Theor Appl Genet (1999) 99:578–586

J.E. Frey · H. Müller-Schärer · B. Frey · D. Frei

Complex relation between triazine-susceptible phenotype and genotype in the weed *Senecio vulgaris* may be caused by chloroplast DNA polymorphism

Received: 28 December 1998 / Accepted: 20 February 1999

Abstract The weed *Senecio vulgaris* acquired high levels of resistance to triazine herbicides soon after the latter's introduction. As in most weeds, triazine resistance is conferred by a point mutation in the chloroplast *psbA* gene that negatively affects the fitness of its carrier. To assess levels of triazine resistance in *S. vulgaris* field populations, we adopted a PCR-RFLP-based molecular diagnostic test recently developed for the triazine resistance-conferring region of the *psbA* gene of other weeds, including *Brassica napus, Chenopodium* spp. and *Amaranthus* spp., and compared these molecular results to the phenotypic response after triazine application. A highly significant linear correlation was found between phytotoxic symptoms and biomass reduction. Variability in phenotypic response was not only found between populations or inbred lines of *S. vulgaris* but also within replicates of the same inbred line. No clear relationship, however, was found between the DNA restriction pattern and the phenotypic response to triazine application, thereby throwing doubt on the use of such molecular diagnostic tests to track triazine resistance in *S. vulgaris*. Our results indicate that the chloroplast genome of *S. vulgaris* is polymorphic and that the level of polymorphism may be variable within single leaves of individual plants. We discuss the possible genetic basis of this polymorphism and its consequence for the acquisition and inheritance of chloroplast-based traits.

Key words CpDNA polymorphism · Heteroplasmy · *psbA* gene · Triazine resistance · *Senecio vulgaris*

Communicated by. G. Wenzel

J.E. Frey (✉) · B. Frey · D. Frei
Swiss Federal Research Station for Fruit-Growing,
Viticulture and Horticulture, CH-8820 Wädenswil, Switzerland
e-mail: juerg.frey@faw.admin.ch
Fax: +411 783 6432

H. Müller-Schärer
Département de Biologie/Unité d'Ecologie,
Université de Fribourg, Pérolles, CH-1700 Fribourg, Switzerland

Introduction

Senecio vulgaris L. (Asteraceae) is a self-compatible, strongly self-fertilizing short-lived annual with an almost world-wide distribution (Mitich 1995). *Senecio vulgaris* ssp. *vulgaris* var 'vulgaris' is presumed to be the most common and widespread plant taxon (Kadereit 1984). It is reported to be an important weed in horticulture, orchards, ornamentals, tree nurseries and in viticulture in many parts of the world (Cloutier et al. 1991; Cross and Skroch 1992; Müller-Schärer and Wyss 1994). *Senecio vulgaris* was the first species to demonstrate herbicide resistance, i.e. to s-triazine herbicides in the late 1960s (Ryan 1970). Such triazine-resistant populations are now common and widely distributed, especially in Europe and North America (Holt and LeBaron 1990). Resistance to triazine herbicides is inherited cytoplasmically in the vast majority of weed species, including *S. vulgaris*. The gene transferring triazine resistance is located in the chloroplast genome, and transmission is mainly maternal (Scott and Putwain 1981; Darmency 1994, and references cited therein). The mutation involves a single base substitution in the *psbA* chloroplast gene which codes for a photosystem II membrane protein to which triazine herbicides bind (Hirschberg et al. 1984). The expected mutation rate giving rise to gamete transmissible triazine resistance is very low and was estimated at 1×10^{-9} to 1×10^{-12} (Duesing 1983). So far all of the investigations on triazine-resistant species, including isogenic resistant and susceptible lines of *S. vulgaris* have indicated that the triazine resistance mutation is associated with a cost in fitness (Jasieniuk et al. 1996).

Recently, a co-ordinated research project has been initiated in Europe to evaluate the potential of biological control of *S. vulgaris* using the naturalized rust pathogen *Puccinia lagenophorae* Cooke (Basidiomycetes: Uredinales) (Frantzen and Hatcher 1997; Müller-Schärer and Scheepens 1997). Because herbicide-resistant plants displaying reduced fitness may be more susceptible to pathogen infection, biological control may be an important complement to solving the problems encountered

with the increasing herbicide resistance brought about through chemical control of weeds. A potential negative cross-resistance to herbicide-pathogen interactions would further increase joint effects of chemical and biological control measures and also help reduce the build-up of resistance to both herbicides and pathogens (Gressel and Segel 1990).

The purpose of this study was to establish a simple and robust polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based diagnostic tool to assess triazine resistance in *S. vulgaris* and thus to estimate relative occurrence of resistance in field populations subjected to varying levels of herbicide pressure and disease incidence. Such a method has recently been developed by Cheung et al. (1993) for several weed species (*Brassica napus*, *Chenopodium* spp., *Amaranthus* spp.), for which it was highly accurate. We first assessed biomass reductions and phytotoxicity symptoms in response to the application of four levels of atrazine concentrations. Although the two sets of variates generally were in agreement at the "accession" level (i.e. population level), we found intermediate levels of resistance and considerable variation within some of the field populations and inbred lines studied. We then applied the published molecular triazine diagnostic to our plant material and tried to explain the observed phenotypic sensitivity patterns and the corresponding molecular data at the individual plant level. We found a good correlation between phenotypic performance upon triazine treatment and chloroplast genotype in highly resistant plants, but no correlation was found in the plants with intermediate levels of resistance. The underlying genetic mechanisms for this lack of correlation in intermediately resistant plants are discussed, and a re-evaluation of the acquisition, reproduction and inheritance of chloroplast-based traits is suggested.

