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The origin and evolution of weed beets: consequences for the breeding and release of herbicide-resistant transgenic sugar beets

P. Boudry, M. Mörchen, P. Saumitou-Laprade, Ph. Vernet, H. Van Dijk

Laboratoire de Génétique et Evolution des Populations Végétales, URA CNRS 1185, Université de Lille 1, 59655 Villeneuve d'Ascq Cedex, France

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Abstract. Populations of weed beets have expanded into European sugar beet production areas since the 1970s, thereby forming a serious new weed problem for this crop. We sampled seeds in different French populations and studied mitochondrial DNA, chloroplast DNA and life-cycle variability. Given the maternal inheritance of the mitochondrial and chloroplastic genomes and the nuclear determinism of the annual habit, we were able to determine the maternal origin and evolution of these weed beet populations. Our study shows that they carry the dominant allele "B" for annual habit at high frequency. The main cytoplasmic DNA type found in northern weed beet populations is the cytoplasmic male-sterile type characteristic of sugar beets. We were able to determine that these populations arise from seeds originating from the accidental pollinations of cultivated beets by adventitious beets in the seed production area, which have been transported to the regions where sugar beets are cultivated. These seeds are supposedly the origin of the weed forms and a frequently disturbed cultivated environment has selected for annual habit and early flowering genotypes. We discuss the consequences of the weed beet populations for the breeding, seed production and release of herbicide-resistant transgenic sugar beets.

Key words: Life cycle variability – Cytoplasmic DNA markers – *Beta vulgaris* L. – Weeds

Introduction

Many plant species can be found both as crop and weed (Harlan 1987). This situation can be due to the evolution of a cultivated type to a weed type, the selection of cultivated forms from weedy races (Pickersgill 1981) or a change of habitat of a wild form closely related to a cultivated species (Keller 1989). In all these cases, this phenomenon involves evolution from a cultivated or a wild type to a weed type. This evolutionary change depends on the existing genetic variability within the species, its distribution among types and gene exchanges between each type. The study of gene exchange between cultivated species and wild relatives is becoming more and more important in view of the possible release of engineered plants as commercial varieties (Ellstrand and Hoffman 1990). The appearance of new invasive forms as a result of hybridization between related species has recently been reviewed by Abbott (1992), and gene exchange from the crop to the weed has often led to a more aggressive weed (Barrett 1984). However, very few studies are available on the risk assessment of the appearance of new invasive forms due to hybridization between genetically modified crops and wild relatives (Williamson et al. 1990).

In beet (*Beta vulgaris* L), three types can be distinguished: cultivated types, wild types and adventitious or weed types. These are fully inter-fertile and are considered to be sub-species of the species *Beta vulgaris* L. (De Bock 1986; Ford-Lloyd 1986; Ford-Llyod and Hawkes 1986). Cultivated types (*B.v.* ssp. *vulgaris* L.) include sugar beet, fodder beet, table beet and leaf beet. Wild beets (*B.v.* ssp. *maritima* (L.) Arcangeli) of northern Europe are restricted to the coastlines, but in the Mediterranean area they can grow more inland (De Bock 1986). Adventitious and weed beets can be

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Correspondence to: P. Boudry

found either on waste and fallow land or in fields as weeds (Horsney and Arnold 1976). Beet is one of the few major European crops to be geographically sympatric with its close wild relatives.

