Towards plastid transformation in rapeseed (*Brassica napus* L.) **and sugarbeet** (*Beta vulgaris* L.)

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CONTENTS

2.4-D	2,4-dichlorophenoxyacetic acid
A	adenine
B5	medium of Gamborg et al
BA	6-benzyladenine
BAP	6-benzylaminopurine
bp	base pairs
C	cytosine
ºC	Celsius grade
CAT	chloramphenicol acetyltransferase
CIP	calf intestine phosphatase
cm	centimeter
cpDNA	chloroplast DNA
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
et al.	and others
etc	*et cetera*
G	guanine
g	gramme or gravity
GA_3	gibberellin A_3
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour
IAA	indole-3-acetic acid
i.e.	that is
IR	inverted repeat
IPTG	isopropyl-D-thiogalactopyranoside
kb(p)	kilobase(pairs)
l	liter
LSC	large single copy region
µ	micro-
M	molarity
MES	2[N-morpholino]ethane-sulfonicacid
min	minute
ml	milliliter
mm	millimeter
mM	millimolarity
mOsm	milliosmolarity
MS	medium of Murashige and Skoog
NAA	α-naphthaleneacetic acid
ng	nanogramme
nPG	*n*-propylgallate
nt	nucleotide
ORF	open reading frame
PEG	polyethylene glycol
PCR	polymerase chain reaction
rpm	rounds per minute
SSC	small single copy region
T	thymine
TAL	thin alginate layer
TIBA	2,3,5-triiodobenzoic acid
T_M	melding temperature
U	unit, enzyme activity
W	watt
w/v	weight per volume
X-Gal	5-Bromo-4-Chloro-3-Indolyl-β-D-galactopyranoside
X-Gluc	5-Brom-4-Chlor-3-Indolyl-β-glucuronide

1. INTRODUCTION

1.1 Protoplast culture: history and achievements

In 1880 J. Hanstein named the cell content of a plant cell "protoplast", thus the term "protoplast" means all the components of a plant cell excluding the cell wall. There are two ways allowing the removal of the cell wall, mechanical and enzymatic. Protoplasts were first isolated mechanically (Binding, 1966; Bilkey and Cocking, 1982). The mechanical method of protoplast isolation was a time-consuming and difficult procedure, thereby yielding only few protoplasts. Mechanically isolated protoplasts were also not uniform, and only highly vacuolated and large cells could be obtained. Mechanically isolated protoplasts have been investigated for their osmotic properties, and many efforts were taken to grow and to regenerate them. However, only in rare cases could those protoplasts be cultured and regenerated into entire plants, such as *Funaria hygrometrica*, a moss (Binding, 1966). Other attempts to isolate protoplasts from higher plants failed for many years until an enzymatic method was discovered. Cocking (1960) used an extract of hydrolytic enzymes from fungi to release tomato protoplasts from root tips. Although cell wall degrading enzymes are toxic to different degrees and might affect the physiology of the cells (Patnaik et al., 1982), the enzymatic removal of the cell wall became the method of choice to isolate large numbers of uniform protoplasts. Protoplast divisions and regeneration to intact plants were first achieved on lower plants. Binding (1966) was the first to report the successful regeneration of moss plants from protoplasts. In 1971 tobacco leaf protoplasts (Takebe et al., 1971) were regenerated into whole plants, thereby proving totipotency for higher plant cells (Vasil and Hildebrandt, 1965). Protoplasts can be isolated from different sources, like leaves, petioles, stems, roots, cotyledons, hypocotyls, pollen, cell suspensions, callus etc. (Vasil and Vasil, 1980). Many important factors may influence protoplast survival and their further development (Vasil, 1976). As it was mentioned above, those are cell wall

degrading enzymes (Patnaik et al., 1982) and source of protoplasts (Vasil and Vasil, 1980). Protoplast density (Eriksson, 1985), composition of nutrients in the media (mineral and organic elements) (Arnold and Eriksson, 1977; Nehls, 1978; Kao et al., 1973; Caboche, 1980; Kao and Michyluk, 1975), osmotic pressure of isolation and culture media (Vasil and Vasil, 1979; Kao and Michayluk, 1980; Lu et al., 1981), pH (Davey, 1983), light (Banks and Evans, 1976; Santos et al., 1980) and temperature (Zapata et al., 1977; Saxena et al., 1982) conditions and many others are all important for protoplast culture. It has been observed to be of considerable benefit to embed protoplasts in gels like agarose or alginic acid in the presence of Ca^{2+} ions (Brodelius and Nilsson, 1980). Immobilisation resulted in increased viability of the embedded protoplasts in comparison with those grown in liquid culture. Embedding of protoplasts in an alginate gel is one of the mildest procedures of cell immobilisation. It provides a gentle environment to the sensitive protoplasts and protects them, most of all, against mechanical stress. An optimisation of listed conditions permits to obtain a highly efficient, easy and reproducible protoplast culture system. Meanwhile, under optimal isolation and culture conditions it is possible to regenerate a plant from a protoplast in less than two weeks (Dovzhenko et al., 1998a, 1998b).

The main steps of protoplast isolation are summarised in Fig.1.1. After protoplasts are isolated from a variety of tissue and organs, they are purified and collected using filtration, flotation and sedimentation procedures. When required, protoplast density is adjusted, and protoplasts are cultured using different culture systems. Since the first successful shoot regeneration of higher plants was reported, about 200 species of *Spermatophyta* have been regenerated from protoplasts to whole plants, among them important species of legumes (Puonti-Kaerlas and Eriksson, 1988), cruciferous plants (Kartha et al., 1974), cereals (Fujimura et al., 1985), and woody plants (Vardi and Spiegel-Roy, 1982).

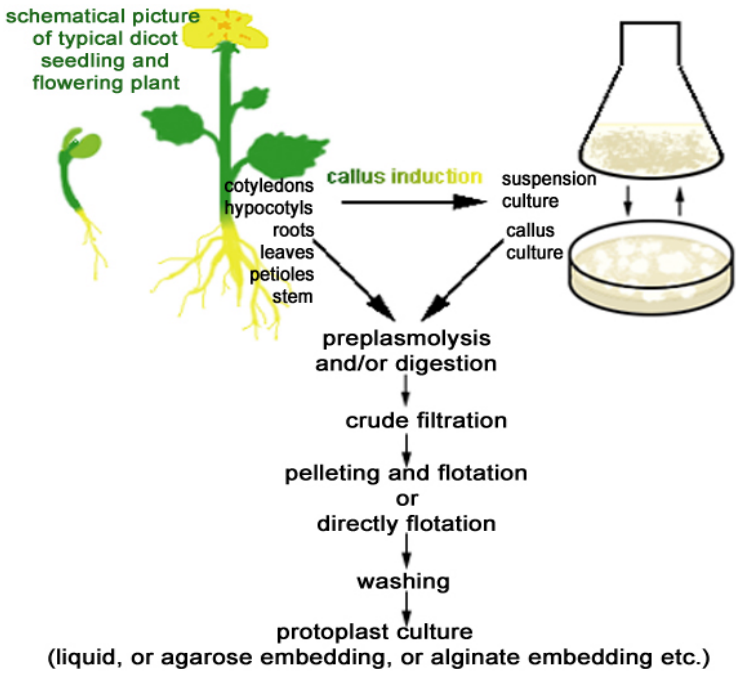


Fig. 1.1. General scheme of protoplast isolation from higher plants.

Enzymatic digestion and high yield of uniform protoplasts, totipotency and the possibility to obtain entire plants or cell lines from single cells allowed the use of protoplasts as a very convenient source for development and establishment of many techniques in modern plant cell biology. Plant protoplasts are instrumental for studies on cell organelles (Lloyd et al., 1980; Fowke and Gamborg, 1980; Galun, 1981), on membrane transport in plants (Taylor and Hall, 1976; Guy et al., 1980), on cytodifferentiation processes and cell development (Kohlenbach et al., 1982a), on plant virus functions and interaction (Cocking, 1966; Nagata et al., 1981). Intraspecific (Lazar et al., 1981; Bonnett and Glimelius, 1983), interspecific (Carlson et al., 1972; Gleba and Hoffman, 1978; Sidorov and Maliga, 1982) and intergeneric (Schiller et al., 1982) hybridisations by somatic cell fusion are possible owing to the development of protoplast culture systems. Protoplasts are suited for direct (Morikawa et al., 1986) and indirect (Thomzik and Hain, 1990) gene transfer into the nucleus and recently also the plastid chromosome (Golds et al., 1993).

1.2 Rapeseed: general information, history of protoplast culture

The name rapeseed (or oilseed rape or colza) refers to a plant species within the genus *Brassica*. Many of the *Brassica* species are economically important as a source of edible oil, condiments, vegetables and cattle fodder. A closely related species is *Arabidopsis thaliana*, one of the most important model plants in modern plant cell and molecular biology. The main virtue of oilseed rape is its high content of oil (40%). Rapeseed, like soybean and palm, is an important source of edible oil, and about 13% of world's edible oil output is produced from the crop (Thomzik, 1993). Additionally, it is the fourth most important source of protein for animal feed. Coarse colza meal contains up to 45% of high quality protein (Downey and Röbbelen, 1989). Canola is a genetic variation of rapeseed developed by Canadian plant breeders. Canola is characterised by a low level of saturated fatty acids. The *B. napus* variety "Tower" was the first "double low" variety with reduced both, erucic and glucosinolate levels. Anti-nutritive glucosinolates affected the meal quality of rapeseed. Oilseed rape is an important target for crop improvement by genetic engineering, and the development of efficient protoplast culture is one of the methods allowing to achieve this aim.

