to a 2 mL centrifuge tube. To the monosome suspension 750 μL TRizol™ was added, and the sample was vortexed and incubated for 10 min at room temperature. Subsequently, 150 μL of chloroform/isoamylalcohol was added; the sample was vortexed and centrifuged for 20 min at 20,000 g at room temperature. The supernatant was transferred into a 2 mL
centrifuge tube containing 1 mL of isopropanol, and the RNA precipitated for 30 min at -20°C. Afterwards, the RNA was centrifuged for 40 min at 20,000 g at 4°C and the pellet was washed with 75% (v/v) ethanol. The pellet with the ribosome footprints was suspended in 20 μL of H₂O. The RNA concentrations were measured using a NanoDrop 1000 spectrophotometer, and the RNA was stored at -80°C.

**Denaturing RNA-PAGE for RNA purification:** RNA was separated on denaturing 12% polyacrylamide gels [30% (v/v) Acryl/Bis™ (19:1), 1x TBE buffer (89 mM Tris pH 8.0, 89 mM boric acid, 2 mM EDTA), 80% (w/v) urea, 0.1% (w/v) ammonium persulfate (APS), 0.6% (v/v) tetramethylethlenediamine (TEMED)]. The RNA sample was dried out using a concentrator plus centrifuge (Eppendorf) and dissolved in 40 μL of loading buffer [90% (v/v) deionized formamide, 20 mM Tris pH 7.5, 20 mM EDTA, 0.04% (w/v) xylene cyanol, 0.04% (w/v) bromphenol blue]. After the incubation for 5 min at 45°C, the sample was denatured for 10 min at 75°C. For each lane, 30-40 μg of RNA dissolved in 40 μL loading buffer was loaded and 4 μL of Dynamarker Prestain Marker for small RNA Plus served as marker for the gel. The gel was electrophoresed in 1x TBE buffer at a constant power of 30 W per gel until the dye front reached the end of the gel. During the run, the gel apparatus was connected to a cold machine and cooled to 8°C (Pharmacia & Upjohn), which avoids gel melting and, especially for small nucleic acids, leads to better electrophoresis results by decreasing mobility rates. Then a region representing RNAs between 20 and 50 nt was excised and the RNA was eluted from the gel in 4 mL TESS solution per lane [10 mM Tris (pH 8.0), 1 mM EDTA pH 8.0, 0.1 M NaCl, 0.2% (w/v) SDS] overnight in a rotator at 4°C. The solution was transferred to a new tube containing 4 mL phenol/chloroform/isoamyl alcohol (25:24:1) and 2.5 μL GlycoBlue™, vortexed and centrifuged for 5 min at 1,700 g at room temperature. Afterwards the supernatant was transferred into a new tube and RNA was precipitated at -20°C overnight by the addition of 2.5 volumes of 100% (v/v) ethanol. The next day, the sample was centrifuged at 15,000 g at 4 °C for 1 h and the pellet was eluted in 500 μL water. Subsequently, the eluted ribosome footprints were transferred to a new tube containing 100 mM NaCl and 500 μL phenol/chloroform/isoamyl alcohol, vortexed and centrifuged at 15,300 g at room temperature for 20 min. Then the supernatant was transferred to a new tube containing
chloroform/isoamyl alcohol, vortexed and centrifuged at 15,300 g at room temperature for 20 min. To precipitate the RNA, the supernatant was transferred into new tubes with 2.5 volumes of 100% (v/v) ethanol and incubated overnight at –20°C. The next day, the sample was centrifuged at 20,000 g at 4°C for 1 h. Afterwards the pellet was washed with 75% (v/v) ethanol, centrifuged at 20,000 g at 4°C for 10 min, and eluted in 20 µL of water. The size distributions of the ribosome footprints were measured using the Bioanalyzer using the Agilent small RNA Kit, and the concentrations were determined with the Qubit 4 Fluorometer using the Qubit™ RNA Assay Kit. The ribosome footprints were stored at –80°C.

Validation: A detailed validation of the ribosomal profiling protocol established for evening primrose is reported in Kozul (2019) and will be published in a subsequent communication.