Materials and methods

Plant material

Senecio vulgaris plants originating from Switzerland and the United States, composed of six inbred lines (including two backcrossed lines) and one field-collected population, were investigated. We used two inbred lines of *Senecio vulgaris* (ssp. *vulgaris* var 'vulgaris') from Switzerland, ELS (CHI, Unterehrendingen, CH) and CH II (Charrat, CH), as described by Wyss (1997) and four inbred lines of *S. vulgaris* that were kindly provided by Jodie Holt (University of California, Riverside, USA) and that have previously been studied and described by McCloskey and Holt (1990): R (triazine-resistant parental *S. vulgaris* biotype, originally collected in Washington, USA), S (triazine-susceptible parental biotype, originally collected in Oregon, USA), $R \times S_{BC6}$ (triazine-resistant sixth generation backcrossed *S. vulgaris* biotype with R cytoplasm and S nuclear genome) and $S \times R_{BC6}$ (triazine-susceptible sixth generation backcrossed *S. vulgaris* biotype with S cytoplasm and R nuclear genome). For comparison, *S. vulgaris* seeds of a field population from Praz, FR, Switzerland (PRAZ), and seeds of each of two populations of *Solanum nigrum* L. (SOLNI 1, 2) and *Amaranthus retroflexus* L. (AMARE 1,2) from field sites near Nyon, VD, Switzerland, were incorporated into the analysis. To assess the abundance of cpDNA polymorphism, we included field populations of *S. vulgaris* from two locations in the Netherlands (DUN; LIEN) and one from the UK (LAN) (as described in Wyss 1997) and 12 individuals of *Chenopodium album* (CHEAL) from Switzerland in this study.

Sampling and herbicide treatment

Plants from the different origins (called "accessions" in the following) were sown in seed trays on 14 December 1995 and kept in the greenhouse of the Department of Biology, University of Fribourg. Two weeks later, 12 plants per "accession" were transferred singly to plastic pots, 9 cm in diameter and filled with nutrient amended peat (Floragard TKS2). The pots were placed randomly on a tray in the greenhouse and kept at a 16-h day (approximately 250 µmol m^{-2} s^{-1} at 23°C and 60% humidity) and a 8-h night (at 16°C and 80% humidity).

On 11 January 1996, when the plants had reached on average six true leaves, the third or fourth leaf of each plant was removed and cut in half along the middle vein; one-half was stored in liquid nitrogen, the other half at –20°C in a freezer for later molecular analysis. On the same day, 3 randomly selected plants per "accession" were treated with one of four triazine concentrations (Gesaprim, 50% atrazine), corresponding to 0.25 kg a.i. ha^{-1}, 1.5 kg a.i. ha^{-1} (normal field concentration), 10 kg a.i. ha^{-1} and a control without atrazine. For each treatment, plants were arranged on a surface of 0.62 m^2 and sprayed for 31 s, corresponding to 500 l ha^{-1}, using an aerospray [Birchmeier 2000, standard nozzle (0.16) with 1 ml s^{-1} at 200 kPa and a spray distance of 30 cm]. Effects of the herbicide were assessed on four occasions (3, 6, 9 and 12 days after application). Phytotoxic symptoms of all plants were individually ranked according to the EWRS classification scheme for plant tolerance (scaling from 1 to 9; with 1 indicating no symptoms/healthy plant and 7–9 heavy damage to total kill) (Anonymous 1992). On 25 January 1996, i.e. 14 days after the herbicide application, the number of leaves was counted and above ground biomass assessed after drying at 60°C for 36 h.

DNA extraction procedure

DNA of individual plants was extracted using a DNA extraction protocol modified after Labarca and Paigen (1980). Leaf disks of 1–2 mm^2 in area were cut out of the leaf halves stored in the freezer and homogenized in 100 µl of 2×TNE lysis buffer (20 m*M* TRIS, pH 7.4, 20 m*M* EDTA, 2 *M* NaCl) in a 500-µl Eppendorf tube using a sterile plastic pestle (Kontes); the tube was then heat-treated immediately for 10 min at 85°C. Extracted DNA was quantified using a TKO 100 fluorometer (Hoefer) and frozen at –20°C for later use. On average, 100 ng DNA per leaf disk was obtained with this DNA extraction method. Three microliters of the extraction solution, corresponding to approximately 1–5 ng template DNA, was used for amplification. The remainder of each leaf and the second half of the leaves that was stored in liquid nitrogen were separately extracted using a plant DNA extraction kit (Qiagen), and the extracted DNA of both leaf halves was pooled. Within-leaf variation in the level of cpDNA polymorphism was assessed by comparing the pooled DNA vs. DNA from the leaf disks.

