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THEORETICAL AND APPLIED GENETICS

Dwarfism and male sterility in interspecific hybrids of *Epilobium*

1. Expression of plastid genes and structure of the plastome

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Summary. Reciprocal differences for male sterility, dwarfism and morphological traits have been studied in intra- and interspecific crosses of five *Epilobium* species. Male sterility occurred in two interspecific hybrids with *E. montanum* as the male parent while dwarfism has been found to varying degrees in three interspecific crosses with *E. watsonni.* In contrast to transient differences in plant height and leaf morphology in reciprocal hybrids of the cross between *E. hirsutum* and *E. parviflorum,* male sterility and dwarfism persistently occur as reciprocally different traits which may be influenced by determinants of the cytoplasm. The molecular characterization of the plastid DNA of the parental lines and the F_1 hybrids indicate that the plastome of male sterile and dwarf plants is identical to that of the female parents. Furthermore, in spite of these developmental disturbances, the expression of plastid genes coding for polypeptides of thylakoid-membrane complexes is unchanged. Thus, it seems unlikely that the genetic compartement of the plastids is responsible for the expression of the male sterile or the dwarfed phenotype.

Key words: *Epilobium* – Male sterility – Dwarfism – Plastid DNA – Gene expression

Introduction

In early studies (Baur 1909; Correns 1909) the main arguments for cytoplasmic genetic factors were differences in the progeny of reciprocal crosses. These investigations used variegated male and female parents. The next step in the investigation of differences in reciprocal crosses was the examination of non-variegated interspecific hybrids in some Onagraceae. In *Oenothera* (J. Schwemmle 1938; B. Schwemmle 1962) and *Epilobium* (Renner and Kupper 1921; Michaelis 1940, 1950, 1959; Michaelis and Dellinghausen 1942) phenotypical features influenced by the genetic material of plastids and mitochondria have been described. For *Epilobium* Michaelis showed that genetic factors of the cytoplasm have an extraordinarily strong influence on the phenotype. It is rather unfortunate that the strain *Epilobium hirsutum* Jena which was used by Michaelis is no longer available. As it had certain cytoplasmic peculiarities Prof. P. H. Raven conducted an extensive search in Europe and America, but it could not be found.

Recently the possibility of special molecular features of the genetic material of the plastids of *Epilobium* was analysed in ten different species. The gene arrangement of plastomes and their inheritance has been reported (Schmitz and Kowallik 1986a, b). Here, for the first time, cytoplasmically inherited traits of *Epilobium* are examined at the molecular level. In interspecific combinations where differences between reciprocal hybrids occur, the structure of the plastome and the expression of plastid genes are investigated.

Materials and methods

Plant material and crossing experiments

Seeds of the Eurasian species *E. hirsutum, E. lanceolatum, E. montanum, E. parviflorum,* and the North American species *E. watsonii* were obtained from plants which had been repeatedly self-crossed and thus could be regarded as homozygous. The origin and source of all species has been described elsewhere (Schmitz and Kowallik 1986a).

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The seeds were germinated under controlled conditions, and the individual plants were grown in greenhouses under natural lighting. The crossing experiments were made during 1980–1986. Since the *Epilobium* species mentioned above are self-fertile, the anthers were removed prior to anthesis. After pollination, the blossoms were protected by glassine bags. The same parental plants were used for reciprocal crosses.

Phenotypical analysis of the offspring was carried out for morphology and colour of different parts of the plants at different developmental stages. Pollen viability was determined by staining prefixed pollen grains from open anthers according to Alexander (1969). A minimum of 30 F_1 hybrids of each cross were analysed. When differences between reciprocal F_1 hybrids were encountered, the crosses were repeated and the progeny examined again to ensure that the reciprocal differences were consistently observed.

Isolation of nucleic acids from plastids

The isolation and purification of morphologically intact chloroplasts from *Epilobium,* and the preparation of high molecular weight ptDNA has been described previously (Schmitz and Kowallik 1986a). Plastid RNA (ptRNA) was extracted from plastids according to Westhoff et al. (1981); it was separated from the DNA by repeated precipitation in a 2 M solution of LiCl at 4 °C and was stored in sterile TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA) at –70 °C.