NGS library preparation and sequencing
To prepare the ribosome footprints for the next-generation sequencing (NGS) library preparation, 5’-termini phosphorylation of the ribosome footprints was performed using the T4 PNK kit (polynucleotide kinase, Thermo Fisher Scientific), following the manufacturer’s instructions. Total RNA for RNA-seq libraries was isolated as described above. Subsequently, Ribo-seq and RNA-seq library preparations were performed according to the manufacturer’s protocols both for the LL and HL treatments in three replicates using the NEXTflex Small RNA-Seq Kit v3 and Zymo Research Zymo-Seq RiboFree Total RNA Library Kit, respectively. To provide details for the Ribo-seq library preparation, to 10.5 µL phosphorylated ribosome footprints 100 ng 3’-4N adenylated adapter and 5’-4N adenylated adapter were ligated, followed by the reverse transcription of the 3’- and 5’-adapter ligated RNA into a first strand synthesis product. The PCR products were subsequently amplified (18 cycles; 10 sec at 98°C, 30 sec at 60°C, 15 sec at 72°C) by using a universal primer and different barcoded primer for each sample. Afterwards a gel-free size selection and clean-up was performed. The size distribution of all final libraries was determined with the Bioanalyzer using the Agilent High Sensitivity DNA Kit, and the concentration was measured with the Qubit 4 Fluorometer using the
Qubit™ dsDNA HS Assay. Multiplexing and sequencing of the NGS libraries were performed at the Max Planck Institute for Molecular Genetics (Berlin, Germany) using an Illumina® Nova Seq 6000 machine (SP flow cell for Ribo-seq and S1 flow cell for RNA-seq libraries, 100 bp single-end reads).

**NGS data analysis**

Quality control of Ribo-seq (accessions GSM5288039 to GSM5288050) and RNA-seq (accessions GSM5288051 to GSM5288068) sequencing data, both included into the GEO Series GSE174154, was performed using FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). 3’ adapter trimming was applied to both data sets using Flexbar v2.5 (Dodt et al., 2012) with default parameters and the corresponding adapter-barcode-combined sequences. To remove a potential PCR bias from the Ribo-seq data set, the four-base 3’- and 5’-unique molecular identifiers (UMI) were removed from the read sequences and attached to the read identifier in order to make them available for further usage, while sustaining correct alignment of the reads. All Ribo-seq data, as well as RNA-seq data for computing translation efficiency, were then aligned against plastome I (I-johSt = AJ271079.4) using STAR v2.7.0a with slightly adapted indexing parameters (--genomeSAindexNbases 8, --genomeChrBinNbits 17) (Dobin et al., 2013). Subsequently, samtools v1.10 was used to remove secondary alignments, to sort and to index them, and to generate mapping, and gene-wise count statistics (flagstat & idxstat, respectively). Conversion of transcriptomic bam files (harboring only reads mapped to exons) to bed files was done using bamToBed from the bedtools v2.29.2 (Quinlan and Hall, 2010), adding read length as additional column. Both file formats, bam and bed, contain coordinate-based information for reads mapped against a set of sequences. BAM is a compressed binary version of the Sequence Alignment/Map format (SAM) and BED (Browser Extensible Data) files contain less but the most important information for read alignments compared to the BAM format. See https://genome-euro.ucsc.edu/FAQ/FAQformat.html#format1 and https://github.com/samtools/hts-specs/blob/master/SAMv1.pdf respectively for format specifications and details. Usable reads for all follow-up analysis were defined by the following criteria: (i) removing tRNA and rRNA-associated reads as well as reads shorter
than 20 bases and longer than 50 bases; (ii) read de-duplication based on chromosomal location and same UMIs (by sorting bed files on the columns scaffold_id, start/end position and both columns having the UMIs using groupBy to take only the first entry of each group identical entries (-o first); (iii) remain only reads with mapping quality equal to 255 (means unique mapped in the context of the STAR mapper). Resulting bed files were loaded into R to generate different types of plots.