During the 1970s, adventitious beet populations expanded in Europe and became a serious weed problem in sugar beet fields (Boiteau and Christmann 1977). The United Kingdom, Ireland, Germany, Belgium, Spain and France are the main European countries directly affected by the expansion of the area troubled by weed beet. In the United States, the presence of weed beets were reported as early as 1920 (Carsner 1928), and they expanded quickly into California (Johnson and Burtch 1959; McFarlane 1975). Many methods, both chemical (difficult at the intra-specific level) and mechanical, have been proposed to control these new weeds, but the problem persists and is getting worse (Longden 1976; Pichenez and Guiraud 1977; Martens et al. 1977). This expansion appears to be linked to recent changes in agronomic and breeding practices: the use of selective herbicides instead of hand or mechanical weeding, short rotations, the moving of seed production areas to southern Europe and the development of triploid varieties (Evans and Weir 1981). Many sugar beet producers are waiting for transgenic varieties resistant to total herbicides to become available in order to eliminate weed beets. Such genetically modified beets have already been developed by several plant breeding companies (De Greef et al. 1989; Dhalluin et al. 1992; Saat and De Laat 1992).

The main phenotypic difference between cultivated and weed beets is their earliness of flowering. Cultivated beets are biennials: they normally do not flower during their first year but need a long period of vernalization. Weed beets, however, are annuals: their early flowering allows them to reproduce in the frequently disturbed cultivated environment. These different life cycles can be due to (1) the absence of any vernalization requirement, a trait determined by the major "bolting" gene B (Munerati 1931; Owen 1954); (2) a low vernalization requirement, a quantitative character permitting floral induction following cold days in spring (Lasa 1977).

The principal hypotheses concerning the origin of populations of weed beets are based on these above-mentioned points (Horsney and Arnold 1976; Desprez 1980; Evans and Weir 1981). The first hypothesis proposes an origin in seed production areas and is based on the possibility of accidental hybridization between cultivated seed parents (genotype "bb") and annual adventitious beets (genotype "BB") present in certain seed production areas. Hybrids carrying the dominant allele for annual habit "B" would then produce seeds, generating the new weed beet populations (Boiteau and Christmann 1977). The occurrence of just such a possible hybridization was recently confirmed by Santoni and Bervillé (1992). The second hypothesis, called "backward selection", postulates their formation in situ in crop fields of cultivated beet, from cultivated beet itself. In this case, the weeds have "bb" genotypes, although very sensitive to vernalization factors, and selection would have decreased the quantitative requirement for vernalization (Johnson 1954). Pollen transport from coastal wild populations to cultivated fields has also been suggested as being involved in weed beet evolution (Horsney and Arnold 1979).

As the origin of these recently expanded weed beet populations is controversial, the objective of this research is to study various weed beet populations using cytoplasmic and nuclear genetic markers. The combined use of cytoplasmic and nuclear genetic markers has been successful in recent studies in determining the origin of new invasive forms in for example, sunflower (Rieseberg et al. 1990). In our study, the variability of mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) was studied using restriction fragment length polymorphism (RFLP) methods. The maternal transmission of organelles allowed us to determine the maternal origin of the studied plants, and to subsequently compare their types with those known in cultivated beets and French wild coastal populations. Among these types, the cytoplasmic male sterility (CMS) type is known to be restricted to cultivated beets (Powling and Ellis 1983; Mikami et al. 1985; Bonavent et al. 1989; Saumitou-Laprade et al. 1991, 1993). The B allele is known to be absent in cultivated beets and present in some French coastal and inland populations. Therefore, on the same plants, we studied the variability of two life-cycle characters, vernalization requirement and earliness flowering both of which are traits determined by nuclear genes. Earliness in "B" genotypes is known to be a highly heritable quantitative trait ($h^2 \approx 0.8$; estimation from inland south-western French populations under greenhouse conditions; Van Dijk and Boudry 1992).

The synthesis of these data on cytoplasmic and developmental variability clarifies the origin and evolution of the populations studied. This allows us to distinguish between the various hypotheses concerning the origin of weed beets. Consequences in assessing the risks of releasing of transgenic sugar beets are discussed in the light of these results.