Electrophoresis, transfer and labelling of nucleic acids from plastids

The conditions for restriction enzyme analysis and electrophoretic separation of ptDNA fragments have been described elsewhere (Schmitz and Kowallik 1986a). PtRNA was heat denatured in formamide and formaldehyde according to Carmichael (1980) and separated on formaldehyde gels (1% agarose). The size of individual RNA species was determined using the BRL RNA ladder as length standard. Nucleic acids were transfered to nitrocellulose filters (Schleicher and Schüll, BA 80) according to Thomas (1980). Cloned ptDNA fragments containing polypeptide genes of the spinach plastome were generously provided by Prof. R. G. Herrmann, Munich. The following DNA fragments were used as probes: (1) a 719 bp Eco RI-Sac I fragment containing the 5' half of the gene for the β-subunit of the ATPase from nucleotide –435 to 285 (atpB); (2) an internal 1200 bp Eco RI-Pst I fragment from the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (rbcL); (3) an internal 1680 bp Bam HI fragment from the gene for the chlorophyll a apoprotein of photosystem I (psaA); (4) a 194 bp Eco R I-Dde I fragment containing the 5' half of the gene for cytochrome b_{559} (psbE) from nucleotide –5 to 189 and (5) an internal 1160 bp Bam HI-Xba I fragment from the gene for the chlorophyll a apoprotein of photosystem II (psbB). Hybridization was carried out for 24 h in 65% formamide, 5×SSC and 0.1% SDS at 42 °C. Using a Quanta II screen (DuPont) and Kodak X-ray film XAR-5, exposure time was 4–7 days.

Results

More than 30 intra- and interspecific crosses between five species of the genus *Epilobium* (Table 1), section *Epilobium* were made in order to analyse cytoplasmically inherited features. Reciprocal hybrids of most of these crosses exhibited the same intermediate phenotype with some features resembling more the paternal or maternal parent. Notable reciprocal differences were only obtained in five crosses for pollen fertility and the growth pattern (Table 2). In contrast to these, differences observed in plant height and shape of the leaves in the reciprocal cross between *E. hirsutum* and *E. parviflorum* (Table 2) were transient. They diminished towards the end of ontogeny.

Fig. 1 a, b. Developmental differences of hybrids from reciprocal crosses between *E. hirsutum* and *E. watsonii* **a** with *E. hirsutum* as the pistillate parent **b** *with E. hirsutum* as the staminate parent; this hybrid exhibits pronounced dwarfism (bar: 1 cm)

The hybrids *E. hirsutum* × *E. montanum* and *E. parviflorum* × *E. montanum* were male sterile whereas the reciprocal hybrids produced normally viable pollen. The anthers of *E. hirsutum* × *E. montanum* did not contain any pollen grains. In contrast the hybrid *E. parviflorum* × *E. montanum* sometimes produced small quantities of inviable pollen as revealed by staining (see Materials and methods) and test pollinations. In the hybrid *E. hirsutum* × *E. montanum* male sterility occurred along with heterosis.

In most crosses in which *E. watsonii* was involved, the growth pattern of the offspring was affected. Generally, the growth was more impaired when it was the female parent. When it was the male parent, dwarfism increased in severity in the order *E. lanceolatum, E. par-*

Table 1. Phenotype of *Epilobium* species used in the crossing experiments. PH = Plant height; EAS = Epidermal appendices of the stem; LO = Leaf outlines[a]: elliptic (el), lanceolate (la), oblanceolate (ob), ovate (ov); EAL = Epidermal appendices of the leafs; LM = Leaf margins: Entire (e), serrulate (sl), biserrate (bs), serrate (s); FD = Flower diameter; STS = Stigma shape: entire (e), four-lobed (fl), irregularly lobed (il); FP = percentage of fertile pollen grains