Polymerase chain reaction

PCR was performed in a total reaction volume of 50 µl, containing 10 m*M* TRIS-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatine, 200 µM dNTPs, 1.5 Units *Taq* polymerase (Oncor Appligene, Basel), 1–5 ng template DNA and 0.5 µ*M* each of the two fluorescence-labelled, sequence-specific primers P1 (5′–ATG-AGGGTTACAGATTTGGTC-3′) and P2 (5′-AGATTAGCACG-GTTGATGATA-3′) described by Cheung et al. (1993). The DNA was amplified on a Perkin-Elmer model 9600 thermal cycler using the following protocol: a 5-min denaturation at 95°C, then 40 cycles of 45 s at 94°C, a 45-s annealing at 55°C and a 1-min exten-

sion at 72°C. The amplification was completed by a final 7 min extension step at 72°C. Five µl of the amplification product was electrophoresed on a 1.5% agarose gel run at 9 V/cm for 60 min to control amplification success.

Restriction analysis

Four microliters of the amplification product was incubated with 2 U of *Mae*I (Boehringer) in 10 µl total volume of incubation buffer (20 m*M* TRIS-HCl, pH 8.0, 250 m*M* NaCl, 6 m*M* MgCl2, 7 m*M* 2-mercaptoethanol) at 45°C for 3°h, followed by a 5-min inactivation of the restriction enzyme at 70°C. Of the digested DNA 2 µl was analysed together with an internal standard (Tamra 500, Perkin-Elmer) by capillary electrophoresis on an ABI310 Genetic Analyzer (Perkin-Elmer). The amplification products of several samples with polymorphic restriction patterns were purified by running 20 µl on a agarose gel, cutting out the 277-bp product and extracting the DNA in 30 µl of high purity H_2O (Milli-Q) using QIAquick gel extraction columns (Qiagen). These purified templates were then used for restriction analysis for a comparison to the results obtained with unpurified amplification products to assess any potential effects of star activity.

Sequencing and sequence analysis

A dye-terminator cycle-sequencing reaction (Perkin-Elmer) was performed on the purified 277-bp amplification fragments and the products then sequenced on an ABI310 Genetic Analyzer (Perkin-Elmer). For the purpose of comparison, corresponding sequences of ten plant types (listed in the legend of Fig. 2) were downloaded from Genebank and aligned to the rice (*Oryza sativa*) sequence using the computer programme GENEJOCKEY II (Biosoft).

Results

Phenotypic sensitivity

Analysis of variance showed significant effects of plant accession, herbicide dose and its interaction term on phytotoxicity symptoms and biomass ($P<0.001$). Plant responses greatly varied among the accessions, ranging from complete resistance (even to the highest herbicide concentration that corresponds to approximately 7 times the field rate) to high susceptibility (above 80% biomass reduction even at approximately 16% field rate), thus indicating various levels of herbicide resistance (Table 1). In addition, variability in plant responses within the three replicates was found within some of the field populations as well as, unexpectedly, within some of the inbred lines of *S. vulgaris*, as indicated by coefficients of variation of up to 88%.

Overall, biomass reduction and phytotoxicity symptoms were highly correlated (r_s=–0.83, $P<0.001$). A highly significant negative linear correlation was found in all accessions showing susceptibility to the herbicide (Table 1).

Restriction patterns

Amplification using the primers P1 and P2 resulted in a single fragment of the expected size of 277 bp in all

Table 1 Effect of atrazine herbicide application on various "biotypes" of *Senecio vulgaris* (SENVU), *Amaranthus retroflexus* (AMARE), and *Solanum nigrum* (SOLNI): 1=control; 2=0.25 kg ha^{-1}; 3=1.5 kg ha^{-1} (field rate); 4=10 kg ha^{-1}

Plant origin[a]	Code	Biomass				Phytotoxicity symptoms				r_s[b]	Percentage susceptible cpDNA type[c]			r_s[d]
		g/plant	% control							Overall				Overall
		1	2	3	4	1	2	3	4		2	3	4	
SENVU inbred lines														
R	USA	0.73	151.6	131.4	107.6	1.0	1.0	1.0	1.3	0.04^{ns}	0.0	0.0	0.0	–
R×S	USA	1.53	112.7	94.6	75.9	1.0	1.0	1.0	1.0	–	0.0	0.0	0.0	–
S	USA	1.96	66.3	47.5^{f}	7.4	1.0	1.7	2.0	4.7	–0.84**	88.1^{h}	88.8^{i}	88.4	–0.19^{ns}
S×R	USA	0.56$^{g\ e}$	51.1^{g}	26.2^{h}	14.4	1.0	3.7^{g}	3.3^{f}	8.0	–0.83**	88.5^{i}	58.5	87.0	–0.47^{ns}
ELS	CH	1.52	16.9^{h}	19.7	3.3^{f}	1.0	4.3	4.0	6.3	–0.93***	86.6^{f}	86.8^{f}	88.0^{g}	–0.26^{ns}
CH II	CH	1.31^{f}	85.4^{g}	47.2	34.3^{f}	1.0	1.0	2.0^{g}	1.3^{f}	–0.43^{ns}	92.6^{g}	92.4^{h}	91.8^{g}	0.52^{ns}
Field populations														
SENVU, PRAZ	CH	0.99	43.4^{f}	6.4	3.4^{g}	1.0	2.7	5.7	8.3	–0.97***	85.1^{f}	85.4^{f}	87.6	–0.31^{ns}
AMARE 1	CH	0.93	67.6^{f}	63.3^{f}	70.5	1.0	1.0	1.0	1.0	–	0.0	0.0	0.0	–
AMARE 2	CH	0.86	68.5	72.3	99.7	1.0	1.0	1.0	1.0	–	0.0	0.0	0.0	–
SOLNI 1	CH	1.80	32.3^{h}	28.3^{h}	6.6^{f}	1.0	2.0^{g}	3.7	5.3	–0.89***	78.9^{g}	80.2	79.9	–0.19^{ns}
SOLNI 2	CH	1.58^{f}	128.6	134.7	106.1^{f}	1.0	1.0	1.0	1.0	–	0.0	0.0	0.0	–