Materials and methods

Plant material

Seeds from 85 mother plants were sampled from 12 inland populations in two types of regions in France (see Fig. 1):

– South-western France (3 populations: Nérac, Lectoure 1, Condom; plants sampled in different locations around Lectoure were added as "Lectoure 2"): an area where several beet breeding

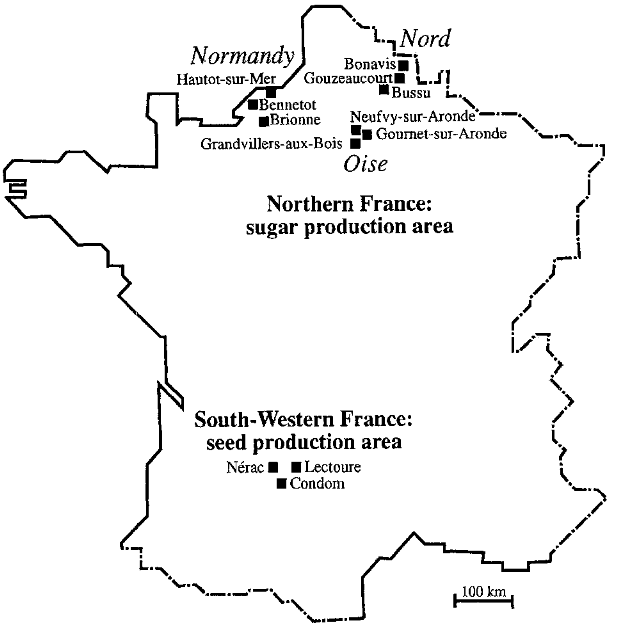

Fig. 1. Location of the 12 French inland populations studied

companies have been producing seed for over 40 years. In this region adventitious beets may be seen along roadsides, on waste or follow land and as weeds in sunflower fields.

- Northern France (three sub-regions): Normandy (3 populations: Brionne, Bennetot, Hautot-sur-Mer), Oise (3 populations: Neufvy-sur-Aronde, Gournet-sur-Aronde, Granvillers-aux-Bois), and Nord (3 populations: Bussu, Gouzeaucourt, Bonavis): in these sugar beet production areas, weed forms are only present in sugar beet fields, where they cannot be killed by selective herbicides.

Life-cycle variability

A total number of 765 seeds collected from 61 mother plants (considered to represent half-sib families as the species is self-incompatible) were sown synchronously, and the 558 germinated seedlings (mean number and standard error per family: 8.6 ± 0.4) were grown in pots under homogeneous greenhouse conditions. Annual habit was detected by growing plants under long days (16–18 h) at a temperature between 20 °C and 25 °C. Under such photothermal conditions, only individuals carrying the B allele are able to bolt and flower; "bb" genotypes remain vegetative as they require vernalization for flowering induction (Margara 1960). Plants were classified as flowering or non-flowering 130 days after germination. Proportions of flowering plants were compared using the Chi-square test. Earliness of flowering time was recorded as the number of days between germination and anthesis and compared using an analysis of variance.

Mitochondrial RFLPs

Characterization of mitochondrial genetic information was performed by RFLP methods and non-radioactive detection as described in Saumitou-Laprade et al. (1993). Each of the 85 half-sib families was represented by one offspring. Hybridization with three probes, *atp6* (Dewey et al. 1985), *pBv4* (Saumitou-Laprade et al. 1993), and N_{vulg}/N2, on total DNA digested by restriction enzyme *Eco*RI permitted the differentiation of the six known mitochondrial types in beets. The N_{vulg}/N2 probe is the differential fragment of the mitochondrial N2 *Eco*RI restriction profile we isolated directly after electrophoretic separation from an agarose gel (using the QIAEX kit QIAGEN, according to the protocol provided for large DNA fragments of 5–45 kb). This new probe allows us to distinguish the N_{vulg} type from the N2 type in total DNA extracts.

Chloroplast RFLPs

Characterization of chloroplast genetic information was performed on the same plants as above by rapid RFLP methods using complete non-radioactively labelled cpDNA as a probe hybridized on restricted total DNA extracts (Forcioli et al. in press). *Eco*RV, *Hin*dIII and *Sma*I restriction profiles were revealed, as they are known to be potentially polymorphic (Mikami et al. 1985; Kishima et al. 1987, Saumitou-Laparade et al. 1991; Forcioli et al. in press).