Since the first report on successful isolation, culture and regeneration of complete plants from rapeseed mesophyll protoplasts (Kartha et al., 1974), oilseed rape protoplasts are one of the most favourite models in somatic cell hybridisation or transformation. Rapeseed protoplasts from microspore-derived haploid plants (Thomas et al., 1976; Kohlenbach et al., 1982b), leaves (Kartha et al., 1974; Li and Kohlenbach, 1982; Pelletier et al., 1983), cotyledons (Lu et al., 1982), hypocotyls (Glimelius, 1984; Spangenberg et al., 1985; Thomzhik and Hain, 1988), roots (Xu et al., 1982) and stem cortex (Klimaszewska and Keller, 1985) were isolated and regenerated into the whole plants. This demonstrates totipotency of plant cells from different origins. Direct somatic embryogenesis has been obtained from mesophyll protoplasts isolated from androgenetic canola plants (Li and

Kohlenbach, 1982). An efficient and reproducible regeneration procedure for rapeseed protoplasts, especially hypocotyl protoplasts (Glimelius, 1984), was an important prerequisite for the use of somatic hybridisation and transformation. *B. napus* cybrids of different varieties and intergeneric cybrids of *B. napus* and *Raphanus sativus* have been regenerated after protoplast fusions in PEG containing solution (Pelletier et al., 1983; Thomzik and Hain, 1988). Direct DNA transfer by electroporation (Guerche et al., 1987) and transformation by *Agrobacterium tumefaciens* (Thomzik and Hain, 1990; Thomzik, 1993) have been demonstrated for protoplasts of oilseed rape. Nevertheless, plant regeneration from protoplast-derived calli of *B.napus* is dependent on the genotype used and often of low efficiency (Thomzik and Hain, 1988). A genotype-independent and highly efficient regeneration protocol is so far not available.

1.3 Sugarbeet

1.3.1 Sugarbeet is an important crop

Sugarbeet (*Beta vulgaris* L.), which belongs to the family *Chenopodiaceae*, is one of the most important arable crops. Sugarbeet is a biennial plant species. Around 35%– 40% of world's sugar output is produced from sugarbeet (Winner, 1993). *In vitro* and protoplast culture of sugarbeet has been studied for about 30 years. Despite the large economic value of the crop, especially in the northern hemisphere, and the rather long period of investigations it is still very difficult to engineer sugarbeet plants containing new, agriculturally important traits, such as herbicide, pesticide and disease resistances, increased sugar content in the roots, cytoplasmic male sterility etc.. Engineering sugarbeet plants with beneficial traits is tedious and time-consuming by conventional breeding and classic genetics. Because sugarbeet is an allogamous, heterozygous and biennial crop plant, it takes up to 8 backcrosses to get plants with improved traits using the methods of classic genetics. Thus, the development of effective systems for the micropropagation of plants in tissue culture or regeneration from protoplasts in concert with efficient

transformation methods could be a more efficient system.

1.3.2 Tissue culture

The first experiments on tissue culture of sugarbeet were done about 30 years ago (Butenko et al., 1972). In the beginning the tissue culture of beets has been applied for two purposes: vegetative propagation (Coumans-Gills et al., 1981; Saunders, 1982) or screening for somaclonal variants/mutants with useful traits (Hooker and Nabors, 1977; De Greef and Jacobs, 1979). As mentioned above, sugarbeet is an allogamous and heterozygous crop plant, therefore the micropropagation allows to maintain interesting genotypes. Direct shoot formation from different plant tissues and/or organs is widely used to achieve this aim, while indirect regeneration needs to be developed to obtain variants/mutants. Indirect regeneration includes an additional step of callus induction and the development of conditions for shoot and/or embryo formation. In the early 1970-s root formation from callus was described, but regeneration of whole plants was limited, infrequent and of a very low efficiency (Butenko et al. 1972; Welander, 1974; Hooker and Nabors, 1977).

Attempts to regenerate whole plants from sugarbeet callus can be classified in the following way:

1) infrequent or non-reproducible regeneration from spontaneously forming friable callus during *in vitro* shoot culture. Short or long periods of regeneration activity for this friable callus (white or green) were observed (De Greef and Jabobs, 1979; Saunder and Daub, 1984);
2) organogenesis from habituated compact callus. Here, only root formation, but no shoot regeneration was observed (De Greef and Jacobs, 1979; Van Geyt and Jacobs, 1985);
3) reproducible induction of friable regenerable callus. Several alternative systems with successful regeneration of sugarbeet plantlets were described (Catlin, 1990; Jacq et al., 1992; Snyder et al., 1999)

It is important to note, that plant regeneration was observed only from friable callus. Data on sugarbeet callus formation and its organogenic activities are summarised in Table 1.1.

Table 1.1. Sugarbeet callus: sources, morphology, and hormone composition of regeneration media, and type of organogenesis

Authors	Genotypes, tested/regene-rated	Callus source	Callus morphology	Regeneration media (hormone composition), mg/l	Organo-genesis
Hooker and Nabors, 1977	1/1	embryos	compact and heterogeneously coloured,	BAP 5 + TIBA 0.5 or 5	roots
		cotyledons, hypocotyls	compact green, friable brown		roots buds
De Greef and Jacobs, 1979	1/1	leaf pieces	compact	kinetin or BAP 0.1-1 + GA_3 0.1-1	roots
			friable line	kinetin 1 + GA_3 0.2	distorted leaves and plantlets
Saunders and Daub, 1984	7/2	shoot cultures	friable white	BAP 0.25, 1 or 5 + IAA 0 or 0.3	leaf structures and shoots
Van Geyt and Jacobs, 1985	7/7	leaves, petioles, hypocotyls	compact white	BAP; zeatin; NAA; 2.4-D 0; 0.1; 0.3; 0.5; 0.7; 1; 2 in combination of one cytokinin and one or two auxins	roots
		shoot base	friable	hormone free, BAP or zeatin 1 or more	distorted leaves and plantlets
Saunders and Doley, 1986	5/5	leaf pieces	friable	hormone free, BA 1	buds
Tetu et al., 1987	4/4	a)*auxin induced:* petioles, roots	friable white, compact green	2.4 D 1 or IAA 1 or NAA 1 or NAA 1 + IAA 1	roots
		b) *auxin/ BAP induced:* petioles, roots	compact green	BAP 0.5 + NAA 1	friable white callus with further bud formation
		c) *antiauxin/ cytokinin induced:* cotyledons, roots, petioles, shoot tips, flower buds	friable green	BAP 1 or 3 + TIBA 1 zeatin 1 or 3 + TIBA 1	buds
		d*) multiple-hormone sequence:* cotyledons, roots, petioles	friable green	NAA 1 + BAP 1	somatic embryos and buds

Freytag et al.,1988	6/6	petioles	globular	BA 0.4 + IBA 0.1	shoots and somatic embryos
Catlin, 1990	3/3	cotyledons	compact friable and cream coloured	BAP 1	- shoot meristems
D´Halluin et al., 1992	2/2	seedlings	friable nodular	6-BA 2 + IAA 0.1 + GA_3 0.2	embryos
Jacq et al., 1992	6/6	hypocotyls	yellow compact white friable	BAP 1	- shoots
Hall et al., 1996a	1/1	epidermis	friable	BAP 1 µm	embryos
Snyder et al., 1999	1	hypocotyls	friable	BAP 1	embryos, shoots

Concerning direct shoot formation on sugarbeet, explants of different origin have been used with different degrees of success. Typical for this type of shoot formation is regeneration from pre-existing meristems or predetermined cells, which are usually buried deep within the explants (Freytag et al., 1988; Bannikova et al., 1994). Thus, it is complicated to apply such explants to develop for gene transfer methods. Successful shoot formation with different efficiencies has been observed from petioles (Freytag et al., 1988; Krens and Jamar, 1989; Bannikova et al., 1994), cotyledons (Fry et al., 1991), leaves (Bannikova et al., 1994) and epicotyl-derived thin layer explants (Toldi et al., 1996).

1.3.3 Protoplast culture

"Recalcitrant species" are plant species, which are difficult either to regenerate using tissue culture methods and/or to transform with foreign DNA. Until now sugarbeet was a "recalcitrant" crop, particularly with respect to protoplast-based techniques. Only during the last years the situation slowly improved. The earliest protoplast isolations were performed in 1981 (Smolenskaya and Raldugina, 1981), however, the formation of protoplast derived colonies from suspension cultures and leaves was observed only several years later (Szabados and Gaggero, 1985; Bhat et al., 1985; Bhat et al., 1986). In these experiments merely rhizogenesis was obtained. 10 years later after the first protoplast isolation had been successful, fertile sugarbeet plants could be recovered from leaf protoplasts (Krens et al.,

1990). In these experiments *n*-propylgallate (nPG), an inhibitor of lipoxigenase played an important role. It prolongs the period of cell viability and also stimulates sustained cell divisions with subsequent shoot formation. Nevertheless, cell divisions, plating efficiency and regeneration ability varied greatly from one experiment to the other and appeared to be highly accession-dependent. Embedding of protoplasts in alginate gels (Schlangstedt et al., 1992; Hall et al., 1993) allowed an increase in plating efficiency and to improve experimental reproducibility. Callus or suspension cultures (Szabados and Gaggero, 1985; Bhat et al., 1985; Lindsey and Jones, 1989; Bannikova et al., 1994), petioles (Pedersen et al., 1993; Schlangstedt et al., 1994) and leaves (Krens et al., 1990; Schlangstedt et al., 1992; Hall et al., 1993; Lenzner et al., 1995) were used as protoplast source. In these experiments colony formation of two different types was observed: one being friable, the other compact. Formation of colonies of the friable type was obtained from suspension (callus) and leaf protoplasts, however, only protoplast derived colonies from leaves were able to regenerate shoots. Shoot formation from compact colonies was never observed.

Until now, only four laboratories (Steen et al., 1986; Krens et al., 1990; Weyens and Lathouwer, personal communication in Lenzner et al, 1995; Lenzner et al., 1995) succeeded in plant regeneration from sugarbeet protoplasts. Hall et al. (1995, 1996a) recognised that sugarbeet stomatal guard cells are totipotent. Using epidermis explants of sugarbeet it could be demonstrated, that colonies of regenerable type are formed from guard cell protoplasts and that shoot formation occurs from such colonies (Hall et al., 1997). Also, PEG-mediated transformation of guard cell protoplasts and their regeneration into plants has been successful (Hall et al., 1996b). Despite of this significant breakthrough, DNA integration and shoot formation are still genotype dependent processes and are not routine procedures easy to reproduce.

