Present state and future prospects of biotechnology in sunflower breeding

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ABSTRACT

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The cultivated sunflower is considered to be a species very recalcitrant to various methods of biotechnology, although substantial progress has been made in recent years. For example, very efficient embryo culture techniques are available for obtaining numerous wide crosses which can be very valuable for broadening genetic variation in sunflower. In particular, interspecific hybrids have been demonstrated to be a unique source for creating "new" traits, like cytoplasmic male sterility (CMS) or resistance against devastating diseases, such as *Sclerotinia* rot. The successful regeneration of entire plants from cultured somatic tissue or even single cells, i.e. protoplasts, has been demonstrated for specific genotypes. Furthermore, the recovery of androgenetic haploid and doubled haploid plants is basically feasible in sunflower, although the rate of regeneration strongly depends on genotype. Particular progress has been made in the field of "genome characterization", by using both biochemical and molecular markers. In particular, the chloroplast and mitochondrial genomes have been analyzed and described in detail. Such analyses built the foundation for future "marker-based selection" and for the identification and isolation of specific genes as candidates for "genetic engineering".

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops of the world. Its seed oil can be used for human consumption, and also as a raw material for oleochemistry. It can also be used as a substitute for mineral oil in various applications, such as a fuel, a lubricant or an oil for hydraulic systems.

The genetic basis of modern hybrids is comparatively narrow (cf. Arnaud, 1986). Sunflower cultivation is almost exclusively performed with hybrids based on a single cytoplasmic male sterile (CMS) source, discovered by Leclercq (1969) in *Helianthus petiolaris* Nutt. and transferred to *H. annuus*

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germplasm. Due to the exclusive usage of female lines with this cytoplasm for hybrid seed production, all hybrids cultivated worldwide are closely related – at least with regard to their cytoplasm.

Therefore, further expansion of sunflower production may be limited due to epidemic diseases, since sunflower is very sensitive to various fungal pathogens. Consequently, hybrids resistant to the most dangerous pathogens in Central and Northern Europe, e.g. *Sclerotinia sclerotiorum* (Lib.) de Bary and *Botrytis cinerea* Pers. ex Fr. have to be developed for economic cultivation under temperate climatic conditions.

Increased breeding efforts are required in order to broaden the genetic variability of cultivated sunflower. Successful applications of new methods of "biotechnology" can contribute to overcoming the problems of a narrow genetic base, and at the same time accelerate the breeding process. In this field, various techniques have been shown to be relevant for sunflower breeding, e.g. embryo culture ("embryo rescue"), meristem culture, anther and microspore culture, protoplast culture and cell fusion, and molecular techniques including gene transfer ("genetic engineering", cf. Fig. 1).

SEXUAL INTERSPECIFIC HYBRIDIZATION VIA EMBRYO RESCUE

Interspecific hybridization is one possibility of transferring genes for disease resistance or for improved quality from one species to another, e.g. from wild species to cultivated lines. It is also a means of developing new sources of CMS.

In general, interspecific crossability of cultivated *H. annuus* and most annual species is fairly high. However, earlier extensive hybridization programs revealed that almost complete incompatibility exists between cultivated sunflower and diploid ($2n=2x=34$) perennial species, e.g. *H. angustifolius* L., *H. divaricatus* L., *H. giganteus* L., *H. nuttallii* T. & G. and *H. mollis* Lam. (Georgieva-Todorova, 1984). Partial compatibility with *H. annuus* was found for some tetraploids ($2n=4x=68$), e.g. *H. decapetalus* L. and *H. hirsutus* Raf., and hexaploid species ($2n=6x=102$), e.g. *H. resinosus* Small and *H. tuberosus* L. (Georgieva-Todorova, 1984). In these cases, postzygotic incompatibility between the embryo and the endosperm was believed to prevent efficient and direct recovery of interspecific hybrids. Interspecific hybridizations between cultivated sunflower and wild *Helianthus* species have been accomplished utilizing the embryo rescue technique (Chandler and Beard, 1983; Espinasse et al., 1985; Kräuter and Friedt, 1989). For example, new hybrids of *H. annuus* × *H. hirsutus* and *H. scaberrimus* (= *H. pauciflorus* Nutt.) × *H. annuus* crosses were successfully grown on a modified White's medium by Bohorova et al. (1985).