Results

CpDNA variability

Results obtained from the 85 half-sib families are presented in Table 1. Out of the five chloroplast types known in cultivated and wild coastal beet types four were found: three of them, previously identified as types 2, 3 and 4 in Saumitou-Laprade et al. (1991), could be distinguished by *Hin*dIII restriction profiles. Polymorphism of *Sma*I restriction profiles divided type 3 into two types, called 3 and 5 in this study, as has been described by Kishima et al. (1987). *Eco*RV patterns were found to be monomorphic. The four cpDNA types we found were therefore named types 2, 3, 4 and 5.

Type 4 was the most frequent cpDNA type found in Northern populations: 45 out of 51 individuals; the other 6 individuals were found to be type 3. The reverse situation was found in south-western populations, where type 3 was the most frequent type: 21 out of 34 plants. Two other cpDNA types (2 and 5) were found at low frequencies. These two types also appear to be present in some wild French coastal populations (Kishima et al. 1987; Bonavent et al. 1989; Saumitou-Laprade et al. 1991; Forcioli et al. in press).

MtDNA variability

The results obtained for mtDNA variability for the same 85 plants mentioned above are also presented in Table 1. Figure 2 gives an example of a hybridization profile of individuals obtained using the *pBv4* probe and the enzyme *Eco*R1, which allowed the differentiation of four types (for this purpose named a–d). Of the six mitochondrial types already known in beets (Saumitou-Laparade et al. 1993), five were found. Two plants from the Lectoure 1 population (south-western France) exhibited a new mitochondrial type not yet described, which we named "H". This new type was distinguished using the N_{vulg}/N2 probe.

Table 1. Cytoplasmic DNA types, as defined by a combination of cpDNA and mtDNA types, found in the inland beet populations studied and their comparison to the types known in sugar beet and french wild coastal beets. Observed cpDNA types were distinguished using labelled complete cpDNA as a probe hybridized on total DNA extracts restricted by *Hin*dIII, *Sma*I and *Eco*RV enzymes and are named according to Saumitou-Laprade et al. 1991 (see also text). Observed mtDNAs were distinguished using combined results from the probes *atp*6, *pBv*4 and TO/N2 on *Eco*RI restriction profiles; they are named according to Saumitou-Laprade et al. 1993 (see text)

	Cytoplasmic DNA types						Total number of plants
CpDNA types	2	3	3	4	5	5	
MtDNA types	S	N_{vulg}	N2	S_{vulg}	H	N3	
Sugar beet[a]	–	O-type	–	CMS	–	–	/
French wild coastal populations[a]	+	+	+	–	–	+	/
Inland populations							
South-western France							
Nérac	1	5	–	5	–	–	11
Lectoure 1	–	4	–	–	2	2	8
Lectoure 2	1	5	–	1	–	–	7
Condom		7	–	1	–	–	8
Total for south-western France	2	21	–	7	2	2	34
Northern France							
Nord:							
Bussu	–	1	–	11	–	–	12
Gouzeaucourt	–	–	–	1	–	–	1
Bonavis	–	–	–	1	–	–	1
Oise:							
Neufvy-sur Aronde	–	4	1	3	–	–	8
Gournet-sur-Aronde	–	–	–	4	–	–	4
Grandvillers-aux-Bois	–	–	–	9	–	–	9
Normandy:							
Brionne	–	–	–	8	–	–	8
Bennetot	–	–	–	4	–	–	4
Hautot-sur-Mer	–	–	–	4	–	–	4
Total for northern France	–	5	1	45	–	–	51

[a] Data from Mikami et al. 1985; Bonavent et al. 1989; Saumitou-Laprade et al. 1991, 1993; Forcioli et al. in press

In the south-western inland populations, five mitochondrial types were found: S_{vulg}, N_{vulg}, S, N3 and H. N_{vulg} is the most frequent type, being present in 21 plants out of 34, 7 plants carry the S_{vulg} type. In the northern inland populations, only three mitochondrial types were observed: S_{vulg}, N_{vulg}, and N2. S_{vulg} is largely dominant, with 45 out of 51 plants carrying this type.