1.3.4 Transfer of foreign DNA to sugarbeet cells

The possibility to integrate foreign DNA (genes or chromosome fragments or complete genomes) is a major goal in modern plant cell biology and biotechnology. Sugarbeet is one of the most recalcitrant crop species with respect of genetic modifications. The first attempts to insert foreign DNA in sugarbeet were done on hairy root cultures and protoplasts. Electroporation conditions and transient expression of treated protoplasts were established (Lindsey and Jones, 1987; Joersbo and Brunstedt, 1990). Further, transient gene expression in shoot apical meristems of sugarbeet seedlings could be observed following particle bombardment (Mahn et al., 1995). Stable transformation of sugarbeet protoplasts was performed by electroporation, but the transformed colonies had no regeneration activity (Lindsey and Jones, 1989). Paul et al. (1990) obtained transgenic hairy roots, induced by *Agrobacterium rhizogenes*. The biolistic method was tested as well, and transient (Mahn et al., 1995) and stable transformation (Ingersoll et al., 1996), but no shoot regeneration from transformed lines, was observed. The first transformed shoots of sugarbeet have been obtained using an *Agrobacterium*-mediated transformation procedure. However, the transformation efficiencies were low with a maximal efficiency of 1% for transformation of cotyledons (Krens et al., 1996), also genotype dependent and required special skills in the laboratories in which transformation experiments were done. Embryogenic friable callus from either seedlings (D'Halluin et al., 1992) or leaf disks (Ben-Tahar et al., 1991) or hypocotyls (Snyder et al., 1999), shoot base explants (Lindsey and Gallois, 1990) or cotyledon explants (Fry et. al, 1991; Krens et al., 1996) were transformed yielding plantlets that contained foreign DNA. If petiole explants were used for transformation, only compact non-regenerable callus could be produced (D'Halluin et al., 1992). Since Hall et al. (1996a) discovered that stomatal guard cells of sugarbeet retain totipotent capacity, the PEG-mediated method for transformation of beet guard cell protoplasts was successfully demonstrated (Hall et al., 1996b). Recently, the bombardment of regenerable

friable callus (Snyder et al., 1999) was developed, resulting in approximately 8% transformation efficiency.

1.4 Plastid transformation of higher plants

Plant plastids (chloroplasts, chromoplasts, leucoplasts, etioplasts etc.) are cell organelles with two enclosing membranes and contain their own genome (plastome). They are the major biosynthetic centres of the plant cell. Plastids are involved in the synthesis of different important compounds such as carbohydrates, pigments, amino and fatty acids. The plastome is a circular double-stranded DNA molecule and varies in size between plant species from 120 to160 kb. Plastid DNA is highly conserved and frequently contains a large and small copy region (LSC and SSC accordingly) and two inverted repeats (IR_A, IR_B) Fig. 1.2. (Sugiura, 1995).

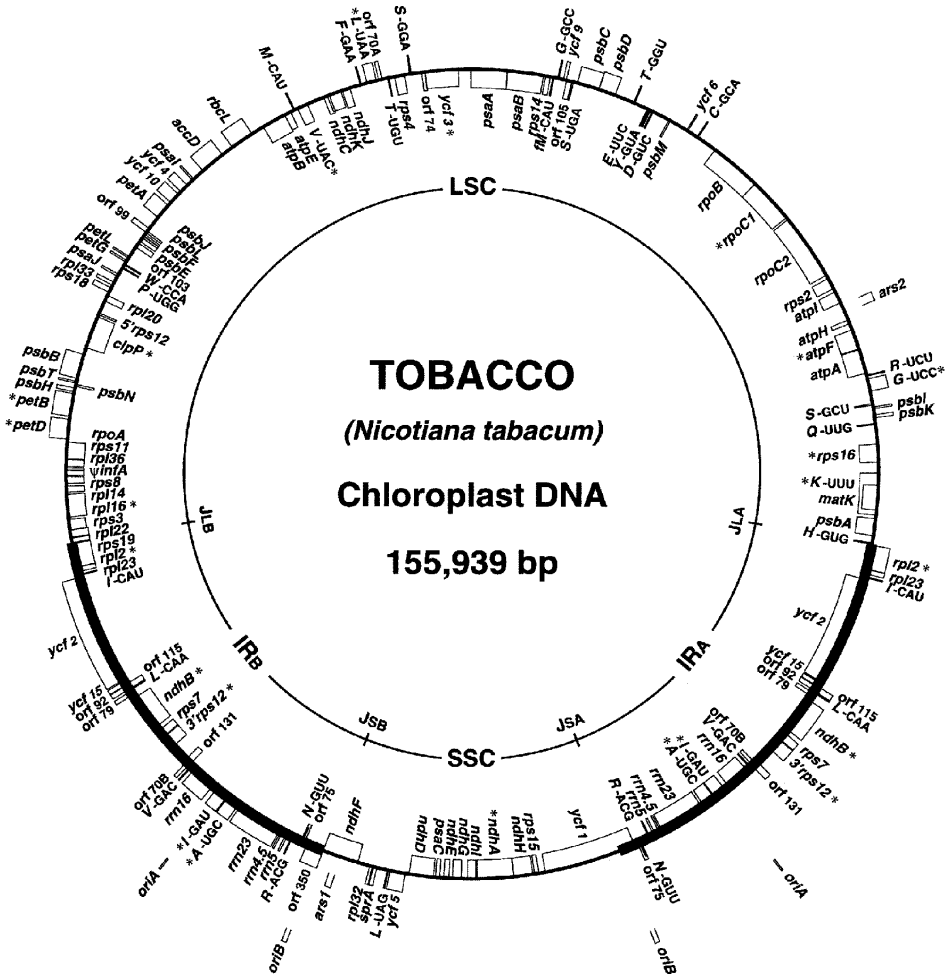


Fig. 1.2. Gene map of the circular molecule of plastid DNA of tobacco (the picture was taken from a homepage "Center for Gene Research", University of Nagoya, Japan; Sugiura, 1998).

A plant mesophyll cell contains 10000-50000 copies of the plastid DNA molecules (Bendich, 1987). For tobacco (Shinozaki et al., 1986), liverwort (Ohyama et al., 1986), rice (Hiratsuka et al., 1989), black pine (Tzudzuki et al., 1994), maize (Maier et al., 1995), *Arabidopsis thaliana* (Sato et al., 1999), *Oenothera elata* (Hupfer et al., 2000), *Lotus japonicus* (Kato et al., 2000) and spinach (Schmitz-Linneweber et al., 2001) the complete plastomes are sequenced.

The plastids of higher plants are an attractive target for genetic engineering. Transformation of the plastome has several advantages over nuclear transformation and has become an important tool for both, basic and applied higher plant research. These are:

1) plastids are mostly maternally inherited, which prevents pollen-mediated outcrossing and, thus, uncontrolled transfer of the transgenes into the environment (Maliga 1993, Daniell et al., 1998);
2) the high copy number of plastid chromosomes per cell makes feasible high levels of protein expression and accumulation (McBride et al., 1995; Staub et al., 2000);
3) gene integration into the plastome occurs via homologous recombination, therefore it is possible to target specific sites precisely and avoid position effect or effects due to multiple integration events. Genes are uniformly expressed and, futher, it is possible to modify or inactivate plastid genes (Medgyesy et al., 1985; Fejes et al., 1990; Svab et al., 1990; Kanevski and Maliga, 1994; Eibl et al., 1999);
4) the plastome has a prokaryotic gene organisation, therefore several genes can be transcribed in one operon (Staub and Maliga, 1995)
5) gene silencing does not occur in plastids and transgene expression is stable (Sidorov et al., 1999);

Stable plastid transformation of higher plants is usually achieved in the following way:

- introduction of a vector containing homologous flanking areas and a selectable marker by particle bombardment or PEG-treatment of protoplasts (Svab et al., 1990; Svab and Maliga, 1993; Golds et al., 1993; O'Neill et al., 1993)
- integration of the transforming DNA into the plastome by two homologous recombination events (Svab and Maliga, 1993);
- elimination of the wild-type genome copies under selection pressure (Kofer et al., 1998).

Both, the biolistic method (Svab et al., 1990) and the PEG method (Golds et al., 1993) could be successfully used to integrate DNA into the chloroplast genome sequence. While the biolistic method uses DNA-coated particles which are shot through the enveloping double membrane of the chloroplast, the mechanism by which DNA is transported into the chloroplast by PEG-treatment is unclear. Both methods have shortcomings and depend on the regeneration capacity of the targeted tissue or protoplasts. In the case of the PEG transformation system it requires also protoplast culture experience (Kofer et al., 1998).

The most frequently used selectable marker is spectinomycin resistance, based either on integration of 16S-rDNA nucleotide sequences containing point mutations (Svab et al., 1990) or on the expression of aminoglycoside-3´-adenyltransferase (*aad*A gene) (Svab and Maliga, 1993). Selection of plastid transformants by kanamycin resistance, based on the expression of the neomycin phosphotranferase (*npt*II gene) has also been reported (Carrer et al., 1993; Carrer and Maliga, 1995). Recently the betaine aldehyde dehydrogenase (BADH) gene from spinach was used as a selectable marker (Daniell et al., 2000). Reporter genes of chloramphenicol acetyltransferase (CAT) (Daniell et al., 1990), β-glucoronidase (*uid*A, GUS) (Ye et al., 1990; Eibl et al., 1999) and green fluorescent protein

(GFP) (Hibberd et al., 1998; Sidorov et al., 1999; Khan and Maliga, 1999) have been transiently or stably transformed in plastids. Stable plastid transformation of higher plants has been so far reported for two species of the genus *Nicotiana*, *N. tabacum* (Svab et al., 1990; Svab and Maliga, 1993; Golds et al., 1993) and *N. plumbaginifolia* (O'Neill et al., 1993), two cruciferous species, *Arabidopsis thaliana* (Sikdar et al., 1998) and rapeseed (Chaudhuri et al., 1998), potato (Sidorov et al.1999) and the cereal species rice (Khan and Maliga, 1998). Plastid transformation is a rapidly developing area of plant molecular and cell biology, which allows to investigate the functionality, regulation and evolution of the plastid genome, interaction between different cell compartments (Staub and Maliga, 1993; Rochaix, 1997; Kavanagh et al., 1999) and to use the plastids in plant biotechnology (McBride et al., 1994, 1995; McBride and Stalker, 1999; Kota et al., 1999; Staub et al., 2000; Iamtham and Day, 2000; Lössl et al., 2000).