Epilobium species	Origin	PH (cm)	EAS	LO	EAL	LM	FD (mm)	STS	FP %
E. hirsutum L. (1)	Cordoba, Spain	90–110	+ +	la	+ +	sl	2.5–3.0	fl	100
E. hirsutum L. (2)	Siena, Italy	90–110	+	la	+ +	sl	2.6–3.1	fl	100
E. lanceolatum Seb. et Mauri (3)	Barcelona, Spain	80–100	±	la	±	s	1.1–1.3	e	96
E. montanum L. (4)	Berlin, FRG	70–90	–	ov	–	bs	1.2–1.4	fl	92
E. parviflorum Schreb. (5)	Catalania, Spain	40–60	±	ob	±	sl	1.0–1.1	fl	100
E. parviflorum Schreb. (6)	Berlin, FRG	40–60	±	ob	±	sl	1.0–1.1	fl	100
E. watsonii Barbey var. parishii (7)	Berlin, FRG	70–90	±	la	±	sl	0.7–0.9	e	100

[a] The terms for leaf outlines and leaf margins are used according to the definitions of Synge (1956). The number of epidermal appendices is expressed in terms of + and –

Table 2. Phenotype of interspecific hybrids exhibiting differences in reciprocal crosses. Abbreviations are as in Table 1. In crosses with *E. watsonii* the percentage of lethal progeny (1), extreme dwarfs (ed), dwarfs (d), semi-dwarfs (sd) and normal plants (n) is given at the beginning of each line. Intra- and interspecific hybrids without reciprocal differences are described by Schmitz (1985)

Cross		PH (cm)	EAS	LO	EAL	LM	FD (mm)	STS	FP (%)
1×6		60–70	+ +	la	+ +	sl	2.2–2.5	fl	70
6×1		30–40	+	ob	±	sl	2.4–2.6	fl	85
1×4		100–130	+	la	±	sl	2.1–2.3	fl	0
4×1		90–110	+	la	±	sl	2.4–2.7	fl	80
5×4		70–90	–	el	±	bs	1.0–1.2	fl	0
4×5		70–90	–	el	±	bs	1.1–1.3	fl	89
1×7	14% ed	2–5	–	ov	–	e	–	–	–
	68% d	15–30	–	ov	–	e	–	–	–
	18% n	100–110	+	la	+	sl	2.0–2.1	il	55
7×1	80% l	–	–	–	–	–	–	–	–
	20% ed	0.4–0.6	–	ov	–	e	–	–	–
5×7	30% d	3–30	–	el	–	e	–	–	–
	70% n	30–70	–	el	–	sl	1.1–1.3	il	67
7×5	20% ed	0.5–3	–	ov	–	e	–	–	–
	80% d	3–15	–	el	–	e	–	–	–
3×7	15% sd	30–40	±	la	±	s	–	–	–
	85% n	70–80	±	la	±	s	0.7–0.9	e	42
7×3	50% sd	30–40	±	la	±	s	–	–	–
	50% n	90–100	±	la	±	s	1.0–1.1	e	47

viflorum and *E. hirsutum*. Not only the degree of dwarfism between single plants of these crosses differs but the frequency of dwarfism increases from 15% in the cross *E. lanceolatum* × *E. watsonii* to 82% in the cross *E. hirsutum* × *E. watsonii*. The developmental disturbances are expressed to the greatest extent in the offspring of the cross *E. watsonii* × *E. hirsutum* where the plants do not exceed 1 cm height (Fig. 1). All dwarf plants are characterized by wrinkled leaves and the inability to produce flowers. Only in the reciprocal crosses between *E. lanceolatum* and *E. watsonii* are reduced, totally sterile flowers produced. Interestingly, the phenotype of dwarf hybrids can alter during ontogeny. As shown in Fig. 2, renormalized shoots can emerge from dwarf plants which have reached a certain height. The backcross of *E. hirsutum* × *E. watsonii* with *E. watsonii* were not significantly different when compared to the backcross with *E. hirsutum*. The same result was obtained in the other backcrosses with the hybrids *E. parviflorum* × *E. watsonii* and *E. lanceolatum* × *E. watsonii*.