CPM (counts per million reads) normalization was done on the total number of reads that mapped to protein-coding genes of the chloroplast. Gene length normalization to obtain RPKM (reads per kb of exon per million reads) values was applied by summing up the exon lengths for each gene in kb. Translational output and log$^2$ ratio bar plots are based on gene-wise read counting in which only reads greater or equal to 25 and less or equal to 35 were considered. Translational efficiency was computed by dividing the RF ratio by the RNA ratio where the ratios were calculated as follows: RF ratio (Ribo-seq sample1/sample2) and RNA ratio (RNA_seq sample1/sample2).

**Statistical analyses**

All numerical results are reported as mean ± SD. Statistical significance of the difference between experimental groups was analyzed by unpaired t-tests using GraphPad Prism software (Figure 2). Differences were considered statistically significant for $P < 0.05$ or $P < 0.01$. Two-way ANOVA with Tukey post-hoc testing was performed using SigmaPlot 14.0 (Systat Software) (Table 1). Reports are provided in Supplemental File 4. RNA gel blots, immunoblots and run-on analyses were repeated at least twice. Representative data are shown.

**Accession numbers**

The following new GenBank Accession Numbers are reported in the manuscript. Novel chloroplast sequences are listed together with their metadata in Supplemental Table 1: KT881175.1, KX014625.1, MN807266.1, MN807267.1, MN812468.1, MN812469.1, MN812470.1, MN812471.1, MN812472.1, MN812473.1, MN812474.1, MN812475.1, MN812476.1, MN812477.1, MN812478.1, MN812479.1, MN812480.1, MN812481.1, MN812482.1, MN812483.1, MN812484.1, MN812485.1, MN812486.1, MN812487.1,
MN812488.1, MN812489.1, MN812490.1, MN812491.1, MN812492.1, MN812493.1. New Ribo-seq and RNA-seq Accession Numbers of the GEO Series GSE174154 are as follows: GSM5288039, GSM5288040, GSM5288041, GSM5288042, GSM5288043, GSM5288044, GSM5288045, GSM5288046, GSM5288047, GSM5288048, GSM5288049, GSM5288050, GSM5288051, GSM5288052, GSM5288053, GSM5288054, GSM5288055, GSM5288056, GSM5288057, GSM5288058, GSM5288059, GSM5288060, GSM5288061, GSM5288062, GSM5288063, GSM5288064, GSM5288065, GSM5288066, GSM5288067, GSM5288068.

**Supplemental Data**

**Supplemental Text.** Photosynthetic phenotype of AB-I plants, RNA gel blot analyses of psbB operon transcripts.

**Supplemental Figure 1.** Photosynthetic parameters of compatible AB-II and incompatible AB-I plants grown under LL or HL conditions.

**Supplemental Figure 2.** RNA gel blot and RNA-seq analyses of the clpP and psbB operons in compatible (AA-I, AA-II, AB-II) and incompatible material (AB-I).

**Supplemental Table 1.** Accession numbers, genome sizes, genetic information, and corresponding nuclear genotypes of *Oenothera* plastomes used for association mapping.

**Supplemental Table 2.** Chloroplast mRNA editotype and cDNA mapping results of three *Oenothera* species from subsection *Oenothera*.

**Supplemental Table 3.** Origin and collector information for the *Oenothera* strains used in this work.

**Supplemental Table 4.** Chloroplast substitution lines, F1 hybrids, and corresponding wild types used in this work.

**Supplemental Table 5.** Oligonucleotides used for the generation of probes for RNA gel blot and run-on transcription analyses.

**Supplemental File 1.** Multiple sequence alignment of the 46 *Oenothera* plastomes used for association mapping provided in the FASTA format (.txt file).

**Supplemental File 2.** Multiple sequence alignment and annotation of the 46 *Oenothera* plastomes used for association mapping provided as SeqMan Pro project file (DNASTAR) (.sqd file).
Supplemental File 3. Annotation of the alignment consensus of the 46 *Oenothera* plastomes used for association mapping provided in the GenBank format (.txt file).

Supplemental File 4. Two-way ANOVA reports generated using SigmaPlot14 software containing statistical analyses for Table 1, Figure 2B, and Supplemental Figures 2A and 2C (.xls file).