1.5 Research aims

The use of any crop species in plant biotechnology and/or fundamental research is impossible without development of effective, reproducible and routine methods for regeneration and genetic transformation. A successful application of methods for gene transfer depends on the possibility to transform a cell/tissue which can be regenerated into a plant afterwards. While for some species these problems have already been solved, for others the methods have not been established or if available, they are suited just for some genotypes. Genotype dependence concerning methods for regeneration and/or transformation has to be overcome in many species.

The main goal of this investigation was to develop methods for plant regeneration, which could be used for plastid transformation in rapeseed and sugarbeet through either PEG-mediated DNA uptake into protoplasts or delivering DNA-coated gold particles (the biolistic method) into cells/organelles. For a successful solution of the problems the following steps have to be achieved:

1) development of a novel and efficient method for fast protoplast regeneration

Tobacco plants were used for establishing a novel technique. Different factors were checked and optimised, i.e. growth conditions of donor plants, isolation and culture conditions. High efficiency, fast regeneration, reproducibility, applicability to different aims and convenience are the main criteria that would be significant.

2) establishment of rapeseed protoplast culture

Successful shoot regeneration from protoplast derived colonies in rapeseed was done in many laboratories. Unfortunately, shoot regeneration from protoplasts is genotype dependent. Breeding lines, "Westar" and "Drakkar" were tested. The protoplast system should be efficient enough for use in plastid transformation. Different growth regulators should be tested for finding optimal regeneration conditions from protoplast derived colonies, since a low regeneration efficiency on established media was observed (Thomzik and Hain, 1988).

3) test for optimal source tissue/organ for protoplast culture and genetic transformation in sugarbeet

In the literature there is only one report (Hall et al., 1997) about successful and highly efficient protoplast isolation and regeneration from guard cells and their subsequent transformation. Therefore, guard cell protoplasts should be tested for their regeneration capacity and gene transfer by the PEG method. Alternatively, protoplasts from other sources could be examined. Since shoot regeneration in sugarbeet was often observed from various tissues/organs, it is also necessary to test explants of different origin for their regeneration and, thus, to determine the type of explants/tissues/organs suitable for plastid transformation by the biolistic method.

4) plastid transformation in rapeseed and in sugarbeet

Species-specific vectors containing flanks from the rapeseed and the sugarbeet plastid chromosomes with the *aad*A-cassette as a selection marker should be constructed. The PEG method for protoplasts and the biolistic method for protoplast derived colonies or other sources, such as explants of different origin, callus etc., will be tested. Resistant lines can then be selected on medium supplemented with spectinomycin and/or streptomycin. After selection resistant lines will be examined by DNA analysis for the integration of the marker gene into the plastome.

2. MATERIALS AND METHODS

2.1 Chemicals

Substance	Manufacturer
Agar (purified)	Sigma, St. Louis, USA
Agarose (SeaKem, LE)	Biozym, Hameln
Alginic acid (from *Macrocystis pyrifera*)	Sigma, St. Louis, USA
Alkaline phosphatases:	
CIP (calf intestine phosphatase)	Boehringer Mannheim, Mannheim
SAP (shrimp alkaline phosphatase)	Amersham Buchler, Braunschweig
Ampicillin (as Ampicillintrihydrate)	Serva Feinbiochemica, Heidelberg
B5 salts	Sigma, St. Louis, USA
BAP (6-benzylaminopurine)	Sigma, St. Louis, USA
Bacto agar	ICN, Ohio, USA
Bacto trypton	Serva Feinbiochemica, Heidelberg
Cellulase “Onozuka” R-10	Yakult Pharmaceutical Industry, Japan
Desoxynucleotides	Amersham Buchler, Braunschweig
Dimanin C	Bayer, Leverkusen
DNA-Ligase (from Rapid Ligation Kit)	Boehringer Mannheim, Mannheim
DNA-markers:	
λ *Eco*57I/*Mlu* I	MBI Fermentas, Vilnius, Lithuania
*Eco*47I	MBI Fermentas, Vilnius, Lithuania
500 bp	MBI Fermentas, Vilnius, Lithuania
200 bp	MBI Fermentas, Vilnius, Lithuania
DNA-Polymerase:	
Klenow enzyme	Boehringer Mannheim, Mannheim
Taq-polymerase	QIAGEN, Hilden
Pfu-polymerase	Promega,
Driselase	Sigma, St. Louis, USA
Ethidiumbromide	Roth, Karlsruhe
Formaldehyde (35% solution)	Roth, Karlsruhe
Glucose (D(+)-Glucose)	Bader, Deventer, The Nederlands
IAA (indole-3-acetic acid)	Sigma, St. Louis, USA
IPTG (isopropyl-D-thiogalactopyranoside)	MBI Fermentas, Vilnius, Lithuania
Kinetin	Sigma, St. Louis, USA
Macerozyme R-10	Yakult Pharmaceutical Industry, Japan
Mannitol	Sigma, St. Louis, USA
MES (2[N-morpholino]ethane-sulfonicacid)	Sigma, St. Louis, USA

MS salts	Sigma, St. Louis, USA
NAA (α-naphthaleneacetic acid)	Sigma, St. Louis, USA
nPG (*n*-propylgallate)	Sigma, St. Louis, USA
Oligonucleotide	MWG, Ebersberg
Phytagel	Sigma, St. Louis, USA
Proteinase K	Amersham Buchler, Braunschweig
Polyethylenglycol 1500	Sigma, St. Louis, USA
Restriction enzymes	MBI Fermentas, Vilnius, Lithuania
Spectinomycin (as Spectinomycindihydrochloride)	Sigma, St. Louis, USA
Streptomycin (as Streptomycinsulfate)	Sigma, St. Louis, USA
Sucrose	ICN, Cleveland, USA
2,3,5-triiodobenzoic acid (TIBA)	Sigma, St. Louis, USA
Thidiazuron	Sigma, St. Louis, USA
X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-D-galactopyranoside)	Biometra, Göttingen
X-Gluc (5-Brom-4-Chlor-3-Indolyl-β-glucuronide)	Sigma, St. Louis, USA
Zeatin	Sigma, St. Louis, USA

All the other chemical agents which are not included in the list were in p.a. quality and from Baker Chemicals (Phillipsburg, USA), Difco (Detroit, USA), Merck (Darmstadt), Roth (Karlsruhe), Serva Biochemica (Heidelburg) and Sigma (St. Louis, USA).

2.2 Bacteria and vectors

DNA-Vectors:

pGEM-T Easy	(Promega, Madison, USA)
pUC18	(Yanisch-Perron et al., 1985)

Plasmids:

pSL-GUS-INT-PAT	(the *pat*-gene, the *uid* A gene with an integrated STLS1-intron)	Josef Kraus, Planta GmbH, Einbeck, Germany
pUC16 *aad*A	(the *aad*A-gene)	(Koop et al., 1996)

Bacteria for cloning:

"Epicurian *coli* SURE 2"	(Stratagene, Heidelberg)

pUC16 *aad*A contains the aminoclycoside 3´-adenyltransferase (*aad*A) gene from *Escherichia coli* (Goldschmitt-Clermont, 1991) under the control of the tobacco 16S rRNA promoter (16S promoter, Prrn) and flanked 5´ by 26 bp fragment from tobacco *rbc*L-operon and 3´ by the terminator of the *rbc*L-gene of the *Chlamydomonas reinhardtii* plastome.

2.3 Primers

Isolation of the *aad*A-cassette:

*aad*A-li	5´-gct cga gat acc ggt ccc ggg aat tcg ccg tcg-3´
*aad*A-re	5´-ggt taa cgg cgc ctg gta ccg agc tcc acc gcg-3´

Isolation of plastid fragments:

*ycf*3-li	5´-gat tgg gta tgg ctt caa c-3´
*ycf*3-re	5´-cga tca tag gga tca att tc-3´
*trn*V-li (orf131)	5´-cca cgt caa ggt gac act c-3´
*rps*7-re (orf131)	5´-ctg cag tac ctc gac gtg-3´

A detailed comparison of selected fragments has been done with the help of "Blast search" programme (http://www.ncbi.nlm.nih.gov/BLAST/). PCR primers have been designed using a sequence of the tobacco plastome (Shinozaki et al., 1986).

Detection of the *uid*A gene:

*uid*A-li	5´-atg gtc cgt cct gta gaa ac-3´
*uid*A-re	5´-agc aca tca aag aga tcg ctg-3´

Detection of the *aad*A-gene:

*aad*A-li	5´-agc act aca ttt cgc tca tcg c-3´
*aad*A-re	5´-act atc aga ggt agt tgg cgt c-3´

2.4 Methods of recombinant DNA and vector construction

Methods for DNA cloning, such as PCR, restriction, agarose gel electrophoresis, dephosphorylation, blunt-ending and ligation, bacterial transformation were performed in accordance to Sambrook et al. (1989), or in accordance to protocols developed by manufacturers.

2.4.1 Isolation of plasmid DNA

For plasmid isolation in small amounts (~5 µg) for analysis of recombinant bacteria a "rapid alkaline extraction" method (Birnboim and Doly, 1979) modified accordingly to Eibl (1999) was used. Plasmid DNA for cloning and sequencing was isolated using "QIAPrep Miniprep-Kit" (Qiagen, Hilden). DNA was isolated from 3 ml of bacterial culture in LB medium.

