BIOTECHNOLOGY FOR BREEDING OF FLAX
(*Linum usitatissimum* L.)

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ABSTRACT

For an accelerated and simplified selection of desirable characters in breeding of flax an application of various biotechniques would be useful, e.g. (1) somatic cell culture from meristems or calluses for rapid propagation of plants and in vitro-selection, (2) anther- or microspore-culture for regeneration of haploid plants, (3) interspecific hybridization of cultivated species and their wild relatives, e.g. by embryo rescue, (4) culture and regeneration of hybrid protoplasts (asexual hybrids), (5) genetic engineering with isolated protoplasts ("direct gene transfer") and (6) application of genetic engineering via specific vector systems (cf. Fig. 1). Some of these methods and techniques have already been applied in practical flax breeding programmes.

APPLICATION OF CELL- AND TISSUE CULTURE TECHNIQUES

Biotechnology can help to accelerate a breeding programme and improve efficiency of selection as demonstrated in other useful plant species like rapeseed (*Brassica napus*) (cf.[11]). Therefore, plant biotechnology may offer corresponding advantages of accelerating selection processes in a flax breeding programme. For example, cotyledon, hypocotyl, meristem or stem segments have been used as explants for culture initiation in flax[12, 19, 22, 28]. Plant regeneration, e.g. from hypocotyl segments, proved to be highly efficient [12, 19] (Table 3).

Plant cells *in vitro* can undergo spontaneous genetic changes which may be manifested in the regenerated entire plants; this phenomenon has been termed "somaclonal variation"[23]. If a trait of agronomic value can be created and identified in cells *in vitro*, and those cells regenerated to whole plants whose

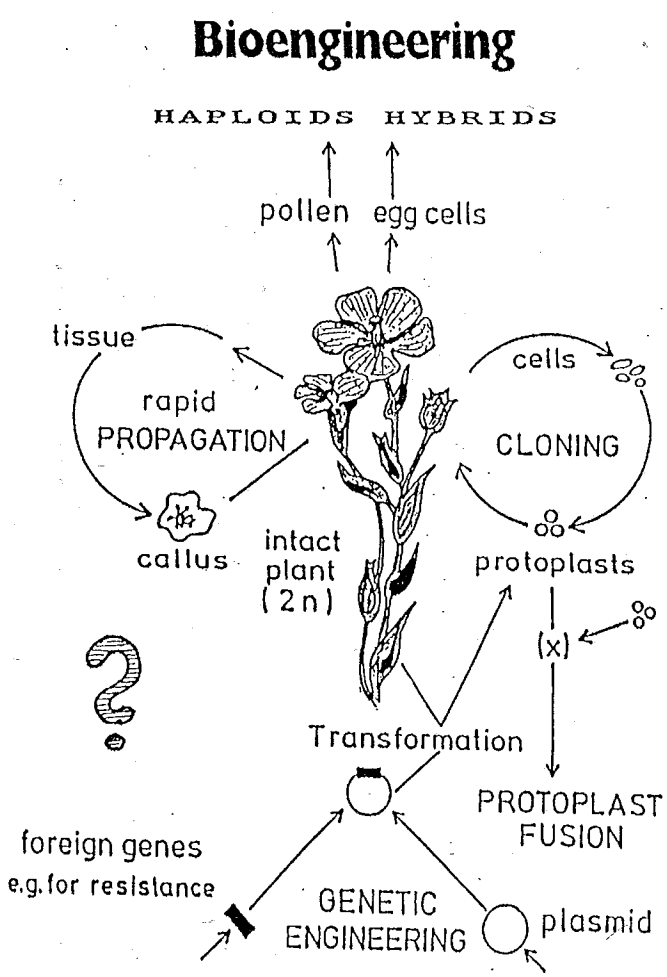

Fig. 1. An overview of biotechniques applicable to flax.

progeny express the trait, it saves time and efforts as compared to conventional breeding procedures. Thus, tissue culture techniques offer a potential for producing varieties by *in vitro*-selection immediately. In flax it was possible to select a salt-tolerant line from induced callus in a high salt environment[26]. The progeny of this line was tested for its performance in normal and saline soil under controlled greenhouse conditions where it showed superiority over its parent variety. This line was also superior in normal, non stressed soil indicating that a non-specific mechanism of tolerance has been activated in culture[25]. However these results obtained under controlled environmental conditions, growing the parent and selected line side-by-side within greenhouse pots, could not be confirmed in field tests where intergenotypic competition was absent[35] (Table 3).