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AUTHOR CONTRIBUTIONS
A.Z., D.K., M.A.S., J.N., F.G., A.F., I.M., and S.G. performed the experimental work. All authors analyzed and discussed data. S.G. designed the study and wrote the manuscript. R.B. participated in writing.
Table 1. Comparison of light-acclimation responses of *O. elata* (AA-I), *O. biennis* (AB-II), and their green chloroplast substitution lines AA-II. Plants were cultivated at either 300 µE m$^{-2}$ s$^{-1}$ or 600 µE m$^{-2}$ s$^{-1}$ actinic light intensity. Values are means ± SD (n = 5-6 plants grown in parallel). Different lowercase letters indicate significant differences ($P < 0.05$) according to two-way ANOVA with interactions followed by Tukey post-hoc testing (Supplemental File 4).

| Parameter | AB-II 300µE- | AB-II 600µE- | AA-I 300µE- | AA-I 600µE- | AA-II 300µE- | AA-II 600µE- |
|-----------|--------------|--------------|-------------|-------------|--------------|--------------|
| Chlorophyll a/b | 4.01 ± 0.06$^a$ | 3.60 ± 0.15$^b$ | 4.08 ± 0.05$^a$ | 4.04 ± 0.16$^a$ | 3.92 ± 0.08$^a$ | 3.74 ± 0.15$^b$ |
| Chl. [mg m$^{-2}$] | 631.0 ± 18.1$^{1ab}$ | 548.6 ± 104.3$^c$ | 668.1 ± 27.1$^{1ad}$ | 688.3 ± 58.8$^d$ | 556.7 ± 8.4$^b$ | 464.4 ± 34.1$^e$ |
| Fv/Fm | 0.79 ± 0.01$^a$ | 0.73 ± 0.08$^b$ | 0.81 ± 0.01$^a$ | 0.74 ± 0.06$^b$ | 0.81 ± 0.01$^a$ | 0.71 ± 0.03$^b$ |
| ETRII [µmol m$^{-2}$ s$^{-1}$] | 172.3 ± 14.0$^a$ | 75.1 ± 30.1$^b$ | 171.5 ± 5.9$^a$ | 137.5 ± 51.4$^c$ | 147.9 ± 13.7$^a$ | 84.4 ± 14.7$^b$ |
| gH$^+$ [s$^{-1}$] | 39.2 ± 2.5$^a$ | 27.4 ± 3.8$^b$ | 43.0 ± 2.9$^{ac}$ | 45.8 ± 11.0$^c$ | 40.4 ± 3.6$^a$ | 33.3 ± 2.8$^b$ |
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**Figure 1.** Distribution of *Oenothera* AA-I, BB-III and AB-II/BA-III species, and compatibility/incompatibility relations upon hybridization.

(A) Native distribution of *Oenothera* A and B genome species (pure colors) and their hybridization zones (two-color pattern). Yellow and magenta gradients indicate occurrence of distinct species or subspecies.

(B) Genetic species concept of evening primroses, based on plastome/nuclear genome compatibility and incompatibility, exemplified for the A and B genome species. Species are defined by their combinations of nuclear and chloroplast genomes (boxed in magenta), and are genetically separated by PGIs that occur upon hybridization and vary in the severity of the hybrid phenotype (BB-I white, AB-I and BB-II yellow-green, AA-III bleaching leaf phenotype).
Figure 2. Light-dependent phenotype and physiology of AB-I plants.

(A) Yellow-green (*lutescent*) leaf phenotype and growth retardation under the high-light (HL) condition (right panel). Plants are pictured at the end of the early rosette stage about 30 days after germination. All molecular genetic and physiological analyses presented in this work were performed at this developmental stage. Bar = 5 cm.

(B) Photosynthetic quantification and photooxidative damage. Left panel: Quantification of the components of the photosynthetic electron transport chain by difference absorbance spectroscopy. Note that AB-I plants under HL condition are not able to perform a typical light acclimation response by strongly increasing the content of all redox-active components of the electron transport chain relative to low-light (LL) conditions. Bars represent mean values ± SD [n = 6-8 plants grown alongside]. Right panel: Severe photooxidative damage of AB-I plants under HL conditions, exemplified by measurement of the maximum quantum efficiency of photosystem II in the dark-adapted state (F0/Fm). Bars represent mean values ±SD [n = 6-8 plants grown alongside]. Different lower case letters indicate significant differences (P < 0.05) according to two-way ANOVA with interactions followed by Tukey post-hoc testing (Supplemental File 4).