Cytoplasmic types

Table 1 presents the distribution of the cytoplasmic types found in the inland populations studied, as defined by a combination of cpDNA and mtDNA types. Six cytoplasmic types were found: cpDNA types 3 and 5 are shared by two mtDNA types within a population (respectively N_{vulg} and N2 in Neuvfy-sur-Aronde; H and N3 in Lectoure 1). Five of these cytoplasmic types have been observed in both cultivated and wild coastal beets (Powling and Ellis 1983; Mikami et al. 1985; Saumitou-Laprade et al. 1991; Forcioli et al. in press). The new mtDNA type H that is associated to cpDNA type 5 defines a sixth cytoplasmic type.

The distribution of cytoplasmic types is very different between south-western and northern populations. The CMS type is the most frequent type found in the northern populations studied (45 out of 51). This type also occurred in south-western populations (7 out of 34), but in the latter the O-type occurs the most frequently (21 out of 34). The other four types are rare and restricted to only 1 or 2 populations.

Life-cycle variability

The study of life-cycles concerns the presence or absence of the vernalization requirement, a trait determined by the major gene "B", and earliness of flowering of "B" genotypes. Our results are summarized in Table 2. The present experiment showed that the inland populations contained a large proportion (86%) of plants capable of flowering without prior vernalization. We were able to conclude, therefore, that the B allele is largely present in all sampled inland populations. As this allele is not present in cultivated types, its origin

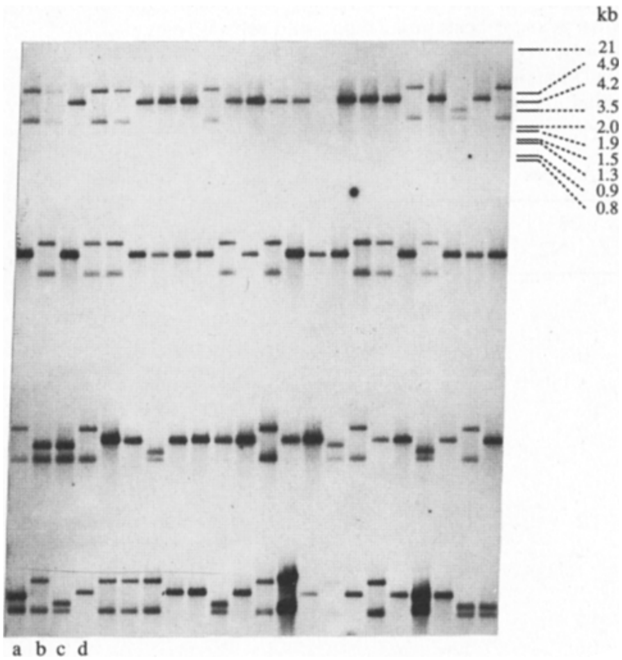

Fig. 2. Southern blot analysis of the *Eco*RI-digested total DNA from 96 individuals. The hybridization was performed with the *pBv*4 probe. Lambda DNA digested with *Eco*RI and *Hin*dIII is shown as a molecular weight marker. Four restriction patterns can be distinguished (a–d) (see Saumitou-Laprade et al. 1993)

has to be in non-cultivated populations. South-western inland populations exhibited the highest percentage (95%) of plants that flowered without previous vernalization, but there were also significant differences between populations in this region ($P = 0.03$). In the northern inland populations, the percentage of flowering plants was significantly lower than in the south-western populations: 82% ($P < 0.0001$). This percentage varied significantly between sub-regions ($P < 0.0001$) and within two of these sub-regions, between populations (Normandy: $P = 0.031$; Oise: $P = 0.035$; Nord: $P = 0.1$).