ns, Not significant; ** $P<0.01$; *** $P<0.001$; [a] See text for details; [b] Spearman rank correlation coefficient between biomass and phytotoxicity symptoms at 8 days after treatment; [c] Median of three *Mael* digestions from each sample; [d] Spearman rank correlation coefficient between percent susceptible cpDNA type and phytotoxicity symptoms at 8 days after treatment (here, only plants treated with the herbicide were considered); [e] Variability within replicates [coefficient of variation, after correction for small sample size (Sokal and Rohlf 1981)]: f: CV 30–50%; g: CV 51–70%; h: CV 71–90%; i: CV >91%

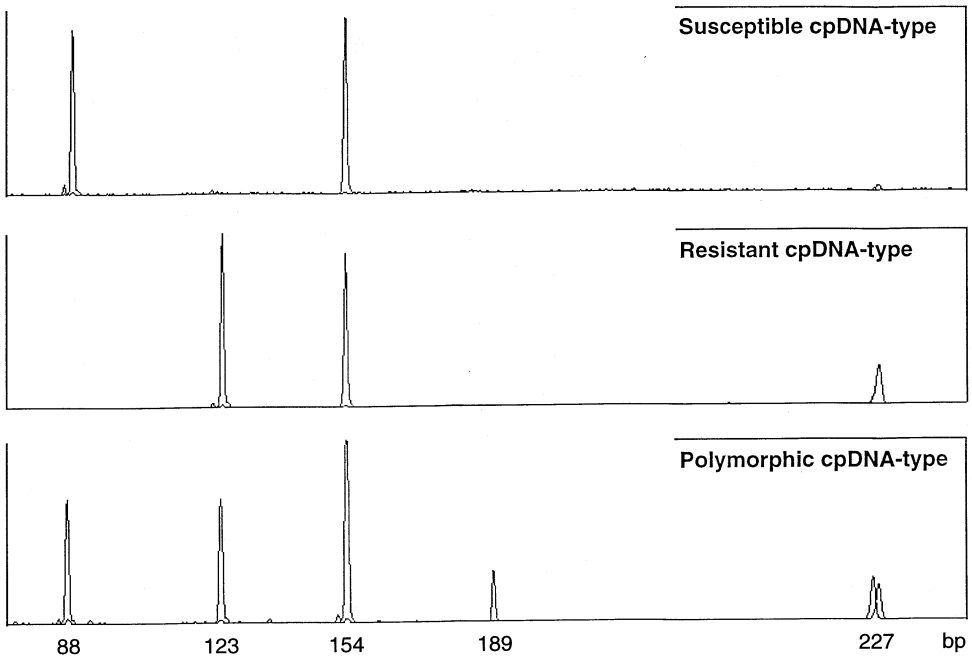

Fig. 1 Restriction patterns obtained after *Mae*I digestion of the amplified *psbA* gene sequence encompassing the triazine resistance conferring point mutation in *S. vulgaris*. The 35-bp fragment is invisible because it contains no labelled primer. A double peak around 277 bp occurs because of a slight variation in the migration of the two different colour labels used for primer labelling

three weed species. Restriction of this PCR fragment with *Mae*I resulted in a total of six DNA fragments (277, 189, 154, 123, 88, and 35 bp) in the entire *S. vulgaris* population. Mapping the fragment lengths suggested a second *Mae*I recognition site in *S. vulgaris* and *S. nigrum* that seemed to be absent in *A. retroflexus* and *C. album* and that generally cut at position 154 (from the 5'end of the amplified fragment) in all individuals irrespective of their triazine resistance status (Fig. 1). Because triazine resistance is achieved by a point mutation that eliminates the *Mae*I recognition sequence at position 88 bp, this resulted in two restriction fragments in individuals carrying the triazine resistance-conferring point mutation and in three restriction fragments in those lacking this point mutation (Fig. 1). The restriction pattern in *S. vulgaris* and *S. nigrum* is thus different from that found in *A. retroflexus* and *C. album*, in which the resistant genotype lacks a *Mae*I recognition sequence in the amplified fragment, whereas the susceptible genotype has a recognition sequence at position 189. Thus, resistant genotypes of both weeds generally produce one fragment of 277 bp (corresponding to the undigested amplification product), while the susceptible genotypes produce two fragments of 189 bp and 88 bp (Fig. 1). Our sequencing results (Genbank accession number AF061287) confirmed the existence of the second *Mae*I recognition sequence in *S. vulgaris* (Fig. 2). Comparison of the *S. vulgaris* sequence to other known sequences of this genome region showed almost complete homology at the amino acid level, with polymorphisms being constrained to silent mutations (Fig. 2). The triazine resistance-conferring point mutation also occurs in *Brassica napus* (Reith and Straus 1987), and the second *Mae*I recognition sequence at position 123 bp has also been reported in Alfalfa, Broadbean, and Tobacco (see Fig. 2).