(C) Blue-native PAGE independently confirming the reduction of the electron transport chain complexes in AB-I under HL. Protein extracts equivalent to 30 μg chlorophyll were loaded in each lane. This experiment was performed two times independently with similar results.
Figure 3. Molecular genetic analyses of the psbM - petN spacer region in compatible (AA-I, AA-II, AB-II) and incompatible (AB-I) material under HL and LL conditions.

(A) Sequence context and insertions/deletions (blue triangle) in the spacer that are specific to plastome I. Arrows indicate transcription start sites.

(B) and (C) RNA gel blot analyses of psbM (B) and petN (C) transcript accumulation under LL and HL conditions. These experiments were performed three times with similar results.

(D) Immunoblot analysis of PetN accumulation under HL. N.t. = Nicotiana tabacum wild type, ΔpetN = petN knockout in N. tabacum (Hager et al. 1999). 100% corresponds to 5 µg chlorophyll equivalent. Tobacco control lines were grown in tissue culture as described in Methods. The experiment was performed independently three times with similar results.
A 144-bp deletion specific to plastome I

clpP

Transcription start site (AA-I, AB-II, AB-I)

TATA box

PpsbB-175 transcription start site (spinach)

PpsbB-52 processing site (AA-I, AB-II, AB-I)

psbT psbH

pbf1

psbB

RNA

psbB petB rrn16 rrn23

psbT psbH

pbf1 (HL)

RNA

petA (HL)

RNA

psbB psbT pbf1 psbH petB petD psbB psbT pbf1 psbH petB petD

Ribosomal footprints

Total RNA

Translation efficiency

Ribosomal footprints

Total RNA

Translation efficiency

AA-I

AA-II

AB-II

AB-I

LL

HL

LL (HL)

RNA

psbB

RNA

psbB petB rm16 rm23 pbf1

AB-I (LL)

AB-I (HL)

F

Pbf1 (LL)

5 kD

Pbf1 (HL)

5 kD

G

AA-I

AA-II

AB-II

AB-I

H

AA-I (HL) vs. AA-II (HL)

Log2 ratio

[AA-I (HL) / AA-II (HL)]

Ribosomal footprints

Total RNA

Translation efficiency

AB-I (HL) vs. AB-II (HL)

Log2 ratio

[AB-I (HL) / AB-II (HL)]

Ribosomal footprints

Total RNA

Translation efficiency
Figure 4. Regulation of the psbB operon in compatible (AA-I, AA-II, AB-II) and incompatible (AB-I) plants under HL and LL conditions.

(A) Physical map of the region in the chloroplast genome containing the clpP and psbB operons. The 144-bp deletion in the intergenic spacer upstream of the psbB operon promoter is indicated. Transcription start sites and mRNA processing sites are indicated by arrows. Note that pbf1 (encoded on the opposite strand) is transcribed from its own promoter.

(B) RNA gel blot analysis of psbB transcript (representative of the whole psbB operon; also see Supplemental Figure 2).

(C) 5'-RACE with and without TAP treatment, a method to map transcription start sites and RNA processing sites of the psbB operon. For details, see Methods.

(D) Run-on transcription analysis of the psbB operon (psbB, petB), including appropriate controls (rrn16, rrn23, and pbf1).

(E) RNA gel blot analysis of pbf1 transcripts.

(F) Immunoblot analysis of Pbf1 accumulation. 100% corresponds to 3 µg chlorophyll equivalent.

(G) RNA gel blot analysis of petA transcript accumulation, serving as a control for a gene outside of the psbB operon.

(H) Ribosomal profiling and translation efficiency of the psbB operon of AA and AB genotypes under HL conditions. Note a tendency of increased translation in AA-I vs. AA-II (left panel). In turn, under the presence of the B genome, translation of psbT and pbf1 is reduced in incompatible AB-I plants when compared to compatible AB-II (right panel).

(B) to (G) With the exception (F) of all experiments were performed independently three times with similar results. (F) was performed two times with similar results.

(H) The experiment is based on three replicates from a pool of about 20 individuals per genotype. Bars represent mean values ±SD.