The results concerning earliness of flowering in "B" genotypes are also given in Table 2. The earliest plant flowered 31 days after germination. The mean and standard error of age at flowering time in south-western populations is 46±0.6 days. This trait varied between the three populations ("Lectoure 2" was excluded from the test as being plants sampled in different locations) ($P = 0.05$) and was significantly different in northern population: 54±0.6 days ($P < 0.0001$). there were significant differences between northern sub-regions ($P < 0.0001$) and within 2 of these sub-regions, between populations (Normandy: $P = 0.0001$; Oise: $P = 0.83$; Nord: $P = 0.001$).

Discussion

By combining our results for cytoplasmic DNA and developmental traits in the studied inland populations we were able to test hypotheses concerning the origin of these populations. We can distinguish between the various hypotheses previously proposed for the origin of weed beets in sugar production areas. These results will have consequences on the control of weed beet populations, sugar beet breeding and the risk assessment of transgenic sugar beets.

Maternal origin of inland beet populations

Two main types of mtDNA are found in modern cultivars: S_{vulg}, which is characteristic of the CMS type (Owen 1945) and used world-wide for the production of hybrid varieties, and N_{vulg}, O-type, used as a male-sterility maintainer in parental seed production. In wild coastal populations, one finds the N_{vulg} type and four other types: N1, N2, N3 and S (Saumitou-Laprade et al. 1993). Four cpDNA types have been described in wild beets; a fifth type has been detected only in CMS-cultivated cytoplasm (Mikami et al. 1985; Bonavent et al. 1989; Saumitou-Laprade et al. 1991). The CMS-cultivated cytoplasm type can therefore be easily distinguished by its characteristic mtDNA and cpDNA types.

The mtDNA and cpDNA types we found in the inland populations studied are present in cultivars or wild coastal populations. The association between cpDNA types and mtDNA types was found to be very strong, as would be expected due to maternal inheritance of both organelles (Mikami et al. 1985): no recombination between the two genomes occurs. Cytoplasmic type, defined by both cpDNA and mtDNA, therefore appears to be a highly informative marker for the assessment of the origin of the inland populations studied.

The CMS type is the main cytoplasmic type in weed beet populations of the sugar beet production areas. As this cytoplasmic type (cpDNA type 4/mtDNA S_{vulg} type) has never been observed over a large number of French coastal accessions (Mikami et al. 1985; Bonavent et al. 1989; Saumitou-Laprade et al. 1991, 1993; Forcioli et al. in press), we have to reject seed transport from coastal populations as being the principal means by which northern inland populations originated. As the CMS type is the cytoplasmic type carried by all sugar beets in sugar production areas, we confirm the hypothesis of a cultivated maternal origin for the northern inland populations. A few plants were found with O-type cytoplasm: they could have derived from old cultivars carrying this cytoplasmic type or by the seed transport of south-western adventitious types accidentally collected with sugar beet seed in seed

Table 2. Developmental cycle variability in the inland beet populations studied. Percentages of plants without vernalization requirement and earliness and flowering are compared to those known in sugar beets and French wild coastal beets