Witrzens et al. (1988) described a method for the culture and regeneration of plants from callus of cultivated sunflower and an interspecific hybrid, *H.*

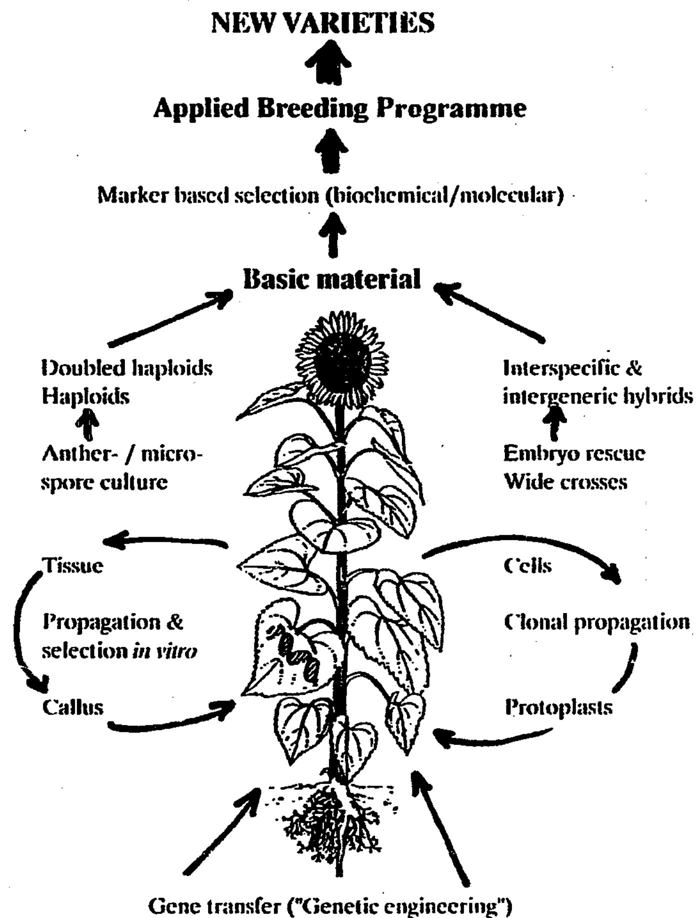

Fig. 1. Overview of biotechnology applicable to sunflower.

annuus × *H. tuberosus*, where immature embryos were the only explant which consistently gave regenerable callus in the genotypes tested. Difficulties often found in such experiments were the premature initiation of flowering of regenerating shoots and the frequent occurrence of "vitreous" plantlets which could not be successfully grown into mature plants. Culture improvements were achieved by partially replacing inorganic nitrogen with amino acids and by adding 10, 30, and 100 μM phloridzin, esculin, and naringin to the medium (Witrzens et al., 1988).

Average success of interspecific hybridization, i.e. recovery of hybrid plants, has been reported by Kräuter et al. (1991), and summarized in Table 1. In this hybridization program, a total of 34 different interspecific cross combinations were successfully raised via in vitro embryo culture by using a solid

Recovery of interspecific hybrids (F_1) via embryo rescue[a]

Cross combinations	278
-successful	123 (44%)
In vitro culture of embryos	1178
-globular stage	141 (12%)
-young heart stage	495 (42%)
-differentiated stage	542 (46%)
Plants recovered (rate)	481 (41%)

[a]From Kräuter (1990).

culture medium (9 g/l agar, pH 5.6) containing the usual macro-and micronutrients, supplemented by myo-inositol (100 mg/l) and with 10 g/l sucrose (Kräuter, 1990). Individual cross combinations are listed in Table 2. The potentially useful cross combinations are being repeated in order to create broad genetic variation for disease resistances (Dahlhoff et al., 1991).

The recovery of interspecific hybrids using in ovulo embryo culture has recently been reported by Espinasse et al. (1991). In particular, several rare hybrids of cultivated sunflower (female) and *H. maximiliani* Schrod. were obtained. Such hybrids have also been reported by Kräuter et al. (1991). Other highly interesting new hybrids are those with *H. nuttallii* (NUT 1517 and NUT 2000) reported by Vasiljevic et al. (1991). The latter authors also attempted to recover many other interspecific combinations which so far have been more difficult to obtain.