In addition, a cellular selection procedure has been used in order to identify herbicide resistance in callus cultures initiated from hypocotyl segments of linseed by using a selection medium containing chlorsulfuron, the active compound in the herbicide Glean^R. Callus from several progeny showed resistance to chlorsulfuron, indicating heritability, i.e. sexual transmission of this novel trait[17] (Table 3).

Table 1. Influence of flax genotype and induction medium on anther response (%) and plant regeneration[29]

Cultivar	Medium A1[1]		Medium A5[2]	
	%ac[3]	%cs[4]	%ac	%cs
Antarès	6.8	4.0	5.3	20.0
Ariane	1.3	0	14.0	0
Atalante	1.0	0	9.4	12.5
Bionda	0.7	0	12.7	0
Hella	5.0	0	17.0	0
Linda	3.7	0	6.0	7.7
$\bar{x}$	3.1	0.7	10.7	6.7

[1] after SUN[40] modified, [2] after FOROUGHI-WEHR *et al.*[9] modified;
[3] % anthers producing callus, [4] % calluses producing shoots.

Culture systems for single cells like microspores or protoplasts are one of the most essential requirements to incorporate "genetic engineering" into plant breeding procedures. Considerable progress in protoplast culture and regeneration has been reported for *Linum* species in recent years[1, 2, 24]. In *Brassicaceae*, *Solanaceae* and other genera, it is possible now to obtain "haploid" plants reproducibly through microspore- or antherculture[20, 33]. Plant regeneration has also been achieved from flax anthers cultured in our laboratory via callus[29] (Table 1). The application of haploidy-techniques would be more

efficient, if it were possible to induce direct microspore embryogenesis. Recently direct embryogenesis was observed from flax microspores, but the embryos remain to be regenerated to intact plants (NICHTERLEIN and UMBACH, unpubl.; cf. Table 3).

Table 2. Some *Linum*-Species used for interspecific hybridization experiments[30]

Species	2n = [1]	TGW(g)[2]	Oil(%)[3]	C18:3(%)[4]	Hybrids[5]
L. africanum	30	4.0	39.2	42.0	+
L. alpinum	18	3.2	30.2	55.6	
L. anglicum	32	1.4	33.0	52.7	
L. angustifolium	30	1.7	35.1	57.5	+
L. austriacum	18	1.5	25.5	48.7	
L. bienne	30	1.4	29.8	62.5	+
L. companulatum	28	0.8	40.6	19.5	
L. catharticum	16	0.1	35.3	12.1	
L. crepitans	30	4.1	37.4	56.3	+
L. flavum	30	0.9	41.2	21.2	
L. grandiflorum	16	1.5	31.4	50.5	
L. hologynum	18	1.3	30.6	55.2	
L. monogynum	30	4.4	26.7	54.4	
L. narbonensis	30	5.0	34.6	53.3	+
L. usitatissimum	30	7.5	37.5	55.0	

1) somatic chromosome number; [2] approx. 1,000 seed-weight; [3] oil content in dry seed; [4] proportion of linolenic acid in the oil; [5] viable and fertile interspecific hybrids with *L. usitatissimum* obtained, already (+).

Haploid plants carry a single set of chromosomes in their somatic cells. By doubling this chromosome set spontaneously or artificially, e.g. with colchicine, homozygous doubled haploid (DH) lines are obtained. Such homozygous inbred lines are the ultimate product of traditionally long-term breeding and selection processes in self pollinating species like flax. With the aid of the "haploidy-technique" mentioned above, several years can be saved in a breeding programme (cf.[39]).