Plasmid DNA in larger amounts for nuclear or plastid transformation was isolated using Qiagen-Maxiprep columns (Tip 100 to Tip 500; Qiagen, Hilden). After DNA purification through the columns and isopropanol and ethanol precipitations following the protocol, additional DNA purification was performed. To dried DNA pellets 1,1 ml of water was added. After DNA dissolving during shaking for 1 hour at 37ºC solution was transferred in new 2ml plastic tubes, 550µl in each. Sodium acetate (pH5.2, 0.1 volume) and ethanol (100%, 2.5 volume) were added and DNA precipitated. DNA was washed twice with ethanol 70% and after drying dissolved in TE (pH5.6) or in sterile water to get a final concentration of ~2 µg/ml and stored at -20ºC.

LB medium		**TE-buffer**	
NaCl	10 g/l	Tris-HCl, pH 8.0	10 mM
Peptone	10 g/l	EDTA	1 mM
Yeast extract	5 g/l		

2.4.2 Dephosphorylation of linearised vector DNA

An optimised protocol for efficient dephosphorylation was developed. Approximately ~0,5 U SAP (shrimp alkaline phosphatase) was added to blunt-end linearised DNA (5µg) and the mixture was incubated for 30 min at 37°C. Then DNA was purified with QIAquick PCR Purification Kit (Qiagen, Hilden) and resuspended in 30-50µl of 1 x conc. "Calf Intestine Phosphatase" (CIP) buffer (Boehringer Mannheim, Boehringer). Afterwards 0.2U of CIP were added and the mixture was incubated for 30 min at 37°C. Additional 0.1U of CIP were supplied

again and the incubation temperature was increased to 56°C. The duration of incubation was the same as in the previous step. Phosphatase activity was completely inactivated by adding SDS (final concentration 0.5%), EDTA (final concentration 5 mM) and proteinase K (final concentration 100 µg/ml). Inactivation continued for 30 min at 56°. Afterwards DNA was extracted with P/C/I mixture (phenol/chloroform/ isoamylalcohol, 25:24:1, pH 8.0), then with only phenol and with only chloroform using phase-lock plastic tubes (5 Prime → 3 Prime, Inc., Boulder, USA) in each extraction step. Dephosphorylated DNA was purified with QIAquick PCR Purification Kit (Qiagen, Hilden)

2.4.3 "Blunt-ending" of linearised DNA and "blunt end" and "sticky end" ligation.

Linearised DNA (a vector and/or isolated fragment) with 5´-protruding ends was blunted by "fill in"-reaction with Klenow polymerase according to the protocol (MBI Fermentas, Vilnius, Lithuania). For ligation, approx. 100 ng of vector DNA (pSB or pSB-*Acc*I, or pRS) and fragment (the *aad*A-cassette) in amounts corresponding double molar (for "blunt end") or equal molar (for "sticky end") concentrations like vector DNA were mixed and ligated with "Boehringer rapid ligation Kit" (Boehringer-Mannheim, Boehringer) for 30 min at 20ºC. Ligation products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden) and eluted with sterile water.

2.4.4 Transformation of *E.coli*

Ligation products were transformed in electrocompetent cells "Epicurian *coli* SURE 2" (Stratagene, Heidelberg). Following the standard protocol (Stratagene, Heidelberg) about 20 ng of DNA from ligation reactions were mixed with 50µl of competent cells, transferred to 2 mm cuvettes and transformed by the use of the electroporation method for EasyjecT Plus electroporator (EquiBio, Ashford, United Kingdom). Test transformations with the standard plasmid (pUC18) resulted in a

transformation efficiency over than $1 \cdot 10^9$ cells per 1 µg DNA.

2.4.5 Cloning of PCR-fragments

The plastid fragments from sugarbeet and rapeseed, homologous to tobacco fragment *trn*V-*rps*7 (nucleotides (nt) 140126-142640), were amplified using *Pfu* DNA polymerase (Promega, Madison, USA). After they have been extracted from 1%-agarose gel with QIAquick Gel Extraction Kit (Qiagen, Hilden), A-tailing was performed using *Taq* DNA Polymerase (QIAGEN, Hilden) according to the producer's protocol (Promega, Madison, USA). Products of reaction were purified using QIAquick PCR Purification Kit (QIAGEN). Purified products of A-Tailing reaction were ligated with pGEM-T Easy vector using Rapid Ligation Kit (Boehringer-Mannheim, Boehringer) and purified again in the same way. After transformation, bacteria were plated to LB agar plates containing 100µl of 10 mM IPTG, 100µl of 2%-X-gal and 75 mg/l ampicillin. Using the blue-white selection system white colonies were selected. Positive colonies were confirmed by restriction of DNA from white clones with *Not*I (Fig. 2.1).

2.4.6 Cloning of transformation vectors with the *aad*A-cassette

The *aad*A cassette was either PCR-amplified or excised with *Sma*I and *Ksp*AI. The PCR product was blunt-ended with Klenow polymerase and the *aad*A-cassette, obtained either by PCR or by cutting out with restrictases, was dephosphorylated and ligated into primary vectors (pSB or pRS). Suitable integration sites for vectors were found with the programme Vector NTI Version 4.0.2. Colonies were selected on agar-solidified LB medium supplemented with 75 mg/l of ampicillin and 100 mg/l of spectinomycin. Orientation of the inserts was confirmed by restriction analysis with *Bam*HI, *Cfr*42I, *Eco*32I, *Hind*III, *Not*I and *Pvu*I.

2.5 Methods of DNA analysis

The standard analytical methods used in this work were described by Sambrook et al. (1989). When it was necessary protocols from manufacturers were used.

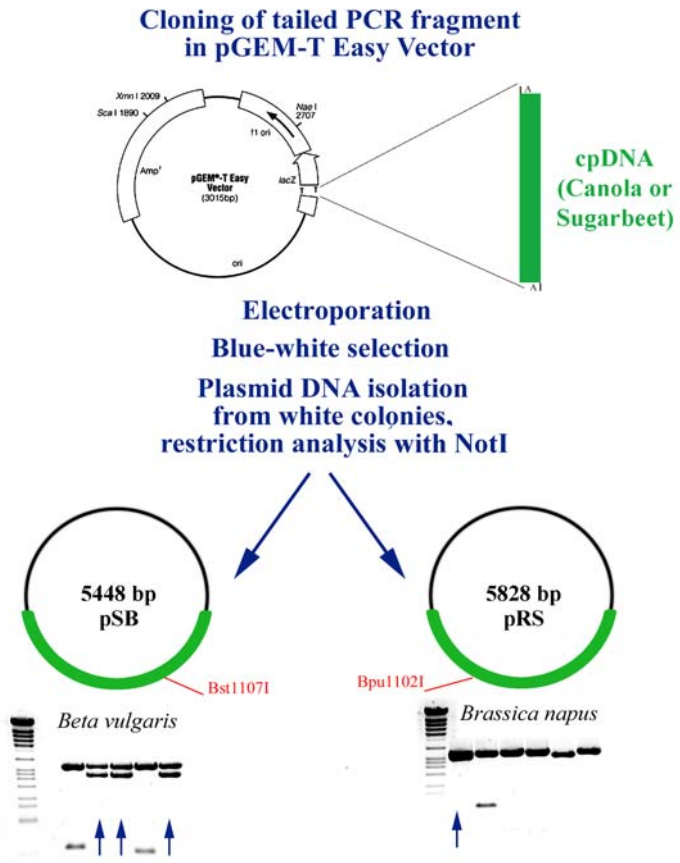


Fig. 2.1. Insertion of sugarbeet and rapeseed plastid fragments in vector pGEM-T Easy.

2.5.1 PCR (polymerase chain reaction)

Standard conditions for PCR are presented below. PCRs were done using hotlid HYBAID PCR Express thermocycler Ready for Gradients Thermoblocks (Hybaid Ltd., Ashford, United Kingdom) and chemicals from PCR Kit for *Taq* DNA polymerase (Qiagen, Hilden) or chemicals for *Pfu* DNA polymerase (Promega, Madison, USA). In the case of difficulties to obtain expected amplification products, which could be due to a low specificity of used primers, experiments on optimisation of PCR conditions were performed. Different melting temperatures (Tm) and magnesium chloride concentrations were tested.

Step	**Recommended conditions**	
Denaturation	1-3 min	94°C
3-step cycling (30-35 cycles)		
Denaturation	1 min	94°C
Annealing	0.5 min	(Tm-5)°C
Extension	1 min/kbp (2 min for *Pfu* polymerase)	72°C
Final extension	5-10 min	72°C

Standard components	**Concentration in reaction**
Template DNA	0.1-10 ng
DNA-Polymerase buffer	1x
$MgCl_2$	1.5 mM
Primer 1	0.5 µM
Primer 2	0.5 µM
dNTP mix	200µM of each dNTP
Tag DNA Polymerase	0.5 U
Total volume (adjusted with distilled H_2O	50 µl

2.5.2 DNA-sequencing

DNA from vectors pSB and pRS was sent for sequencing to Toplab (Martinsried). Cloned plastid fragments were sequenced (Appendix 1).

2.5.3 DNA isolation from plant tissues

DNA from tobacco, rapeseed and sugarbeet was isolated with "DNeasy Plant Mini Kit" (Qiagen, Hilden). DNA isolated this way was applied for PCR and Southern analysis. 100-200 mg of leaf material were used for DNA extraction. To increase final concentration, the amount of elution buffer was reduced by a factor of two.

2.5.4 Southern hybridisation

Plasmid pSL-GUS-INT-PAT was used as a template for restriction-mediated generation of α^{32}P-dCTP labelled probes. Digested plant DNA was electrophoresed in 20 cm agarose-gel for at least 24 h at 30V and afterwards transferred to N+ Nylon membrane (Amersham Buchler, Braunschweig) with the capillary-blot-method. 0.4M NaOH was used as the medium for transfer. DNA was fixed to the membrane with UV-light in "UV-Stratalinker 1800" (Stratagene, Heidelberg). Prehybridisation and incubation with radioactive probes was performed in hybridisation buffer (Church and Gilbert, 1984) at 63°C overnight. After washing, the membrane was developed for about 1 night on Biomax-Film (Kodak). Signals were detected with a Phosphoimager (Fujifilm BAS 1500).