Many interspecific F_1 hybrids are usually sterile (cf. Table 2). In these cases, chromosome doubling using colchicine can facilitate selfing and/or backcrossing. For example, submerging *H. annuus* × *H. bolanderi* hybrid seedlings for 5 h in a colchicine solution of 150 or 250 mg/l, supplemented by 0.02 mg/l DMSO (pH=5.4) in the dark, without moistening the roots resulted in about 30% doubled sectors and highly increased fertility of the derived amphiploids (Jan and Chandler, 1989). However, further studies with respect to the appropriate stage for colchicine treatment and the optimum experimental conditions must be carried out in order to improve ease of application of this method. Alternatively, backcrossing of interspecific hybrids can also be facilitated by storing pollen in liquid nitrogen without loss of pollen viability, as reported by Roath et al. (1988).

Interspecific hybridization using in vitro and in ovulo embryo rescue is an applicable and highly efficient method for obtaining many wide hybrids in the genus *Helianthus*. Furthermore, this technique can be utilized to accelerate a sunflower breeding program, by obtaining four to five generations in one year (cf. Azpiroz et al., 1987).

Interspecific hybrids (F_1) obtained via in vitro embryo culture[a]

Female×Male	Fertility[b]	Physiological type[c]
HA89×*H. decapetalus*-B[d]	–	P
HA89×*H. decapetalus*/Dijon[e]	+	A
HA89×*H. originalis*/Giessen	+	P
HA89×*H. angustifolius*/Giessen	+	P
HA89×*H. resinosus* #1545[d]	+	P
HA89×*H. nuttallii* #239[d]	–	A
HA89×*H. nuttallii* #329[d]	–	A
HA89×*H. nuttallii* #103[d]	+	P
HA89×x*H. laetiflorus*/HUNG.[f]	+	A
HA89×x*H. laetiflorus* #558[d]	+	A
HA89×*H. argophyllus*/Gatersleben[g]	+	A
HA89×*H. tuberosus* #1705[d]	+	A
HA89×*H. bolanderi* #775[d]	–	A
HA89×*H. debilis*/Basel[h]	–	A
HA89×*H. strumosus* #1934[d]	+	A
HA89×*H. giganteus* #1897[d]	–	P
HA89×*H. grosseserratus* #1630[d]	–	–[k]
HA89×*H. mollis* #1948 (MOL/Wien)[i]	+	A
HA89×*H. rigidus* #1843[d]	–	A
HA89×x*H. laetiflorus*/Giessen	+/–[m]	A
HA89×*H. maximiliani* #40, #42, #44[d]	+	A
Baso×*H. mollis* #1948 (MOL/Wien)[i]	+	A
Baso×*H. debilis*/Basel[h]	+	A
H. nuttallii #239[d]×HA89	+	P
H. nuttallii #329[d]×HA89	+	P
H. xlaetiflorus #558[d]×HA89	+	P
H. argophyllus/Gatersleben[g]×HA89	+	A
H. debilis/Basel[h]×HA89	+	A
H. debilis/Basel[h]×sf-135	+,–[m]	A
H. divaricatus #1885[d]×HA89	–	–[k]
H. decapetalus/HUNG.[f]×sf-135	+,–[m]	P
H. annuus spp. *lenticularis*/Gatersleb.[g]×sf-135	+	A
H. niveus spp. *canescens* #1409[d]×sf-135	+	A

[a]After Kräuter et al. (1991); [b]+ =fertile, – = (male) sterile; [c]A=annual, P=perennial; [d]from Dr. D. Skoric, Institute of Field and Vegetable Crops, Novi Sad, Yugoslavia; [e]from Jardin Botanique, Dijon, France; [f]from Hortus Botanicus, Va'cra'to't, Hungary; [g]from Zentralinstitut für Genetik und Kulturpflanzenforschung, Gatersleben, Germany; [h]from Botanischer Garten der Universität Basel, Switzerland; [i]from Botanischer Garten der Universität Wien, Austria; [k]dead before flowering; [m]reduced pollen.