INTERSPECIFIC GENE TRANSFER

Utilization of germplasm from wild species via interspecific hybridization is another interesting supplementary tool for introducing foreign useful genes into cultivated plants. It can help to create new genetic variability when genes for desirable traits are not available in crop species. Wild species have commonly been used as a source disease resistance, modified and improved quality, and other important characteristics. There are many reports on interspecific hybrids in various genera, e.g. *Brassica*[14, 41], *Helianthus*[13, 21], *Solanum* and *Triticum*[7, 38].

The genus *Linum* includes a large number (more than 150) of distinct wild species. For example, many wild *Linum* species are characterized by striking differences in fatty acid composition as compared to cultivated linseed (Table 2). Therefore, interspecific hybrids between flax cultivars and wild species can provide useful material for subsequent selection. Unfortunately, in many combinations of cultivated and interesting primitive wild species, hybrids have not been obtained, yet[15, 30, 37]. This can be due to incompatibility during endosperm or embryo development (postfertilization barriers), where the embryo begins to develop but degenerates prior to full maturity. Isolation and culture of hybrid embryos ("embryo rescue") may circumvent such postzygotic barriers in interspecific hybridization.

For an application of the embryo culture technique the initial steps of an ordinary sexual hybridization have to be carried out first. About 7-10 days after pollination, depending on it's developmental stage, the immature embryo has to be excised out of the ovulum and plated on suitable medium. This medium enhances the growth of the embryo as well as shoot and root formation[6, 34] (Table 3).

Table 3. Cell- and tissue culture techniques for flax

Explant(s)	Result	Reference(s)
cotyledon, hypocotyl, meristems, or stem segments	culture initiation	12, 19, 22, 28
hypocotyl segments	efficient plant regeneration	12, 19
induced callus	salt-tolerant line	25, 26, 35
hypocotyl callus	Glean^R resistance	17
anther callus	plant regeneration	29
microspores	direct embryogenesis	(unpubl.)
interspecific embryos	shoot & root formation	6, 30, 34
protoplasts	culture & regeneration	1, 2, 24

Hybridization of *L. usitatissimum* to various other species, like *L. africanum*, *L. angustifolium*, *L. bienne*, *L. crepticans* and *L. narbonensis*, were entirely successful in that viable and fertile hybrid plants could be regenerated which opens new possibilities for further improvements of cultivated flax. However, all attempts of hybridization of *L. usitatissimum* to other species like *L. campanulatum*, *L. catharticum* and *L. flavum* were unsuccessful so far due to

incompatibility of pollen and style (prefertilization barriers). Respective interspecific hybrids could possibly be recovered by protoplast fusion techniques. In this case the cell-walls of somatic tissue of both parents have first to be removed enzymatically. The resulting protoplasts are subsequently treated with special agents, like PEG (polyethylenglycol), or by electroshock for attachment and final fusion of the cells. A successful fusion product will include the entire genetic material of the fused cells, i.e. parental species. This fusion product, the newly formed hybrid protoplast ("heterokaryon"), can be regenerated to a hybrid plant if plated on suitable culture medium and maintained under appropriate growing conditions. The requirements for fusion and regeneration have already been established for *Linum* species[1, 2, 24] (Table 3).

APPLICATION OF GENETIC ENGINEERING

Modern gene technology or "genetic engineering" can be considered as a distinct area of biotechnology, which includes the identification, isolation, possible modification, multiplication ("cloning"), and transfer into a foreign "genetic background", i.e. the cell of a related or unrelated plant. For the final success of "genetic transformation", the expression of the respective manipulated gene(s) from a donor into the receptor plant has to be accomplished.

Most of the requirements for an incorporation of genetic engineering into basic and applied flax breeding research are already available. For example, functional vector-systems for gene-transfer are disposable; the vector systems of the soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* are established tools for transferring genetic information into dicotyledoneous plants[10, 36]. Other vector systems have been developed, including the direct ("vector-free") transfer of genes into protoplasts[4, 31, 32] or into microspores[8]. However, the most commonly applied and successful transformation technique of higher plants is the *Agrobacterium* system[36]. Flax tissue was shown previously to be accessible to wild type *A. tumefaciens* strain T37[16](Table 4).