Intra-individual polymorphism in cpDNA

The occurrence of a second *Mae*I recognition site in the amplification product of *S. vulgaris* was an important feature in revealing the occurrence of DNA polymorphism in the amplified fragment. Incomplete digestion is generally used as an explanation for cpDNA restriction patterns that would otherwise imply polymorphism. Because the quality of each single restriction reaction could be assessed in our assay, polymorphism has to be inferred where, for example, both the 123-bp and the 88-bp fragments co-occurred (Fig. 1). The fragment patterns obtained from the purified 277-bp fragments were identical to those obtained using the unpurified amplication product, indicating that the amplification buffer did not induce *Mae*I star activity (data not shown).

Intra-individual cpDNA polymorphism was found in all field populations and in the inbred lines of *S. vulgaris* (Table 2). Polymorphism was also found in *S. nigrum* and in *C. album*, but not in *A. retroflexus*. On average, 83% of the *S. vulgaris* individuals from field populations and over 78% of those from the inbred lines were polymorphic with respect to their cpDNA (Table 2). No cpDNA polymorphism was found in the inbred lines selected for triazine resistance. However, a preliminary assessment in 24 individuals of an earlier generation of the R and R×S lines revealed 37% polymorphic individuals showing at least small amounts of the susceptible cpDNA type (data not shown). The difference in cpDNA types was also very pronounced in *S. nigrum*, where the triazine-resistant phenotypes completely lacked the 88-bp fragment diagnostic for the susceptible cpDNA type, whereas all of the triazine susceptible phenotypes were polymorphic, containing both the 88-bp and the 123-bp fragment (Table 2).