ASEXUAL HYBRIDIZATION USING SOMATIC CELL FUSION

The establishment of protoplast culture in *Helianthus* and the regeneration of entire plants are essential for somatic cell fusion. Basic studies on proto-

TABLE 3

Some important features of methods used for plant regeneration from protoplasts in *Helianthus*

	Chanabe et al. (1991)	Burrus et al. (1991)
Genotype:	*Helianthus petiolaris*	PTO24, i.e. tissue culture selected line out of SFM-3 (cms/*H. petiolaris* Nuttall// cms HA89 backcross)
Source material:	Hypocotyl	Hypocotyl
Protoplast preculture:	16h, 25 °C, dark, TL liquid medium	None, protoplasts put directly into agarose
Method of main culture:	Agarose beadculture (both)	
	Protoplast suspension: 5×10^4/ml (both)	
	TL-medium, i.e. L4 medium of Lenée and Chupeau (1986) with 0.5 g/l sucrose and 0.1 mg/l 2,4D	L4M, i.e. liquid medium of Lenée and Chupeau (1986) with casamino acids (1 g/l) and 2,4D (0.1 mg/l)
Medium for plant regeneration from callus:	MS salts plus $NA_2FeEDTA$ (10 m*M*), MS vitamins plus casamino acids (500 mg/l), sucrose (30 g/l), NAA (1), BAP (1), GA_3 (0.1 mg/l), pH 5.6	R501, i.e. MS medium with casamino acids (500 mg/l), sucrose (30 g/l), NAA (1), BAP (1), GA_3 (0.1 mg/l), pH 5.6

plast culture of wild and cultivated *Helianthus* species have been carried out by Bohorova et al. (1986) and Lenée and Chupeau (1986). Guilley and Hahne (1989) determined the conditions which allow the repeated regeneration of green, nodular, vigorously growing calli from isolated sunflower mesophyll protoplasts. Use of CAYLA cellulase and pectinase was found to be the best for protoplast isolation. Although cell divisions were achieved, the method has so far failed to regenerate plants.

Recently, the successful regeneration of plants from hypocotyl-derived protoplasts, capable of being grown in soil, has been reported for the first time in *Helianthus* (Burrus et al., 1991; Chanabe et al., 1991). Protoplasts were produced from two sunflower cultivars and three wild species. After inclusion in agarose droplets and culture on a TL-medium with 2,4D, loose colonies and "embryoids" were obtained. Finally, after two transfers, shoot formation and subsequent rooting were obtained from only one callus derived from *H. petiolaris* (for a summary and comparison of methods cf. Table 3). High protoplast "regeneration potential" has also been reported for *H. nuttallii* and *H. divaricatus* by Bohorova (1991). However, plant regeneration from cultivated sunflower protoplasts remains very difficult (G. Hahne, pers. commun., 1991). Many basic problems, e.g. "genotypic effects", vitrification and rooting, need to be solved before the regeneration of entire plants from protoplasts becomes routine. The use of protoplasts for genetic transformation will be discussed further in the biochemical and molecular methods section.

TISSUE CULTURE FOR RAPID PROPAGATION AND SCREENING IN VITRO

There are several reports of shoot regeneration from cultured hypocotyls, cotyledons, or leaf pieces with or without callus formation (Greco et al., 1984; Paterson and Everett, 1985; Kräuter and Friedt, 1991). In particular, Bohorova et al. (1985) obtained shoot organogenesis from pith parenchyma and shoot apical explants of the interspecific hybrid *H. annuus* × *H. decapetalus*; shoot apices from *H. annuus* × *H. hirsutus* and *H. annuus* × *H. tomentosus* (= *H. resinosus*) hybrid plants underwent shoot organogenesis. However, other authors were not able to regenerate plants from cotyledon- and hypocotyl-derived embryoids (e.g. Piubello and Caso, 1986). Recently, Pugliesi et al. (1991) regenerated "somaclonal variants" from callus-derived adventitious buds induced from in vitro culture of cotyledons of seven sunflower genotypes. Both, well-known and "new" genetic variation – mainly for morpho-physiological traits – were described (S. Baroncelli, pers. commun., 1991).

Direct somatic embryogenesis and plant regeneration from immature embryos of the hybrid 'cmsHA401' × 'RHA699' (Restorer) have been obtained by Finer (1987) after growing immature zygotic embryos on a high-sucrose (12%) medium. Regenerated plants matured and were harvested. By a similar procedure, Jeannine and Hahne (1991) were able to regenerate fertile

plants. Furthermore, Pelissier et al. (1990) developed a method to obtain somatic embryos from hypocotyl epidermis and parenchyma cell layers. Somatic embryos were used to produce secondary embryos, which were regenerated into plants. Prado and Berville (1990) were able to induce somatic embryogenesis from suspension cultures from hypocotyl- and cotyledon-derived calluses. Direct organogenesis was obtained from the original explants, but no regeneration was achieved from somatic embryos. In this context, a remarkably promising technique was established at the Dept. of Agric. Biology, Sect. Genetics, Pisa University. Researchers were able to improve the frequency of shoot regeneration via direct embryogenesis from cultured leaf-pieces (Baroncelli and Pugliesi, pers. commun., 1991).