Recently, genetic transformation of flax has been achieved using disarmed *Agrobacterium tumefaciens* vectors. These are carrying non-oncogenic Ti plasmid-derived vectors, which contain a chimaeric NPT-II gene and a wild type nopaline synthase gene; nopaline assay confirmed transformation of regenerated shoots[3, 27]. But the production of opines as sole evidence for transformation has been questioned, because such compounds have been detected also in non-transformed tissue of several species[5]. Therefore, NPT-II activity and T-DNA analysis were estimated in transformed callus tissue, but unfortunately no data showing NPT-II activity or the presence of T-DNA in regenerated shoots were presented[3]. However, such T-DNA assays in flax are known from

transformation with *A. rhizogenes*. Cotyledon explants inoculated with *A. rhizogenes* formed transformed roots which regenerated transformed shoots. Ri T-DNA encoded opines were detected in the transformed plantlets and Southern hybridization analysis confirmed the presence of T-DNA from the Ri plasmid[42] (Table 4).

Table 4. Status of genetic engineering in flax

Explant	Vector	Results	Reference(s)
Tissue	A. tumefaciens	integration	16
Callus	A. tumefaciens	NPT-II gene transformation, regenerated shoots	3.27
Tissue	A. tumefaciens	glyphosate resistance	18
Cotyledon	A. rhizogenes	transformed roots, regeneration of transformed shoots	42

Stable transformation in progenies of transformed flax plants was also recorded by using an *A. tumefaciens* strain carrying a disarmed Ti-plasmid vector containing a chimaeric NPT-II gene and a glyphoshate resistance plant-derived 5-enolpyruvylshikimate-3-phosphate synthase gene. Transformed shoots could be regenerated from the inoculated tissue and were proven to be transgenic by a combination of callus assay, nopaline assay and progeny tests. An inoculation method with a transformation efficiency of 10% has been developed. This seems to be adequate enough for incorporating genes of agronomic importance, which are either easily selectable (i.e. herbicide or disease resistances) or linked to antibiotic resistance markers[18] (Table 4).

PROSPECTS FOR THE FUTURE

Although conventional plant breeding procedures have been successful in improving useful plants like flax, it still has to be mentioned that such traditional procedures are time-consuming and sometimes not very efficient.

Modern "biotechniques" can further help to enhance the efficiency of breeding for improved yield and quality of "new" crops like flax or linseed. Specific techniques, like "haploidy-steps" or "genetic engineering" may contribute to accelerate breeding programmes through avoidance of long-lasting inbreeding or backcross generations.

For an application of both, haploidy-technique and genetic engineering in applied breeding of flax, several requirements are given already, like the basic culture or transformation techniques. Others, which are required for a more

general application of this technology, like the accessibility of any particular flax cultivar or line, have to be elaborated. However, this limitation should be overcome by intensified applied research in the near future.

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LITERATURE

1 BARAKAT, M.N. and COCKING, E.C. 1983. Plant regeneration from protoplast-derived tissues of *Linum usitatissimum* L. (flax). Plant Cell Rep. 2, 314-317.

2 BARAKAT, M.N. and COCKING, E.C. 1985. An assessment of the cultural capabilities of protoplasts of some wild species of *Linum*. Plant Cell Rep. 4, 164-167.

3 BASIRAN, N., ARMITAGE, P., SCOTT, R.J. and DRAPER, J. 1987. Genetic transformation of flax (*Linum usitatissimum*) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase. Plant Cell Rep. 6, 396-399.

4 CHEN, W.H., GARTLAND, K.M.A., DAVEY, M.R., MULLIGAN, B.J., POWER, J.B. and COCKING, E.C., 1987. Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. Plant Cell Rep. 6, 297-301.

5 CHRISTOU, P., PLATT, S.G., and ACKERMANN, M.G. 1986. Opine synthesis in wild-type plant tissue. Plant Physiol. 82, 218-221.

6 COLLINS, G.B. and GROSSER, J.W. 1984. Embryo culture. In: Cell Culture and Somatic Cell Genetics of Plants, Vol. 1. VASIL, I.K. (ed.), 241-257, Academic Press, New York.

7 COLLINS, G.B., TAYLOR, N.L. and DEVERNA, J.W. 1984. *In vitro* approaches to interspecific hybridization and chromosomemanipulation in crop plants. In: Gene Manipulation in Plant Improvement. GUSTAFSON, J.P. (ed.), 323-383, 16th Stadler Genet. Symp., Plenum Publ.