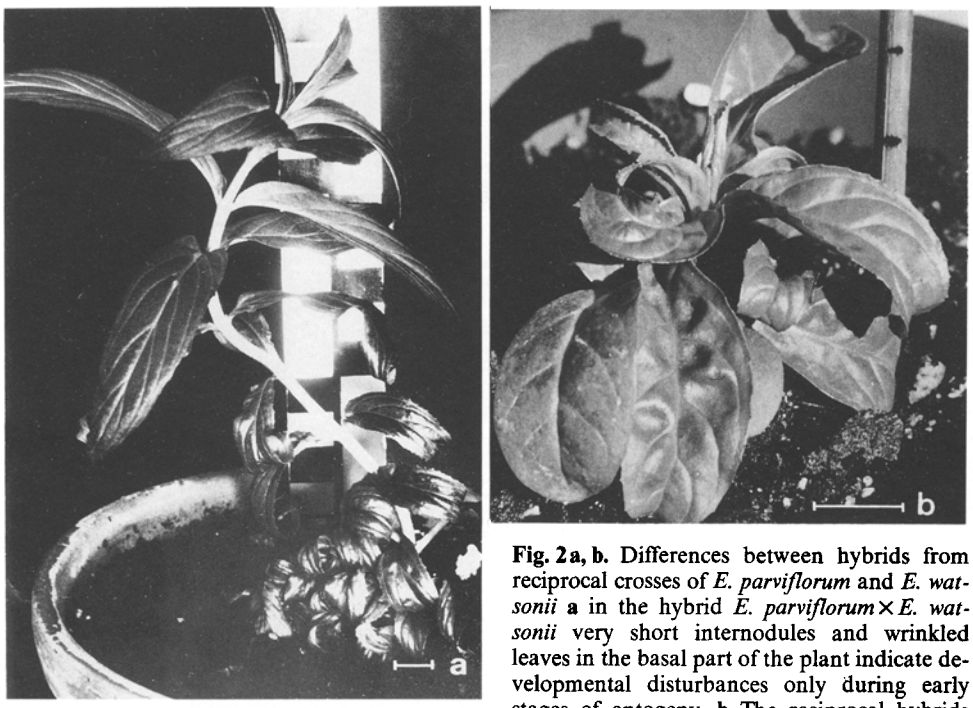

Fig. 2a, b. Differences between hybrids from reciprocal crosses of *E. parviflorum* and *E. watsonii* **a** in the hybrid *E. parviflorum* × *E. watsonii* very short internodules and wrinkled leaves in the basal part of the plant indicate developmental disturbances only during early stages of ontogeny. **b** The reciprocal hybrids persist at a height of about 3 cm (bar: 1 cm)

The offspring of the backcross of *E. hirsutum* × *E. montanum* with the latter alone is male sterile, while in the backcross with the former alone a few fertile plants are found. The same result is obtained in the backcrosses of the male sterile hybrid *E. parviflorum* × *E. montanum*.

The distinctive differences in reciprocal crosses in the occurrence of these developmental disturbances indicate that cytoplasmic factors are involved. For this reason, the DNA and some of the RNAs of the plastids have been examined.

Using suitable restriction enzymes the ptDNA fragment patterns from all *Epilobium* species analysed can be distinguished (Schmitz and Kowallik 1986a). There were no differences in the ptDNA of dwarf hybrids from crosses between *E. watsonii* and *E. parviflorum* as well as from *E. watsonii* and *E. lanceolatum* and normal hybrids; no deviations from the maternal mode of plastid inheritance were encountered (Fig. 3). The same result is obtained by comparing the ptDNA fragment patterns from the male sterile hybrids *E. hirsutum* × *E. montanum* and *E. parviflorum* × *E. montanum*) after digestion with Bam HI (Fig. 4). Digestion of the ptDNAs with Eco RI and Pvu II consistently confirmed these results (data not shown).

Although the ptDNAs of the affected and normal hybrid plants appear identical, alterations in plastid gene expression might account for the apparent cytoplasmic effect. To investigate this possibility, ptRNA was isolated from dwarf and male sterile hybrids and from their female parents. The transcripts from genes representative of both soluble and structural polypeptides have been analysed in hybridization experiments with radioactively labelled-fragments of plastid genes from spinach (Figs. 5 and 6). A single transcript of 1.1 kb from the gene of the cytochrome b_{559} apoprotein and two transcripts of 2.6 kb and 1.9 kb from the gene for the β-subunit of ATPase were detected. In contrast, the genes for polypeptides from the reaction centers of photosystem I and II as well as for ribulose-1,5-diphosphate carboxylase exhibit a more complex transcription pattern. The major transcripts for the rbcL gene range from 1.6 kb to 3.4 kb. Two strong signals with mRNAs of 2.0 kb and 2.2 kb and three further signals with mRNAs up to 5.1 kb are found in a Northern blot with the psbB gene. The psaA gene hybridizes to a major transcript of 6.5 kb and to three minor transcripts of 3.6 kb, 3.1 kb and 2.5 kb.