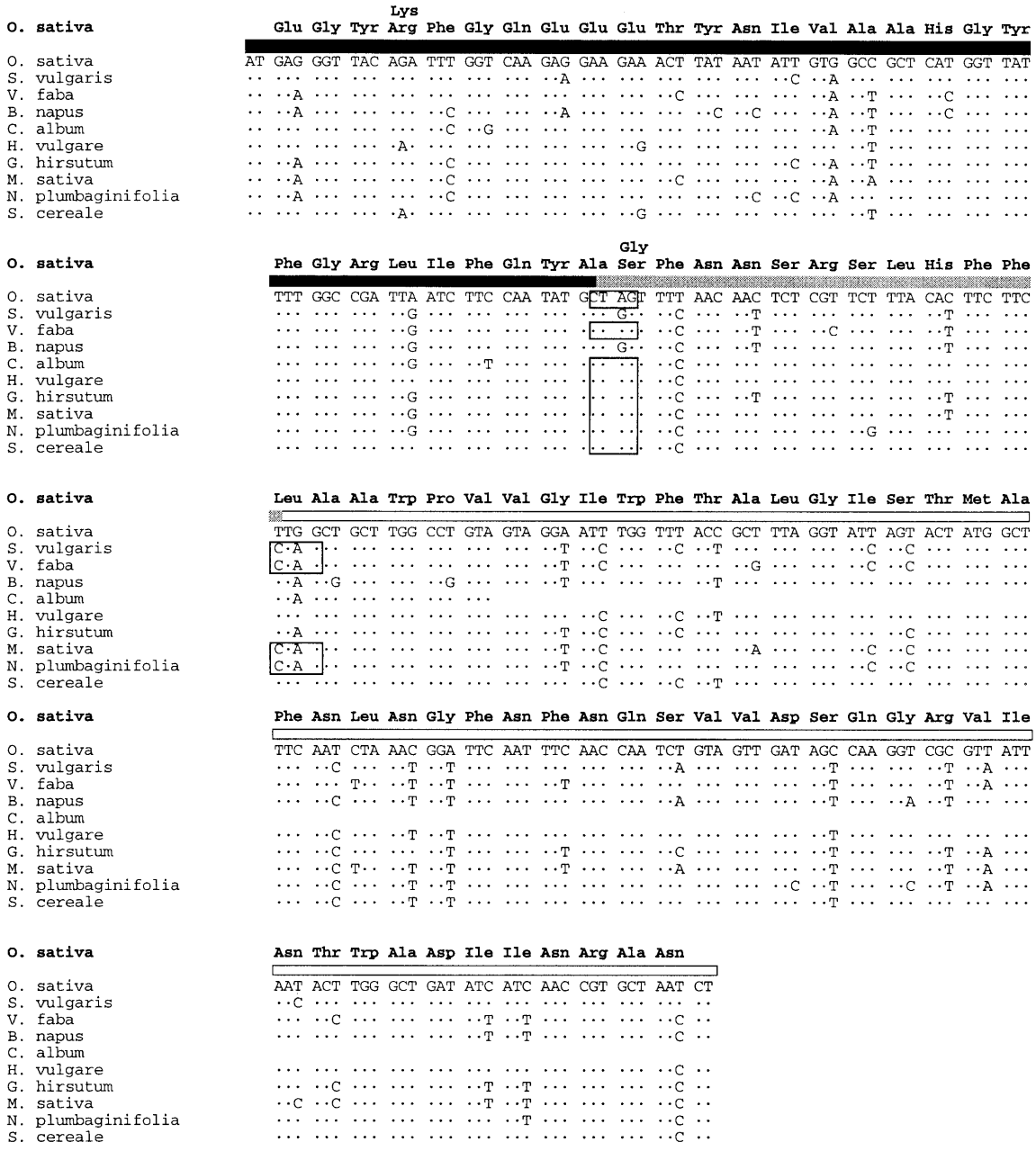

Fig. 2 Alignment of cpDNA sequences encompassing the triazine resistance-conferring region of the *psbA* gene (reference species=*Oryza sativa*, amino acid sequence *above bar*, nucleotide sequence *below bar*). The sequence for *S. vulgaris* was established in this study (Genbank accession number AF061287); the others were downloaded from Genebank [accession number]: Alfalfa (*Medicago sativa* [X04973]), barley (*Hordeum vulgare* [M38374]), *Brassica napus* [M36720], Broadbean (*Vicia faba* [X17694]), *Chenopodium album* [M19053], cotton (*Gossypium hirsutum* [X15885]), rice (*Oryza sativa* [M36191]), rye (*Secale cereale* [X13327]), tobacco (*Nicotiana plumbaginifolia* [X08016]). *Mae*I restriction sites are *boxed* and the restriction fragments are indicated by *bars of different shades*

The difference in the level of polymorphism of cpDNA types between DNA extraction from small leaf disks and that from the remaining leaf tissue indicated that variation in the level of polymorphism occurred within single plant leaves (Table 3). The same result was obtained in a more detailled study that found variation in the level of cpDNA polymorphism within single leaves as well as among different leaves of individual plants (Frey 1999).

Correlation between genotypes and phenotypic sensitivity to atrazine

Overall, we found a significant correlation between the percentage of susceptible cpDNA types within plants

and the phytotoxicity symptoms 8 days after atrazine treatment (Table 1). However, the relationship between the molecular data and the observed phenotypic response after herbicide application was only accurate for the resistant phenotypes, i.e. those with a monomorphic resistant cpDNA type. No significant correlation was found for any of the polymorphic plants, i.e. those plants with variable susceptibility (Table 1). The degree of correlation in general increased with increasing herbicide dose, i.e. with increasing phytotoxicity symptoms (Fig. 3). As the tested plant individuals all originated from a highly selfed inbred line (except the field population from PRAZ), they are genetically very similar. Therefore, this effect indicates compensatory capabilities of polymorphic plants. Polymorphic plants may overcome the deleterious effects of herbicide treatments because they dispose of a limited amount of resistant cpDNA types, i.e. survival may depend on the amount and tissue distribution of resistant cpDNA types. The population CH II was different from the others as it is genotypically polymorphic with a high proportion of susceptible cpDNA types (approximately 70–90%; Table 2), yet its phenotypic performance was that of highly resistant plants (Fig. 3).

Table 2 Abundance of intra-individual cpDNA polymorphism among inbred lines and field populations of *Senecio vulgaris* (SENVU) and among field populations of *Amaranthus retroflexus* (AMARE), *Solanum nigrum* (SOLNI) and *Chenopodium album* (CHEAL). The estimate is conservative as the co-occurrence of the undigested fragment of 277 bp was attributed to incomplete digestion

Species/population	*n*	Polymorphic	
	plants	*n*	%
SENVU inbred lines			
R	9	0	0
R×S	9	0	0
S	9	9	100
S×R	9	9	100
ELS (Unterehrendingen, CH)	9	9	100
CH II (Charrat, CH)	9	9	100
LAN (UK)	12	12	100
SENVU field populations			
PRAZ	9	9	100
DUN (Dünen, NL)	12	9	75
LIEN (Liendern, NL)	12	9	75
Other species			
AMARE 1	9	0	0
AMARE 2	9	0	0
SOLNI 1	9	0	100
SOLNI 2	9	0	0
CHEAL	12	2	17

Discussion

Although generally believed to be uncommon and transitory, enough evidence for chloroplast heteroplasmy has now accumulated that makes it a very plausible mechanism to explain the high degrees of cpDNA polymorphism observed in this study. In recent years, mitochondrial and chloroplast heteroplasmy has been found to be much more common than previously assumed (e.g. Birky et al. 1989 and references therein). Several human disorders originate from a mutation in the mtDNA that occurs in a heteroplasmic state and where the phenotypic appearance of symptoms generally depends on the fraction of mitochondria carrying the mutation. For example, up to approximately 60% mutated mitochondria can