	Flowering without prior vernalization (%)	Earliness of flowering: days from germination to anthesis (x ± SE)	Number of half-sib families	Total number of plants
Sugar beet[a]	0	/	/	/
French wild coastal populations[a]				
Mediterrannean	75	60 ± 2.7[b]	/	50
Atlantic	6	58 ± 1.8[b]	/	590
Channel and North Sea	0	/	/	283
Inland populations				
South-western France				
Nérac	87	48 ± 1.1	5	52
Lectoure 1	100	48 ± 0.9	5	52
Lectoure 2	100	43 ± 1.8	4	26
Condom	96	45 ± 1.4	6	48
Total for south-western France	95	46 ± 0.6	20	178
Northern France				
Nord:				
Bussu	72	59 ± 1.2	12	119
Gouzeaucourt	100	49 ± 2.3	1	16
Bonavis	87	53 ± 2.1	1	15
Oise:				
Neufvy-sur Aronde	63	51 ± 2.7	5	41
Gournet-sur-Aronde	81	50 ± 1.6	4	38
Grandvillers-aux-Bois	83	51 ± 1.7	6	54
Normandy:				
Brionne	98	50 ± 0.7	6	65
Bennetot	100	59 ± 2.2	2	18
Hautot-sur-Mer	93	55 ± 5.8	4	14
Total for northern France	82	54 ± 0.6	41	380

[a] Data from Van Dijk and Boudry 1992

[b] Data adjusted to take into account slightly different environmental growing conditions

production areas, despite the great care of breeding companies. Only 1 plant, carrying the N2 mtDNA type, could have originated from wild coastal populations. Additionally, other cpDNA and mtDNA types known to be present in wild coastal populations were not found in the northern inland populations studied (Forcioli et al. in press).

The maternal origin of the south-western inland populations cannot be defined so clearly because the most frequent type is the N_{vulg} type, which is shared by both cultivated (O-type) and wild coastal beets. Nevertheless, the presence of the CMS type reveals the occurrence of escaped seeds from seed production fields in these wild populations. We therefore conclude that gene flow, via seeds, from cultivated sugar beets to inland populations must have occurred both in the northern sugar production and south-western seed production areas.

Evolutionary history of weed beets

The high frequency of plants without vernalization requirement and very early flowering in the inland beets studied shows the existence of strong, genetically based, developmental differences with cultivated beets. These results enable us to propose an evolutionary history of northern inland weed beet populations. This hypothesis is based on (1) the very high frequency of "B" genotypes in these populations (absence of vernalization requirement), and (2) the risk of formation of hybrid seeds due to the accidental pollination of cultivated seed parents by adventitious beets in seed production areas, as emphasized by Desprez (1980) and Ford-Lloyd and Hawkes (1986). The presence of "B" genotypes in northern inland populations could be explained by accidental pollination between "BB" wild genotypes and "bb" cultivated seed parents, thereby generating "Bb" hybrids. These hybrid seeds are transported and sown in northern sugar production areas. They bolt, flower and produce seeds in the arable field due to the dominance of the annual habit. Selection of the "B" allele and selection of early flowering types happen as a result of natural selection. This scenario is supported by higher frequencies of "B" genotypes and an earlier flowering time in south-western populations than in northern populations, indicating that some cultivated alleles for loci involved in vernalization requirement and earliness of flowering are still present in these northern populations. We therefore support the hypothesis of accidental pollinations of seed production plants by wild beets as the origin of weed beet populations in sugar production areas.

Assessing the risk for the release of herbicide-resistant transgenic sugar beet

The existence of gene exchange via seed and pollen between inland beets and cultivated beets demonstrates the need to study the risk associated with the use of transgenic beets. Varieties resistant to total herbicides are now available from several beet breeding companies and are grown in experimental fields. Some of these total herbicides are used to control weed beets in the highly infested sugar production areas (Martens et al. 1977). This method of control would bring only a temporary solution if gene exchange were to occur between transgenic sugar beets and weed beets. An expansion of these weeds could happen by way of a broadened niche to include all the other transgenic crops resistant to the particular herbicide and, therefore, the efficacy of these herbicide/transgenic resistant crops would soon be dramatically reduced. Seed production of such a herbicide-resistant variety should, therefore, only be carried out in areas where inland wild beets are absent. Bolting beets in sugar production fields should also be strictly eliminated, especially in areas where weed beets are already present. As the annual forms are now present both in seed and sugar production areas, the risks of transgenic material escaping to adventitious or weed beet are considerable.

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