Another technique which requires the aforementioned ability to regenerate plants from cultured somatic tissue via callus is the screening for disease resistance in vitro. In this case, an extension of time in culture would probably be beneficial, since this is known to increase genetic variation within the cultured calluses and among the plants regenerated from them. Two promising methods for screening in vitro have been developed. The first is the application of fungal filtrates to callus cultures in order to screen for resistance to *Phomopsis/Diaporthe* Munt.-Cvet. (Masirevec et al., 1988) or to *Phoma macdonaldi* Boerema (Hartman et al., 1988), the second is the use of oxalic acid in order to screen for resistance to *Sclerotinia sclerotiorum* (Hartman et al., 1988).

Nevertheless, it is necessary to demonstrate that screening in vitro is a reliable method which shows a high correlation with the reaction of the crop in the field. This is a necessary precondition for practical application of screening for resistance in vitro. However, such correlations are lacking so far, both for *Phomopsis* and *Sclerotinia*. In the latter case, even results of different tests applied to entire plants are often contradictory due to the complex nature of plant–pathogen interactions (cf. Tourvieille de Labrouhe and Vear, 1984).

REGENERATION OF ANDROGENETIC HAPLOIDS FOR AN ACCELERATED DEVELOPMENT OF INBRED LINES

Haploid plants can either be obtained via "gynogenesis", i.e. the culture of unfertilized ovaries or ovules in vitro (e.g. Gelebart and San, 1987), or via "androgenesis", i.e. anther or microspore culture in vitro.

An application of the "haploidy technique", which produces either haploids or spontaneously doubled haploids, should allow the breeder to accelerate the breeding process. In addition, the haploidy technique facilitates the selection for characters controlled by recessive genes or genes incorporated from alien species. Several publications reported extensive callusing induced from anthers of various interspecific hybrids cultured in vitro (Bohorova et al., 1985; Mezzarobba and Jonard, 1986, 1988; Kota and Atlagic, 1991; Vas-

iljevic et al., 1991). Successful regeneration of shoots was also reported by Mezzarobba and Jonard (1988), and Gürel et al. (1991a). In the latter case, M2, a medium with half-strength MS macronutrients and complete MS micronutrients, supplemented by amino acids and with high sucrose content (120 g/l, Table 4) proved to yield the best shoot regeneration in most of the genotypes tested.

Bohorova et al. (1985) described direct shoot formation from anthers of *H. divaricatus* and a *H. annuus* × *H. decapetalus* hybrid cultured on a medium with 5 mg/l zeatin. Other hormone combinations, e.g. kinetin (0.2 mg/l) and 2,4D (1.0 mg/l), tended to promote callus formation but failed to induce shoot regeneration. In addition, secondary callus and shoot formation was achieved from culturing stem explants on a medium with 2 mg/l BA, 0.2 mg/l IAA and 20 mg/l adenine. Shoots developed roots on White's medium, even after several transfers and subcultures. However, with repeated subcul-

TABLE 4

Media used in anther culture experiments[a]

Components	Media			
	M1[1]	M2[2]	M3[1]	B1[3]
Macro nutrients	½ MS[b]	½ MS	½ MS	MS
Micro nutrients	½ MS	MS	½ MS	MS
Fe-solution	½ MS	MS	½ MS	MS
Vitamins	M&W[b]	M&W[b]	M&W[b]	W[c]
(mg/l)	vit.B12	vit.B12	vit.B12	mesoinosit.
	0.2	0.2	0.2	500
Amino acids				
Glycine (mg/l)	1.0	2.0	2.0	-
L-Glutamine (mg/l)	-	40	40	-
L-Serine (mg/l)	-	25	25	-
L-Tryptophane (mg/l)	-	1	1	-
L-Cysteine (mg/l)	-	2.5	2.5	-
Organic nitrogen supplement				
Casein hydrolysate (mg/l)	-	-	-	500
Hormones				
NAA (mg/l)	0.5	0.5	0.5	-
BAP (mg/l)	0.5	0.5	0.5	-
Zeatin (mg/l)	-	-	-	5
Sucrose (g/l)	120	120	120	30
Gelrite (g/l)	3.3	3.3	-	3.3
Agar (g/l)	-	-	6	-
pH	5.9	5.8	5.9	5.8

[a](1) Mezzarobba and Jonard (1988), mod.; (2) Mezzarobba, pers. commun.; (3) Bohorova et al. (1985).
[b,c]According to Morel and White (1951) and White (1963), respectively (cited in Gürel et al. (1991a)).

turing, an increase in chromosome number (2n=45–51, 68 and 102) deviating from the diploid number (2n=34) was observed (Bohorova et al., 1985).