In their recent review Hagemann et al. (1985) summarize the transcripts of plastid genes from higher plants so far examined. The size and number of the transcripts from the plastid genes of *Epilobium* correspond largely to those of spinach. The mRNAs for the psaA gene, the psbB gene, the rbcL gene and the psbE gene differ only in a few hundred base pairs between *Epilobium* and spinach. The two transcripts, however, for the atpB gene are 2.8 and 2.6 kb in spinach (Zurawski et al. 1982) but 2.6 and 1.9 kb in *Epilobium*.

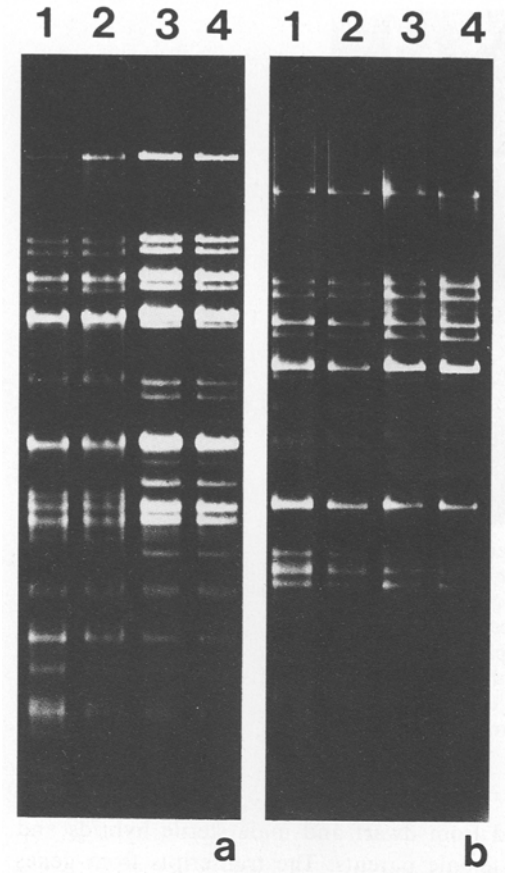

Fig. 3a, b. Restriction fragment patterns of ptDNA from dwarf (*lanes 2, 4*) and normal (*lanes 1, 3*) hybrids after digestion with Bam HI. **a** *E. watsonii* × *E. lanceolatum* (*lanes 1, 2*) and *E. lanceolatum* × *E. watsonii* (*lanes 3, 4*). **b** *E. parviflorum* × *E. watsonii* (*lanes 1, 2*) and *E. watsonii* × *E. parviflorum* (*lanes 3, 4*)