8 DE WET, J.M., BERGQUIST, R.R., HARLAN, I.R., BRINK, D.E., COHEN, C.E., NEWELL, C.A. and DE WET, A.E. 1985. Exogenous gene transfer in maize (*Zea mays*) using DNA-treated pollen. In: Experimental Manipulation of Ovule Tissues. CHAPMAN, G.P. (ed.), 197-209.

9 FOROUGHI-WEHR, B., MIX, G., GAUL, H. and WILSON H.M. 1976. Plant production from cultured anthers of *Hordeum vulgare* L.Z. Pflanzenzüchtg. 77, 198-204.

10 FRALEY, R., ROGERS, S.G. and HORSCH, R.B. 1986. Genetic transformation in higher plants. CRC Crit. Rev. Plant Sci. 4, 1-46.

11 FRIEDT, W. 1988. Biotechnology in breeding of industrial oilcrops – the present status and future prospects. Fat Sci. Technol. 90, 51-55.

12 GAMBORG, O.L. and SHYLUK, J.P. 1976. Tissue culture, protoplasts, and morphogenesis in flax. Bot. Gaz. 137, 301-306.

13 GEORGIEVA-TODOROVA, J. 1984. Interspecific hybridization in the genus *Helianthus* L.Z. Pflanzenzüchtg. 93, 265-279.

14 GLIMELIUS, K., FAHLESSON, J., SJÖODIN, C., SUNDBERG, E., LANDGREN, M. 1988. Fusion of protoplasts as a method to move nuclei and organelles. In: Proc. Int. EUCARPIA Congr. on Genetic Manipulation in Plant Breeding, Helsingør, DK.

15 GREEN, A.G. 1983. Interspecific hybridization in the genus *Linum*. Proc. Austr. Plant Breed. Conf., Adelaide, 302-304.

16 HEPBURN, A.G., CLARKE, L.E., BLUNDY, K.S. and WHITE, J. 1983. Nopaline Ti-plasmid, pTiT37, T-A insertions into a flax genome. J. Mol. Appl. Genet. 2, 211-224.

17 JORDAN, M.C. and McHUGHEN, A. 1987. Selection for chlorsulfuron resistance in flax (*Linum usitatissimum*) cell cultures. J. Plant Physiol. 131, 333-338.

18 JORDAN, M.C. and McHUGHEN, a. 1988. Glyphosate tolerant flax plants from *Agrobacterium* mediated gene transfer. Plant Cell Reports 7, 281-284.

19 KAUL, V. and WILLIAMS, E.G. 1987. Multiple shoot induction *in vitro* from the hypocotyl of germinating embryos flax (*Linum usitatissimum*). J. Plant Physiol. 131, 441-448.

20 KELLER, W.A., ARNISON, P.G. and CARDY, B.J. 1987. Haploids from gametophytic cells – Recent developments and future prospects. In: Plant Tissue and Cell Culture. GREEN, C.E., OMERS, D.A., HACKETT W.P. and IESBOER, D.D. (eds.), 223-241, Alan R. Liss, Inc., New York.

21 KRÄUTER, R. and FRIEDT, W. 1989. Efficient interspecific hybridization in the genus *Helianthus* through embryo rescue. In: Vortr. f. Pflanzenzüchtung 15/I, Proc. 12th EUCARPIA Congr., Göttingen, 15-11.

[22] LANE, W.D. 1979. Influence of growth regulators on root and shoot initiation from flax meristem-tips and hypocotyls *in vitro*. Physiol. Plant. 45, 260-264.

[23] LARKIN, P.J. and SCOWCROFT, W.R. 1981. Somaclonal variation – a noval source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60, 197-214.

[24] LING, Q. and BINDING, H. 1987. Plant regeneration from protoplasts in *Linum*. Plant Breeding 98, 312-317.

[25] McHUGHEN, A. 1987. Salt tolerance through increased vigor in a flax line (STS-II) selected for salt tolerance *in vitro*. Theor. Appl. Genet. 74, 727-732.