The regeneration of six androgenetic plants from anthers of a French variety ('Inra') was reported by Mix (1985). Two of them were shown to be haploid. Another haploid plant was recovered from anthers of cv. 'Luciole'. Alissa et al. (1985) regenerated haploid, polyploid and aneuploid plants from four hexaploid and one diploid wild *Helianthus* species and their interspecific hybrids with different sunflower lines. Best results were observed for a *H. annuus* × *H. resinosus* hybrid (53% of anthers producing plants). No plants could be regenerated from a *H. annuus* × *H. tuberosus* hybrid. The most effective medium proved to be one supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP.

Initial steps to establish a suitable culture method for isolated microspores in our laboratory yielded embryoid formation in very low frequency only and no organogenesis or regeneration up till now (Gürel et al., 1991b). It appears to be very important to give priority to an optimization of the growing conditions of the donor plants and to elucidate the nature of "genotypic" effects in future experiments (Kota and Atlagic, 1991).

Substantial improvements of "haploidy techniques" are still needed, particularly with regard to overcoming pronounced "genotypic effects" and strong environmental (physiological) effects on androgenetic response.

APPLICATION OF BIOCHEMICAL AND MOLECULAR METHODS IN *HELIANTHUS*

Biochemical and/or molecular methods have already been demonstrated to be highly useful for genetic analyses in many crop species. Today, these methods are also known to serve the sunflower breeder as a tool for early identification of important agronomic traits, quality characteristics, disease resistances, or stress tolerance. A large number of investigations on isozymes in many species have demonstrated their utility in basic and applied research (e.g. Goodman and Stuber, 1980; Tanksley and Orton, 1983; Cooke, 1984).

In particular, starch-gelelectrophoresis is a comparatively simple, fast and inexpensive method applicable for the identification of *Helianthus* species, sunflower lines, and hybrids. Rieseberg and Seiler (1990) used this technique for an evolutionary analysis of cultivated sunflower in relation to wild *H. annuus*. They concluded that domesticated sunflower must have evolved from a very limited gene pool.

Contrary to the statement of Georgieva-Todorova (1984), interspecific F_1 hybrids do not always resemble the wild parent(s) morphologically. Instead, it often remains difficult to distinguish a hybrid from either parent. Isozyme determination provides a means of identifying these hybrids. Starch-gel electrophoresis has been successfully used to identify *Helianthus* species, their interspecific crosses, and progenies (cf. Table 5).

TABLE 5

Efficiency of hybrid identification by starch-gel isozyme electrophoresis[a]

Male parent (female HA89)	Number of hybrids	Number of plants identified as hybrids
H. strumosus 1974	4	3
H. tuberosus 1705	19	19
H. tuberosus 5	20	20
H. Xlaetiflorus Hung	7	7
Identification rate:		98%

[a]Source: Heike Köhler, publ. in Dahlhoff et al. (1991).

Molecular methods will certainly play an important role in sunflower breeding in the future. For example, the analysis of Restriction Fragment Length Polymorphisms (RFLPs) seems to be a promising method for the comparison of genotypes. It has already been used for genotype identification in various plant species representing different families, and genera such as *Brassica, Hordeum, Solanum* or *Zea*. In sunflower, the identification of valuable characters such as CMS, stress tolerance, and disease resistance utilizing RFLPs would facilitate selection at young growing stages and at the beginning of a breeding program.

Rieseberg and Seiler (1990) used the RFLP technique for comparing cpDNAs of wild *H. annuus* and cultivated germplasm. They concluded that the chloroplast genome of wild and domesticated forms is almost identical in size and sequence, which supports the conclusions of the isozyme analyses previously mentioned. Earlier restriction analysis of cpDNA by Jansen and Palmer (1987) revealed that a 22-kb inversion marks an evolutionary split in the sunflower family (*Asteraceae*). This inversion corresponds to the 23-kb inverted repeat described in circular cpDNA of sunflower described by Heyraud et al. (1987).