Table 3 Difference in percentage susceptible cpDNA type between the leaf disk DNA extraction and the DNA extraction of the remaining leaf area of each plant sample (median of 3 *Mae*I digestions of each sample; unpaired *t*-test on arcsine square root transformed data)

Species/population	Percentage susceptible cpDNA type (range)		*P*
	DNA of leaf disk	DNA of remaining leaf area	
R	0.0 (–)	0.0 (–)	–
R×S	0.0 (–)	0.0 (–)	–
S	88.8 (74.9–89.6)	94.6 (94.4–95.0)	0.0598
S×R	58.5 (38.9–60.4)	92.4 (91.4–94.7)	0.0024
ELS	86.8 (83.6–89.2)	72.5 (46.3–84.5)	0.1535
CH II	92.4 (86.4–93.5)	71.7 (64.6–88.2)	0.0864
PRAZ	85.4 (85.2–89.5)	93.8 (90.5–94.6)	0.0299
AMARE 1	0.0 (–)	0.0 (–)	–
AMARE 2	0.0 (–)	0.0 (–)	–
SOLNI 1	80.2 (77.8–82.6)	89.3 (80.3–90.3)	0.1337
SOLNI 2	0.0 (–)	0.0 (–)	–

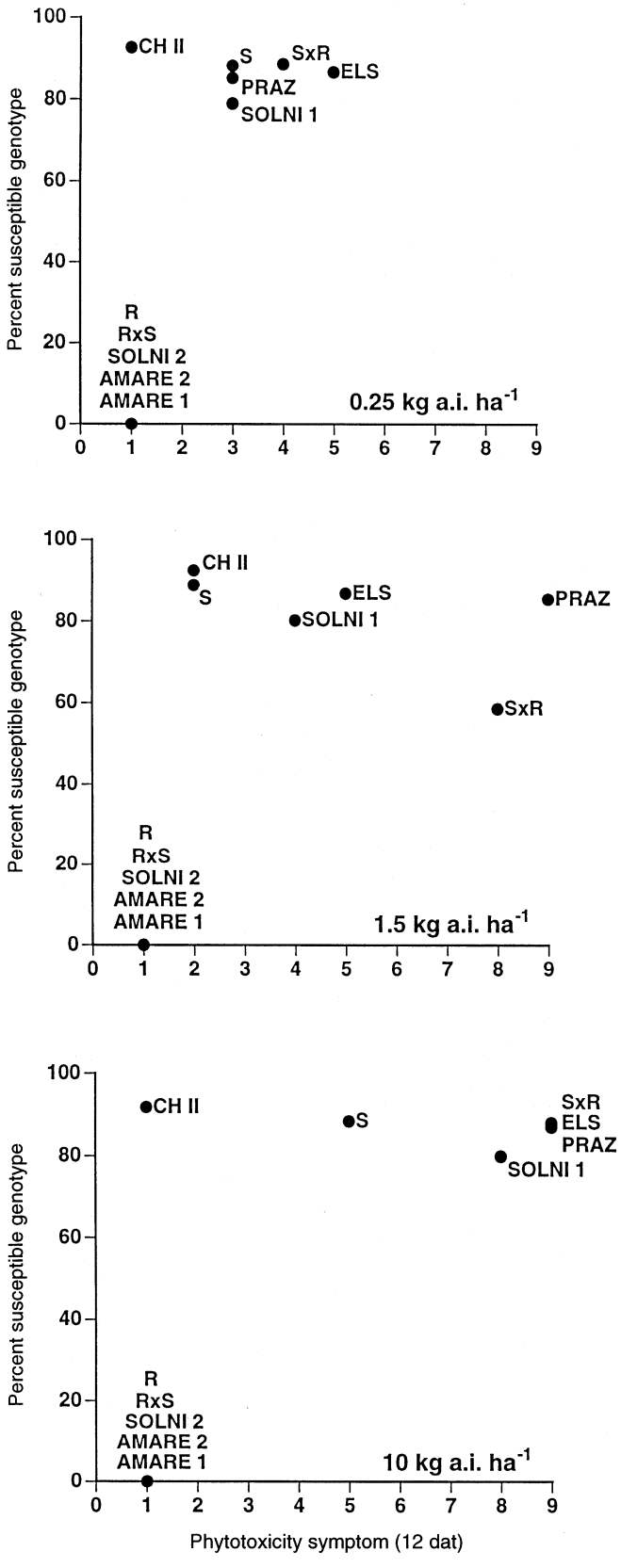

Fig. 3 Genotype assessment (median percentage of susceptible genotype of three *Mae*I digestions per sample) and phytotoxicity symptoms (*dat*, days after treatment) of *S. vulgaris* after application of various concentrations of atrazine (cf. Table 1 for details)

be compensated for by the remaining mitochondria in Leber's Hereditary Optic Neuropathy (Mashima et al. 1995), and about 10% wild-type mtDNA content is reported to fully protect against the phenotypic effects of the MELAS mutation (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; Tanno et al. 1995).