[26] McHUGHEN, A. and SWARTZ, M. 1984. A. tissue-culture derived salt-tolerant line of flax (*Linum usitatissimum*). J. Plant Physiol. 117, 109-117.

[27] McHUGHEN, A., BROWNE, R., KNEESAW, D., and JORDAN, M. 1986. Ti-mediated transformation and regeneration of flax plants *in vitro*. 6th Int. Congr. Plant Tissue and Cell Culture, Minneapolis (Abstracts), 130.

[28] MURRAY, E., HANDYSIDE, R.J. and KELLER, W.A. 1977. *In vitro* regeneration of shoots on stem explants of haploid and diploid flax (*Linum usitatissimum*). Can. J. Genet. Cytol. 19, 177-186.

[29] NICHTERLEIN, K., UMBACH, H. and FRIEDT, W. 1989. Investigations on androgenesis in breeding of linseed (*Linum usitatissimum*). In: Vortr. f. Pflanzenzüchtung 15/II, Proc. 12th EUCARPIA Congr., Göttingen, 25-13.

[30] NICKEL, M., NICHTERLEIN, K. and W. FRIEDT 1989. Results of breeding for modified C18-fatty acid composition in *Linum*. In: Vortr. f. Pflanzenzüchtung 15/I, 12th EUCARPIA Congr.,Göttingen, 15-11.

[31] PASZKOWSKI, J. SHILLITO, R.D., SAUL, M., MANDAK, V. HOHN, B. and POTRYKUS, I., 1984. Direct gene transfer to plants. EMBO J. 3, 2717-2722.

[32] POTRYKUS, I., PASZKOWSKI, J., SAUL, M.W., PETRUSKA, P. and SHILLITO, R.D. 1985. Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. Mol. Gen. Genet. 199, 169-177.

[33] PRAKASH, J. and GILES, K.L., 1987. Induction and growth of androgenic haploids. Int. Review Cytol. 107, 273-292.

[34] PRETOVA, A. 1986. Growth of zygotic flax embryos *in vitro* and influence of kinetin. Plant Cell rep. 3, 210-211.

[35] ROWLAND, G.G., McHUGHEN, A. and McONIE C. 1989. Field performance at saline-affected sites of a somaclonal variant of McGregor flax selected for salt tolerance *in vitro*. Can. J. Plant Sci. 69, 49-60.

[36] SCHELL, J. 1987. Transgenic plants as tools to study the molecular organization of plant genes. Science 237, 1176-1183.

[37] SEETHARAM, A. 1972. Interspecific hybridization in *Linum*. Euphytica 21, 489-495.

[38] SHARMA, D.R. and GILL, B.S. 1983. Current status of wide hybridization in wheat. Euphytica, 32, 17-31.

[39] SNAPE, J.W., SIMPSON, E., PARKER, B.B., FRIEDT, W. and FOROUGHI-WEHR, B. 1986. Criteria for the selection and use of doubled haploid systems in cereal breeding programmes. In: Genetic Manipulation in Plant Breeding. HORN, W., JENSEN, C.J., ODENBACH, W. and SCHIEDER, O. (eds.), 217-229. W. de Gruyter, Berlin – New York.

[40] SUN, H., 1979. Preliminary report on anther culture of flax (*Linum usitatissimum*). K'o Hsueh T'ung Pao exue tong bao 24, 948-950.

[41] YAMASHITA, Y.,TERADA, R., NISHIBAYASHI, S. and SHIMAMOTO, K. 1989. Asymmetric somatic hybrids of *Brassica*: partial transfer of *B. campestris* genome into *B. oleracea* by cellfusion. Theor. Appl. Genet. 77, 189-194.

[42] ZHAN, X., JONES D.A. and KERR, A., 1988. Regeneration of flax plants transformed by *Agrobacterium rhizogenes*. Plant Mol. Biol. 11, 551-559.

Modified version of a paper by K. NICHTERLEIN, M. NICKEL, H. UMBACH and W. FRIEDT, Recent progress and prospects of biotechnology in breeding of linseed (*Linum usitatissimum* L.), Fat. Sci. Technol. 1989 (in press).

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