Analyzing mtDNA, cpDNA, and dsRNA patterns, Brown et al. (1986) speculated that fertile cytoplasm ('CM400' fertile) was associated with a 1.45-kb plasmid. However, Perez et al. (1986) could not detect low molecular weight mtDNA-molecules in cytoplasmic male sterile sunflower lines ("Leclercq's cytoplasm"), while such molecules were present in all of the sunflower lines studied and in *H. petiolaris* ssp. *petiolaris* and *H. petiolaris* ssp. *fallax*. "Leclercq's cytoplasm" is supposed to be derived from *H. petiolaris* and it leads to a breakdown of microsporogenesis due to earlier unknown physiological events (cf. Laveau et al., 1989). Further investigations showed that there is no strict correlation between CMS and the presence of a specific plasmid. It was demonstrated by Crouzillat et al. (1989) that the 1.45-kb plasmid is present in many *H. annuus* materials, in some ecotypes of *H. petio-*

laris, but not in mitochondria of germplasm with the "Leclercq-cytoplasm". However, Perez et al. (1988), who described the nucleotide sequence of this plasmid, were able to detect the plasmid in total cellular DNA of male-sterile sunflower with a comparatively low copy number.

Furthermore, no differences were detected between cpDNAs of *H. annuus* and the "Leclercq-cytoplasm" after digestion with different restriction enzymes. On the other hand, restriction fragment length polymorphisms of the major mtDNA were shown to correspond with cytoplasmic male sterility of sunflower (Crouzillat et al., 1987). Later, Siculella and Palmer (1988) unequivocally demonstrated that sterility caused by the "Leclercq-cytoplasm" is associated with a 12-kb inversion and a 5-kb insertion/deletion near the *atp*A gene in the mtDNA. These findings were recently investigated in more detail and confirmed by Köhler et al. (1991). According to their results, the "Leclercq-CMS" is correlated with the co-transcription of a new open reading frame ('orf') with the *atp*A gene in the mitochondrial genome, as also has been confirmed by Laver et al. (1991). This 'orf' seems to be responsible for an additional mitochondrially encoded polypeptide in CMS sunflower lines (Horn et al., 1991). The 5-kb insertion has already been partially sequenced, and it can further be used for the construction of selectable markers with the aim of an early identification of male sterile lines in a breeding program.

Helianthus genotypes can often be distinguished by restriction analysis of mtDNA, as demonstrated by Heyraud et al. (1987), Crouzillat et al. (1987, 1991) and Serror et al. (1990). For example, molecular relationships were revealed between some CMS lines and the species from which they were derived, e.g. CMS-I/*H. annuus* ssp. *lenticularis* and CMS-F/*H. petiolaris fallax*. On the basis of restriction fragment patterns, a phylogenetic tree was proposed illustrating molecular polymorphism in the mitochondrial genome of *Helianthus*.

Choumane and Heizmann (1988) showed that restriction analysis of nuclear ribosomal genes (rDNA) of *H. annuus* using EcoRI and BamHI can be efficiently used to differentiate *Helianthus* species and populations of *H. annuus*. However, cultivated sunflower lines proved to be identical on the basis of the physical properties of their ribosomal DNA. Kräuter et al. (1991) also used an rDNA probe for the differentiation of sunflower genotypes, wild species and their interspecific hybrids by RFLP. Progress in this field is expected to be rapid with extensive results available in the near future.

APPLICATION OF "GENETIC ENGENEERING"

Basic research for the incorporation of foreign genes into the *Helianthus annuus* genome has already been accomplished. Using disarmed *Agrobacterium tumefaciens* plasmids as vectors, genetically transformed calli have been obtained by Kempf and Hall (1981), Helmer et al. (1984) and Matzke et al.

(1984), while Everett et al. (1987) obtained transgenic plants out of hypocotyl callus.