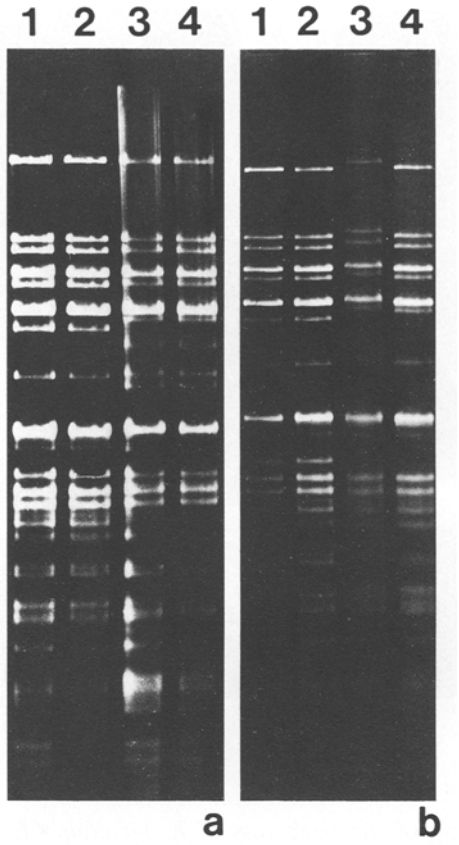

Fig. 4a, b. Restriction fragment patterns of Bam HI digested ptDNA from **a** the male sterile hybrid *E. parviflorum* × *E. montanum* (*lane 4*) in comparison to the fertile hybrid *E. montanum* × *E. parviflorum* (*lane 2*) and to the parental plants *E. montanum* (*lane 1*) as well as *E. parviflorum* (*lane 3*). **b** The male sterile hybrid *E. hirsutum* × *E. montanum* (*lane 4*) in comparison to the fertile hybrid *E. montanum* × *E. hirsutum* (*lane 2*) and the parental plants *E. montanum* (*lane 1*) and *E. hirsutum* (*lane 3*)

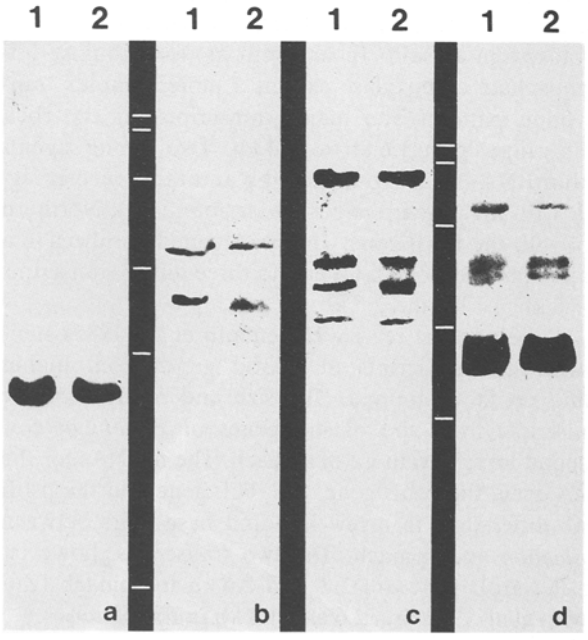

Fig. 5a–d. Northern blots of RNA from dwarf plants. PtRNA from dwarf hybrids (*lane 1*) of the cross *E. lanceolatum* × *E. watsonii* and the female parent *E. lanceolatum* (*lane 2*); RNA was separated on formaldehyde gels (1% agarose) and probed with the radioactively labelled psbE gene (**a**), the atpB gene (**b**), the psaA gene (**c**) and the psbB gene (**d**). The *white bars* between block **a** and **b** as well as **c** and **d** indicate the position of RNA bands used as length standards (9.49 kb; 7.46 kb; 4.4 kb; 2.37 kb; 1.35 kb; 0.33 kb)

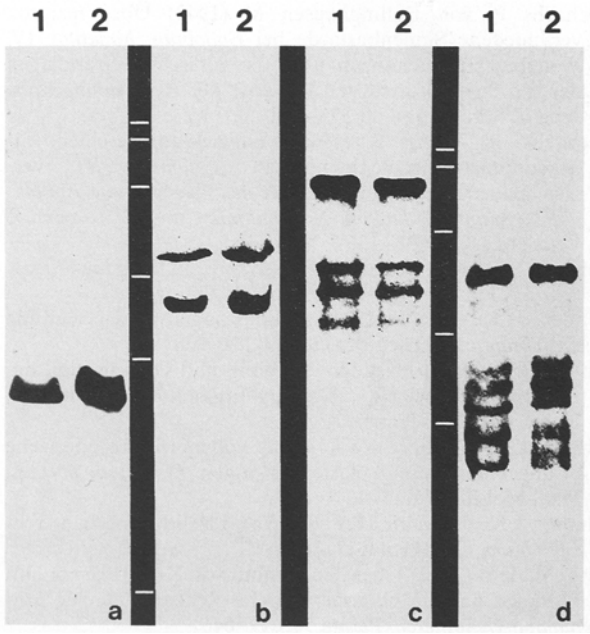

Fig. 6. Northern blots of RNA from male sterile plants. PtRNA from male sterile hybrids (*lane 1*) of the cross *E. hirsutum* × *E. montanum* and their female parent *E. hirsutum* (*lane 2*); RNA separated on formaldehyde gels was probed with the genes described in **Fig. 5** with the exception of block **d** showing a hybridization with the rbcL gene