Hybridisation buffer (Church and Gilbert, 1984)

Na_2HPO_4/NaH_2PO_4 (pH 7.5)	250mM
SDS	7% (w/v)

2.6 Plant material

Tobacco: Two cultivars of tobacco were used in this study as control plant species, i.e. “petite Havana” and “Wisconsin 38”.

Rapeseed: Cv. Drakkar and cv. Westar were used in this study. Seeds were kindly provided by Planta GmbH (Einbeck).

Sugarbeet: Seeds of sugarbeet cultivars “Viktoria” and “7T1308”, aseptic shoot cultures of cultivars “Viktoria”, VRB and 31-188 were used in this study. 47 breeding lines used as donors of leaf explants were grown in a greenhouse (Appendix 2). Both, seeds and plant cultures were kindly provided by Planta GmbH (Einbeck).

2.7 Media and solutions

Solutions for protoplast isolation and immobilisation, and media for protoplast and tissue culture are listed in Tables 2.1, 2.2 and 2.3.

Table 2.1. Solutions for protoplast isolation (preplasmolysis media are not included)

Compound	MMM[a]	MMS[b]	Alg-A[c]	Ca-A[d]	CPW9M[e]	CPW13M	CPW15S	CPW22S	W5[f]
$CaCl_2 \cdot 2\ H_2O$				2940	1480	1480	1480	1480	18400
$CuSO_4 \cdot 5H_2O$					0.025	0.025	0.025	0.025	
KH_2PO_4					27.2	27.2	27.2	27.2	
KI					0.14	0.14	0.14	0.14	
KNO_3					101	101	101	101	
KCl									360
NaCl									9000
MES	1952	1952	1952	1952					
$MgCl_2 \cdot 6H_2O$	2040	4066	2040						
$MgSO_4 \cdot 7H_2O$	2500		2500		246	246	246	246	
Mannitol	ca. 85 g		ca. 85 g	ca. 85 g	9% (w/v)	13% (w/v)	15% (w/v)	22% (w/v)	
Sucrose		ca. 130g							
Glucose									1 g
Alginic acid		28 g							
Agar				10 g					

Amounts are given as mg/l, unless indicated otherwise. All solutions are adjusted to pH 5.8. The last solution is filter sterilised, all other solutions are autoclaved. First through forth medium are adjusted to 550 mOsm.

[a]Magnesium (20mM), MES (10mM), mannitol

[b]Magnesium (20mM), MES (10mM), sucrose

[c]Alginic acid, low viscosity

[d]Calcium (20mM)-agar

[e]Fifth through eighths solutions contain CPW salts (Frearson et al., 1973) with either mannitol (9M, 13M) or sucrose (15S, 22S) (Tomzhik and Hain, 1988; Krens et al., 1990)

[f]W5 salts solution (Menczel et al., 1981)

Table 2.2. Media for preplasmolysis and protoplast culture

Solutions	**F-PIN**[a]	**PIB**[b]	**PIBr**[c]	**PCN**[d]	**F-PCN**[e]	**K8p**[f]	**PC**[g]	**PCB**[h]	**PCBr**[i]
$CaCl_2 \cdot 2\ H_2O$	640	600	420	350	640	600	600	600	420
NH_4NO_3		600				600	600	600	
NH_4Cl			135						135
$(NH_4)_2SO_4$				134					
KH_2PO_4	170	170	85		170	170	170	170	85
KCl		300				300	300	300	
KNO_3	1012	1900	950	2500	1012	1900	1900	1900	950
MES	1952		1000	1952	1952				1000
$MgSO_4 \cdot 7H_2O$	370	300	185	250	370	300	300	300	185
$NaH_2PO_4 \cdot H_2O$				150					
NH_4-succinate[j]	20mM				20mM				
Micro-elements	MS	B5	B5	B5	MS	B5	B5	B5	B5
Inositol	200	200	200	200	200	100	100	200	200
Ascorbic acid		2				2	2	2	
Biotin	0.02		0.02	0.02	0.02	0.01			0.02
Ca-panthotenate	2		2	2	2	1			2
Choline chloride						1			
Folic acid						0.4			
Nicotinamide						1			
Nicotinic acid	2	1	2	2	2		1	1	2
p-Aminobenzoic acid						0.02			
Pyridoxine-HCl	2	1	2	2	2	1	1	1	2
Riboflavin						0.2			
Thiamin-HCl	1	10	1	1	1	1	10	10	1
Vitamin A						0.01			
Vitamin D_3						0.01			
Vitamin B_{12}						0.02			
Citric acid						40	40		
Fumaric acid						40	40		
Malic acid						40	40		
Sodium pyruvate						20	20		
L-Glutamine			100						100
Casein hydrolysate			100						100
Coconut water		20 ml				20 ml	20 ml	20 ml	
Cellobiose						0.25	0.25		
Fructose						0.25	0.25		
Glucose					ca.80 g	ca.68.4g	68.4g	ca. 75g	
Mannitol		ca. 85 g	ca. 85g			0.25	0.25		80 g
Mannose						0.25	0.25		
Rhamnose						0.25	0.25		
Ribose						0.25	0.25		
Sorbitol						0.25	0.25		
Sucrose	ca.130g			ca.130g	20g	0.25	0.25	20 g	20 g
Xylose						0.25	0.25		
2.4-D						0.2	1		
BAP	1			1	1	0.5	0.1	1	
Kinetin									3
NAA	0.1			0.1	0.1	1	0.1	2	1

Amounts are given as mg/l, unless indicated otherwise. All solutions are adjusted to pH 5.8. The last solution is filter sterilised, all other solutions are autoclaved. First through forth medium are adjusted to 550 mOsm.

[a]Fast protoplast incubation *Nicotiana*, vitamin composition after Koop and Schweiger (1985)

[b]Protoplast incubation *Beta*, macrosalts composition after Kao and Michayluk (1975), vitamin composition after Glimelius et al. (1986)

[c]Protoplast incubation *Brassica*, vitamin composition after Koop and Schweiger (1985)

[d]modified from PCN (Koop et al., 1996). Polybuffer 74 was replaced with MES 10 mM

[e]Fast protoplast culture *Nicotiana*, vitamin composition after Koop and Schweiger (1985)

[f]K8p modified from K8p (Kao and Michayluk, 1975) according to Krens et al. (1990). Amino acids were not included

[g] medium composition after Glimelius et al. (1986)

[h]Protoplast culture *Beta*, macrosalts composition after Kao and Michayluk (1975), vitamin composition after Glimelius et al.(1986)

[i]Protoplast culture *Brassica*, vitamin composition after Koop and Schweiger (1985)

[j]Ammonium succinate after Dovzhenko et al. (1998)

Table 2.3. Media for callus induction and shoot regeneration

mg/l	PGoB[a]	MS[b]	MS15B2[c]	MSB1[d] *	RS[e]	SCN[f]	SRN[g]	SRB[h]	SRBr[i]
$CaCl_2 \cdot 2\ H_2O$	300	440	440	440	15	150	420	440	440
$Ca(NO_3)_2 \cdot 4\ H_2O$					708				
NH_4NO_3		1650	1650	1650				1650	1650
$(NH_4)_2SO_4$	400					134			
KH_2PO_4		170	170	170	170		85	170	170
KCl	600								
KNO_3	2000	1900	1900	1900	3000	2500	950	1900	1900
$MgSO_4 \cdot 7H_2O$	500	370	370	370	1233	1233	185	370	370
$NaNO_3$					170				
$NaH_2PO_4 \cdot H_2O$	287,5					150			
MES							1952		
Micro-elements	PGoB	MS	MS	MS	MS	B5	B5	MS	MS
Inositol	100	100	100	100	100	100	100	100	100
Glycine		2	2	2	2			2	2
Biotin				0.01					
Ca-panthotenate				1					
Nicotinic acid	1	0.5	0.5	1	0.5	1	1	0.5	0.5
Pyridoxine-HCl	1	0.5	0.5	1	0.5	1	1	0.5	0.5
Thiamin-HCl	10	0.1	0.1	10,4	0.1	10	10	0.1	0.1
Mannitol							30 g	30 g	
Sucrose	30 g	30 g	15 g	30 g	20 g	30 g	30 g	30 g	30 g
BAP							0.1	2	2
NAA							0.01	1	2
TIBA								1	
Agar	7.5g	8g		8g		8g	8g		
Gelrite									2g
Phytagel			4g		4g			4g	

Amounts are given as mg/l, unless indicated otherwise. All solutions are adjusted to pH 5.8.

[a]after De Greef and Jacobs (1979)

[b]after Murashige and Skoog (1962). Media supplemented with BAP at different concentrations ("con") were named as MSB"con", where "con" is a BAP concentration in mg/l

[c]MS medium (Murashige and Skoog, 1962) with double reduced sucrose concentration and 2mg/l BAP.

[d]after Ben-Tahar et al. (1991). * Medium was used only in the experiment, described in § 3.3.3.

[e]Rapeseed

[f]Shoot culture *Nicotiana*, modified from B5 (Gamborg et al.1968)

[g]Shoot regeneration *Nicotiana*

[h]Shoot regeneration *Beta*

[i]Shoot regeneration *Brassica*

2.8 Seed sterilisation

Three different sterilisation procedures for seeds and leaves were used. Tobacco and rapeseed seeds were sterilised by sterilisation procedure A. Sterilisation method B was used for sugarbeet seeds. Sugarbeet leaves were sterilised in the third way, sterilisation procedure C.

Sterilisation A: seeds were surface sterilised with 70% ethanol (v/v) for 1 min and then treated with 5% (w/v) Dimanin C for 10 min. Afterwards, sterile seeds were washed in autoclaved distilled water in three steps, each for 10 min.

Sterilisation B: seeds were soaked in tap water and incubated in the refrigerator at +4°C overnight. After water was removed, seeds were transferred to 70% (v/v) ethanol (1 min), 35% (v/v) formaldehyde (1 min), 0.05% (w/v) $HgCl_2$ (5 min), 5% (w/v) Dimanin C (10 min), followed by 3 washes in autoclaved distilled water (10 min each).

Sterilisation C: leaves from greenhouse material were cut and surface sterilised with 6% of Chlorbleichlauge (CG CHEMIKALIEN Geselschaft GmbH & Co. KG,) for 5-10 min and washed with sterile water.