More recently, Schrammeijer et al. (1990) attempted the transformation of sunflower cv. 'Zebulon' via co-cultivation of dissected shoot apical meristems from seeds with a disarmed *Agrobacterium tumefaciens* strain harbouring a binary vector carrying genes encoding GUS- and NPTII-activity. Chimeric expression of the two genes was observed in transformed plants, and integration of the foreign DNA in the sunflower genome was confirmed by PCR. However, transformation of shoot meristem cells occurred at low frequencies. Moyne et al. (1989) succeeded in the direct transformation of sunflower protoplasts as demonstrated by the presence of the NPTII marker gene in kanamycin-resistant calli. However, no plants could be regenerated from these calli. In general, the recovery of intact transformed plants by manipulating protoplasts from cultivated sunflower remains the major bottle-neck for an application of "genetic engineering" via protoplasts in sunflower. Further improvements will be necessary for the application of this method in sunflower breeding.

SUMMARY AND PROSPECTS

Sunflower has long been considered a species very recalcitrant to various methods of biotechnology. However, substantial progress has been made in recent years. Highly efficient embryo culture techniques are available for creating numerous wide crosses which can be very valuable for broadening genetic variation in sunflower. Regeneration of entire plants from cultured somatic tissue or even single cells (protoplasts) has been demonstrated for specific genotypes. Further, the recovery of haploid and doubled haploid plants is basically feasible in sunflower, although strongly genotype dependent. Particular progress has been made in the field of "genome characterization", by using biochemical and molecular markers. This builds the foundation for future "marker-based selection" and for the identification and isolation of specific genes as candidates for genetic engineering.

Based on the present state of knowledge, future research activities must focus on the following objectives, in order to provide the basis for an application of respective biotechniques in a sunflower hybrid breeding program (cf. Fig. 2).

(1) The efficiency of somatic tissue culture techniques have to be improved in order to enable the rapid propagation of any kind of sunflower breeding materials – i.e. independent of the respective genotype. This can lead to a gain of time during the breeding procedure and it may help to facilitate the screening for stress tolerance and/or disease resistance in vitro.

(2) Methods for the reproducible regeneration of androgenetic haploids

Breeding scheme of a hybrid variety

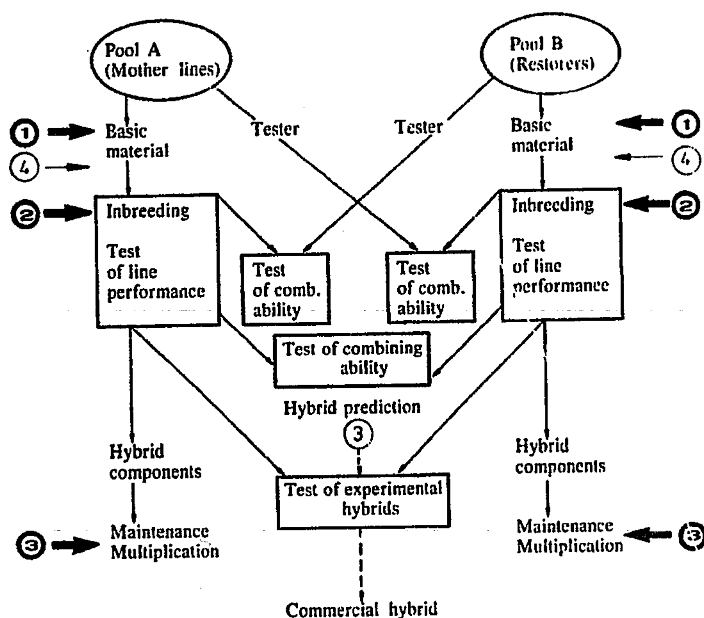

Fig. 2. Possible applications of biotechniques in a sunflower breeding program. Bold figures indicate practical applicability of the respective techniques mentioned in the section " Summary and prospects" and light figures indicate possible future applications.

have to be established as a basis for an accelerated development of doubled haploids, i.e. inbred breeding lines.

(3) Therefore, improved techniques of chromosome doubling must be developed for an easier application of doubled haploids and interspecific hybrids in a sunflower breeding program.

(4) In order to create a better basis for selecting appropriate breeding materials, both sunflower lines as well as primitive *Helianthus* species and subspecies must be described very carefully. Such a detailed characterization and description must not only focus on morpho-physiological characters, but it should also include biochemical and molecular markers in order to construct detailled genetic maps of sunflower.

(5) Finally, applicable techniques for the successful and stable genetic transformation of sunflower must be developed for the future improvement of specific characters which could not be improved by conventional methods, e.g. resistance to *Botrytis* and *Sclerotinia*, and other traits.

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