Discussion

Dwarfism and male sterility are phenotypical traits which may be caused by nuclear genes or by genetic factors in the cytoplasm. In *Epilobium,* cytoplasmically influenced dwarfism occurs in interspecific crosses with *E. hirsutum* Jena (Michaelis 1951, 1962; Michaelis and Wertz 1935). In contrast, in crosses between *E. anagallidifolium* and *E. lactiflorum* dwarfism seemed to result from the action of nuclear genes (Kytövouri 1976). Dwarf hybrids of crosses between *E. watsonii* and three other *Epilobium* species reported in this contribution are probably the result of incompatible combinations of cytoplasmic factors and nuclear genes. The involvement of cytoplasmic factors in the expression of the dwarf phenotype is suggested by qualitative or quantitative differences of this trait in reciprocal crosses. Certainly, reciprocal cross differences are not a sure proof of cytoplasmic effects (Caspari 1948; Michaelis 1954; Oehlkers 1964). But as the backcross *E. hirsutum* × *E. watsonii* with the latter alone as the male parent leads to approximately the same number of dwarf plants as the backcross with the former alone, it is improbable that a mendelian factor accounts for this feature. However, the impaired growth pattern of the backcross hybrids is not simply the result of a maternally inherited factor in the cytoplasm since no significant reciprocal differences were obtained in these backcrosses.

The male sterile phenotype in the hybrids *E. hirsutum* × *E. montanum* and *E. parviflorum* × *E. montanum* is transmitted to all of the offspring when these hybrids are backcrossed to *E. montanum.* These genetic results suggest that male sterility has a cytoplasmic location. The heterosis of *E. hirsutum* × *E. montanum* may also be correlated with cytoplasmic factors since it is not expressed in the reciprocal hybrid. As the backcross of *E. parviflorum* × *E. montanum* with *E. parviflorum* leads again to some fertile plants, it can be assumed that nuclear genes of *E. parviflorum* which are more compatible whith the cytoplasm of *E. parviflorum* than the nuclear genes of *E. montanum* are responsible for the restoration to fertility.

In order to identify the genetic compartments of the cytoplasm which are responsible for the differences in the reciprocal crosses, a molecular analysis of the plastids was undertaken (a further contribution will deal with the mitochondria). In contrast to *Nicotiana* (Kung et al. 1981) no correlation between cytoplasmic male sterility and variations in the ptDNA fragment pattern has been found in *Epilobium.* No deviations from the maternal mode of plastid inheritance and no alterations in ptDNA restriction fragment patterns produced by three frequently cutting enzymes were found in the male sterile hybrids. However, it cannot be excluded that the combination of a plastome with a certain nuclear background may lead to male sterility as described in *Oenothera* (Göpel 1970). Nevertheless, analysis of the transcription of a representative set of plastid genes reveals no significant quantitative or qualitative alterations between fertile and sterile plants equipped with the same plastids. Thus, it is concluded that the hybrid nucleus of the male sterile *E. hirsutum* × *E. montanum* has no traceable effect on the expression of the plastid genes analysed.

While in some cases cytoplasmic male sterility seems to be accompanied by alterations of the genetic material of the plastids (Kung et al. 1981; Vedel et al. 1982; Vedel and Mathieu 1983) and in many cases with rearrangements of the mitochondrial genome (reviewed by Levings 1983), no such specific correlations have been reported for dwarfism. The analysis of plastids from dwarf *Epilobium* hybrids suggests that dwarfism is not accompanied by alterations in the plastome. Furthermore, the expression of plastid genes in dwarfed hybrids does not differ from normal plants. These data suggest that the genetic compartment of the plastids does not play an important role in the expression of the dwarf or sterile phenotype. The next step will be an analysis of mitochondrial DNA and the expression of mitochondrial genes to determine whether this organelle is responsible for these traits.

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