2.9 Seed germination and growth conditions for donor plants

Tobacco: Derooted seedlings were transferred to jars containing 120 ml of SCN medium (Table 2.3). Culture conditions: 25°C, 16 h light, 0.5-1 W/m^2, Osram L85W/25 Universal-White fluorescent lamps.

Rapeseed: Derooted seedlings were cultured on RS medium (Table 2.3) under the same culture conditions as for tobacco plants.

Sugarbeet: Seeds were germinated on MS medium with 2 mg/l BAP (MSB2) or on MS medium containing reduced sucrose concentration (15g/l, MS15B2) for 1 month at 25°C in the dark. Shoot cultures (genotypes VRB, "Viktoria", 31-188) were grown on hormone-free MS medium, or MSB2 (2 mg/l BAP), or MSB1 (1 mg/l BAP). Plants from genotypes "Viktoria" and 7T1308, which had been used to determine regeneration efficiency of different explants, were cultured on hormone-free MS medium (see Table 2.3). Subculture period was four weeks.

2.10 Callus induction from sugarbeet explants and organogenesis

Content of culture media used in these experiments is presented in Table 2.3. Callus was induced from various explants for breeding lines "Viktoria" and 7T1308. Hypocotyl and cotyledon explants were removed from 1 month old seedlings and transferred to MS15B2 medium in the dark. Cotyledons longer than 1 cm were cut perpendicularly to their axis in the middle. Hypocotyls were usually about 1-4 cm in the length and were cut to segments of about 1 cm length. Normally 20-30 cotyledon segments and 50-60 hypocotyl segments were transferred to a petri dish. After small colonies had been formed, they were either transferred to fresh MS15B2 medium (in the dark or in the light) or used directly for experimental purposes (protoplast isolation or particle bombardment).

Callus from root explants (genotype "Viktoria") was induced on MSB2 medium in darkness at 25°C. Callus (genotypes VRB and "Viktoria") from leaves, petioles, or shoot bases was induced on MSB2 medium in the light (photoperiod of 16h/day) at

25°C. When friable callus from explants of different origin appeared (usually after 3-5 weeks of induction) it was used to determine the regeneration efficiency either on MSB0.25 or MS15B2 medium in the light (photoperiod of 16 h/day) or in the dark at 25°C. The regenerated plants were rooted on hormone-free MS medium.

After sterilisation (procedure C) leaves from greenhouse plants of 47 different genotypes were used for callus induction. The procedure is described by Ben-Tahar et al. (1991). Culture conditions and steps are presented in Fig.2.2.

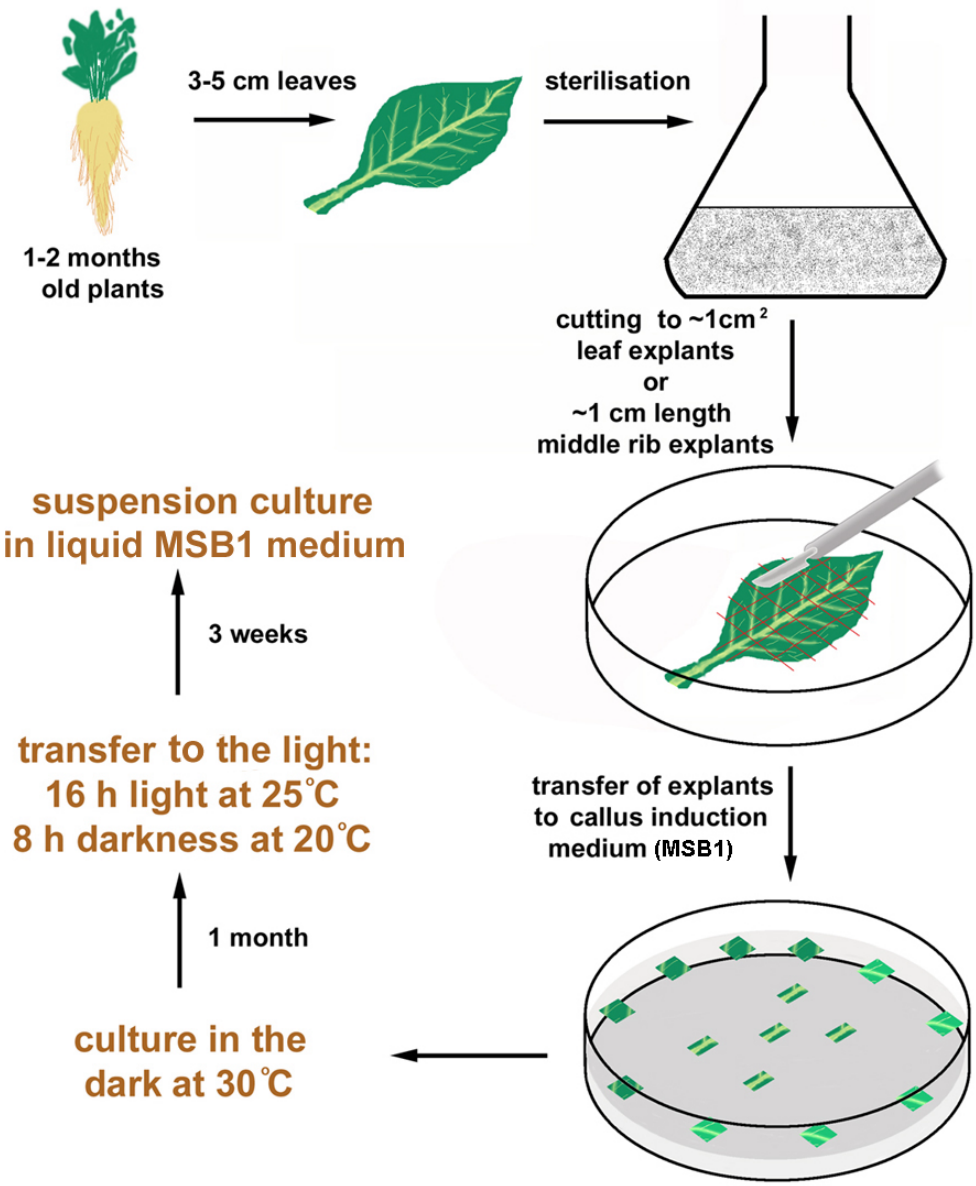


Fig. 2.2. Scheme presenting culture steps and culture conditions for them as described by Ben-Tahar et al. (1991).

2.11 Shoot regeneration from sugarbeet explants

Shoot explants (petiole, leaf and basal tissue explants) were prepared from 20-25 plants of each breeding line tested ("Viktoria" and 7T1308). In the case of seedling explants, those from 50 seedlings were used for both cultivars. 100 explants of each type were tested (20 explants per 9 cm petri dish with 20 ml of medium MSB1). Basal tissue explants were about 0.5 mm in thickness. Other explants were prepared in a way that prevents presence of buds (for petiole explants) and apical meristems (for seedling explants). For this, cotyledons and petioles were cut from seedlings/shoots 1-2 mm below apical or side meristems respectively. Hypocotyls were removed about 2 mm below the epicotyl area.

2.12 Epidermal peelings

Leaves of established sugarbeet cultures (genotypes "Viktoria", VRB and 31-188) growing either on hormone-free MS medium or on medium MSB2 were used for epidermal peelings. Epidermis fragments were isolated manually from the adaxial side of the leaves using a pair of curved forceps. After isolation, fragments were immediately transferred into liquid PCB medium.

2.13 Protoplast isolation, embedding and culture

Protoplast isolation and embedding media are presented in Table 2.1. Protoplast culture media are presented in Table 2.2. Regeneration media for protoplast derived colonies and the medium for rooting are described in Table 2.3.

Tobacco: Leaves from plants about three weeks of age were cut to stripes (approximately 1mm in width) and incubated overnight with 0.25% cellulase Onozuka R-10 and 0.25% macerozyme Onozuka R-10 (Yakult, Honsha, Japan) dissolved in medium F-PIN. Parameters for filtration and purification procedures were as described by Koop et al. (1996), but new media (Table 2.2.) and a novel culture technique were used. Purified protoplasts were resuspended in MMM medium and mixed with the same volume of alginic acid solution (Alg-A), and

alginate embedding was performed in thin alginate layers (the TAL-technique). Protoplast alginate mixtures (by 625µl, $4 \cdot 10^4$ protoplasts) were transferred to the surface of agar-solidified Ca^{2+}-A medium and a polypropylene grid (10x10 meshes, 2x2 mesh size, Scrynel PP2000, K.H.Büttner GmbH, Wasserburg, Germany) was inserted into the alginate. After gels were solidified grids with embedded protoplasts were placed upside down into culture medium (F-PCN) and washed for 2 times with 10 ml F-PCN. Then the grids were transferred to a new petri dish (6 cm in diameter) with 2 ml F-PCN. After protoplast derived colonies were formed, grids were placed on solid F-SRN in Magenta vessels. The regenerated shoots were rooted on hormone-free MS medium.

Rapeseed: Leaves of 3-4 weeks old shoots or cotyledons of 4-6 days old seedlings from two genotypes, "Drakkar and "Westar", were preplasmolysed and digested either as described by Thomzik and Hain (1988) (experiments were performed only with leaf protoplasts) or using a new medium PIBr containing 0.5% (w/v) cellulase Onozuka R-10 and 0.5% (w/v) macerozyme Onozuka R-10 overnight. After the incubation mixture was passed through a 100-µm stainless steel sieve into a 12-ml centrifuge tube, protoplasts were pelleted at 40 g for 10 min. The pellet was suspended with 10 ml CPW13S or MMS. Next steps were performed according to Thomzik and Hain (1988) or Koop et al. (1996). In both cases the TAL-method was used, however different culture media were tested. Leaf protoplasts were cultured either in PC medium or in PCBr. Protoplast density was $4 \cdot 10^4$ pps/grid, volume of culture medium 2 ml. After 7 days of culture period half volume of the medium was replaced by fresh culture medium. When protoplast derived colonies were formed and became visible without a microscope, grids were transferred either to regeneration media described earlier (Pelletier et al., 1983; Glimelius, 1984; Thomzik and Hain, 1988) or to gelrite-solidified SRBr medium 10 days after protoplast isolation. When colonies had grown to a size of up to 2 mm, they were picked and transferred to different media in 6-well dishes to test the optimal hormone composition of regeneration medium. Experiments were

executed with leaf protoplast derived colonies of cultivar "Drakkar". Auxin NAA (0, 0.1, 0.3, 1, 2 and 3 mg/l) with or without GA_3 (0.05 mg/l) was tested in all possible combinations with cytokinins, either with BAP (0, 0.25, 0.5, 1, 2 and 4 mg/l) or with kinetin (0, 0.25, 0.5, 1, 2 and 4 mg/l). Single colonies were transferred into 6-well dishes with 3 colonies per well. For testing regeneration efficiency in 9-cm petri dishes, 100 colonies were tested with 20 colonies per petri dish. Regenerates were transferred to hormone-free MS medium for rooting.