Several hundred chloroplasts may exist within a single plant leaf cell, and each may contain more than 900 copies of DNA (Bendich 1987). Over 10,000 copies of cpDNA may thus occur within a single cell, providing an optimal substrate for generating and maintaining polymorphisms. Classically, biparental chloroplast transmission resulting in chloroplast heteroplasmy has been used to explain the occurrence of cpDNA polymorphism. Biparental chloroplast transmission has been reported for about one-third of all plant genera (Kirk and Tilney-Bassett 1978). In some cases, vegetative segregation is slow, causing heteroplasmy to persist for prolonged periods (Gillham et al. 1991 and references therein). Because phenotypic markers are lacking in most species and because the performance of restriction fragment length polymorphism with low DNA concentrations is rather poor, the frequencey of heteroplasmy and biparental inheritance may have been underestimated (Reboud and Zeyl 1994). Therefore, it is likely that cpDNA polymorphism and heteroplasmy in plants is more frequent than hitherto assumed.

The level of cpDNA polymorphism in *S. vulgaris* is variable. Because the plant individuals of the replicates and those used in the different treatments were all sister plants originating from the same mother plant we expected them to be genotypically similar. However, we found considerable variation within and between replicates, suggesting that the level of intra-individual cpDNA polymorphism is variable (Table 2). In fact, variation between 5% to over 75% resistant cpDNA-types within as well as between leaves of polymorphic plant individuals of *S. vulgaris* was recently found (Frey 1999).

The performance of plants in herbicide bioassays will be determined by the frequency and distribution of resistant chloroplasts, given that susceptible chloroplasts are knocked out by the herbicide treatment. Only those plants will survive that can maintain sufficient photosynthetic activity based on the remaining chloroplast population. Single cells and individual plant parts may have varying amounts of resistant chloroplasts. The accurracy of a molecular diagnostic test to estimate a plant's triazine resistance status may therefore strongly depend on how the sample for DNA extraction was taken. Since we used only 2 mm^2 of leaf area for our DNA extraction, we may have missed the 'average' cpDNA type of plant individuals that will largely determine their performance in a resistance bioassay. Finally, even if the DNA is extracted from entire plants to establish its average cpDNA content, variation among leaves may provide a highly susceptible individual with enough resistant tissue to survive herbicide treatment. Within-plant variation with respect to the level of cpDNA polymorphism may thus ex-

plain those cases in which a phenotypically resistant plant showed a susceptible genotype and vice-versa.

An alternative explanation for the mismatch between phenotypic behaviour and genotype may be nuclear introgression (e.g. Parfait et al. 1998 on mtDNA introgression). If parts of the chloroplast genome containing the amplified sequence have been introduced into the cell nucleus, they will be co-amplified regardless of their function in the nucleus. Thus, the pattern produced by the (active) cpDNA sequence may be confused by the amplification product of the nuclear, potentially inactive DNA sequence. However, this explanation seems to be unlikely as we observed both extremes of purely resistant as well as purely susceptible cpDNA types. If, for example, the resistant genotype had introgressed into the nucleus, the corresponding fragment would co-amplify and, therefore, the purely susceptible cpDNA type would not be observed, and vice-versa. This hypothesis can further be tested by running the analysis using DNA from isolated chloroplasts. A lack of correspondence between phenotype and genotype may also be caused simply by using the wrong genetic marker. For example, the population CH II shows a highly susceptible genotype containing 70–90% susceptible cpDNA types, yet its phenotypic performance is equal to the highly resistant populations. This may be caused by a mutation of T to A or G in the nucleotide triplet normally coding for serine at position 92 bp. This mutation would not affect the *Mae*I recognition site, but it would replace the susceptible amino acid serine by arginine, which may also confer triazine resistance to some degree. This hypothesis can be tested by sequencing. Finally, triazine resistance in *S. vulgaris* may be based on a mechanism that is different from the point mutation in the *psbA* gene. However, the cytoplasmic inheritance of resistance to triazine analogues is well-established for *S. vulgaris* (Scott and Putwain 1981). In fact, the sole exception to the cytoplasmic inheritance of resistance to triazine analogues is velvetleaf (*Abutilon theophrasti*), where it is controlled by a single, partially dominant, nuclear gene (Andersen and Gronwald 1987; Jasieniuk et al. 1996).

In conclusion, we found high levels of polymorphism in a cpDNA sequence determining triazine resistance in *S. vulgaris* that make it necessary to use sophisticated molecular assays combined with thorough sampling strategies to enable accurate molecular typing. The most probable mechanism producing this polymorphism is chloroplast heteroplasmy. Because of this polymorphism, sublethal selection with triazine may induce dramatic frequency shifts in the occurrence of resistant phenotypes in the progeny of surviving plants. This suggests that epigenetic inheritance may play an important role in the evolution of triazine resistance and other cytoplasmically inherited traits in *S. vulgaris* and possibly other weed species.

Acknowledgements We would like to thank Marius Käser for conducting most of the greenhouse studies and laboratory bioassays, Didier Forcioli for his comments on an earlier draft of this manuscript and Gabriela Wyss for helpful discussions and for supplying the seeds for the European seed samples. The American seed samples were kindly provided by Jody Holt. The project was financed in part by the Swiss Office of Education and Science (to a project of COST-816), and by SPP-Environment module Biodiversity (NF-5001-050250). The experiments comply with the laws of Switzerland.

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