Sugarbeet: leaf protoplasts

Leaves from sugarbeet plants grown *in vitro* (cultivars "Viktoria" and VRB) were used for digestion. Donor plants were cultured on MS, MSB2 or MSB1 medium. All media for protoplast isolation and culture contained 0.1 mM *n*-propylgallate (nPG). Protoplast were isolated and purified as described by Krens et al. (1990) and cultured in modified K8p medium (Krens et al., 1990) in thin alginate layers. Other culture medium PCB and PCB0 (minerals from F-PCN and organic from PCB) were tested. Alternatively, the crude protoplast preparation was used for alginate embedding. Digestion in PIB medium containing 1% (w/v) cellulase Onozuka R-10, 2% (w/v) macerozyme Onozuka R-10% and 0.4% (w/v) driselase (Sigma, St. Louis, USA) resulted in a high yield of guard cell protoplasts (up to 90% of total number of intact protoplasts). After one week of culture when small cell clusters of 6-8 cells were already formed half volume of the liquid medium was replaced by fresh medium. Replacement of the medium was continued regularly every 6-7 days. Protoplast derived colonies were transferred to solid MS medium supplemented with 0.25 mg/l of BAP (MSB0.25) for regeneration.

Sugarbeet: callus protoplasts

Friable callus was transferred to an enzyme solution, which consisted of PIB medium + 0.5% (w/v) macerozyme Onozuka R-10 + 0.5% (w/v) cellulase Onozuka R-10. As mentioned above, all protoplast media contained 0.1 mM nPG. The crude protoplast preparation was gently shaken and passed through a 100 µm

steel sieve into a 12-ml centrifuge tube. Protoplasts were washed with PIB by centrifugation for 10 min at 50 g. After the supernatant was removed, the pellet was resuspended in MMS. The volume was adjusted to 10 ml with MMS. MMM (2 ml) was carefully added as top layer, and protoplasts were centrifuged for 10 min at 70 g. Protoplasts were then collected from the interface and transferred into a fresh centrifuge tube. The volume was adjusted to 10 ml with MMM. The protoplast density was determined in a haemocytometer. Then protoplasts were pelleted by centrifugation for 10 min at 50 g. The pellet was resuspended in MMM and protoplasts were embedded and cultured accordingly to the TAL-method at a final density of $3\text{-}6\cdot10^4$ ppl/ml. Grids with embedded protoplasts were washed twice with 10 ml of PCB medium and were cultured in petri dishes (6-cm in diameter) with 2 ml of PCB in the dark. Every week 1 ml of PCB medium was replaced by the same volume of fresh medium. After microcalli had been formed (17-20 days) the grids were transferred to solid MSB2-M3 medium. This medium contains 3 g/l of mannitol in addition to 3 g/l of sucrose for osmotic shock prevention. After 2-3 weeks, enlarged calli were separated and transferred to the light on SRB medium for regeneration. Roots were induced on hormone-free MS medium.

Screening of phytohormone compositions: Protoplast derived colonies were transferred to phytagel- or agarose-solidified MS medium containing different hormone combinations. Cytokinins BAP (0.25, 1 and 2 mg/l) or thidiazuron (0.25 and 1 mg/l) were combined with different concentrations of the auxin NAA (0, 0.25, 1 and 2 mg/l) in the absence or in the presence of the antiauxin TIBA (only 1 mg/l in the case of thidiazuron and 0, 0.25, 1 and 2 mg/l for BAP) in all possible combinations. Protoplast derived colonies (15 colonies per petri dish) were transferred to 9 cm petri dishes and cultured in the dark or in the light, with 3 petri dishes for each hormone combination.

2.14 PEG treatment of protoplasts

The PEG-method of protoplast treatment for plastid transformation was performed as described by Koop et al. (1996) and applied to rapeseed protoplast preparations. DNA of vector pRS-*aad*A was used in these experiments.

Nuclear transformation experiments in sugarbeet were done according to Hall et al. (1997). Plasmid pSL-GUS-INT-PAT, kindly provided by Dr. J.Kraus (Planta GmbH, Einbeck) was used for testing of transformation efficiency in callus protoplast cultures.

2.15 DNA transfer by the biolistic method.

The biolistic method was used for nuclear and plastid transformation of regenerable callus in sugarbeet and for plastid transformation of protoplast derived colonies in rapeseed. For all purposes the same conditions were used. Gold particles, 60 mg (0.6 μm in diameter, Bio-Rad Laboratories, California, USA), were suspended in 1 ml ethanol (100%), and 36 μl of the mixture were transferred into a new plastic tube. After pelleting by centrifugation for 10 sec at 14000 rpm in Eppendorf centrifuge, 25 μg of DNA dissolved in H_2O (volume should be adjusted with sterile water to 255 μl), 250 μl of 2.5M $CaCl_2$ and 50μl of spermidine (Sigma, St. Louis, USA) were added and mixed. The mixture was incubated on ice for 10 min and centrifuged for 1 min at 10000 rpm. After complete removal of the supernatant, the gold was suspended by pipetting and washed twice in 100% ethanol, each for 1 min at 10000 rpm. Microprojectiles coated with DNA were resuspended in 72 μl (5.4 μl per bombardment) of 100% ethanol and stored on ice prior to bombardment. The construction of the bombardment chamber (Model PDS-1000/He Biolistic® Particle Delivery System, Bio-Rad Laboratories, California, USA) is presented in Fig. 2.3. Petri dishes with the targeted material were placed on the middle shelf, stopping screens and macrocarriers containing microprojectiles coated with DNA were placed in the holder and rupture disks of 900 psi were used.

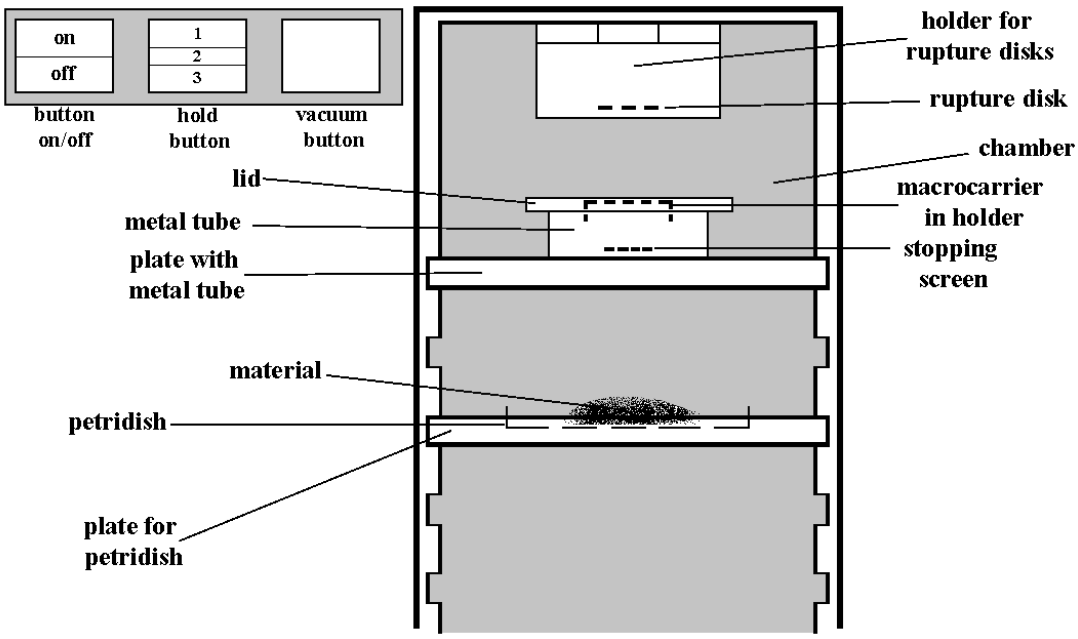


Fig. 2.3. Scheme of the bombardment chamber, Model PDS-1000/He Biolistic ® Particle Delivery System (Bio-Rad Laboratories, California, USA).

2.16 Selection

After PEG treatment: Following the initial 6-8 days of protoplast culture either 100 mg/l spectinomycin alone or in concert with streptomycin at the same concentration were included in liquid and solid media for selection of rapeseed colonies. Bialaphos at a concentration of 1 mg/l was supplied to both, liquid and solid media to select resistant sugarbeet protoplast derived colonies.

After bombardment: Grids with rapeseed protoplast derived colonies were transferred to selection medium (SRBr supplemented either with 100 mg/l of spectinomycin alone or with spectinomycin and streptomycin both at the same time (100 mg/l)) 3 days after the shooting. Recovered colonies were transferred to the same medium in 6-well dishes. Sugarbeet callus bombarded with pSB-*aad*A was transferred either to MSB2 or to MSB0.1 supplemented with 100 mg/l spectinomycin. Resistant colonies were collected after 4-5 weeks of culture and were transferred to identical fresh medium. After resistant colonies were enlarged in size, they were transferred to selection medium with both antibiotics at a

concentration 100 mg/l. After bombardment with pSL-GUS-INT-PAT, sugarbeet callus was incubated for 7-10 days and then it was selected on MSB2 medium supplemented with 1 mg/l bialaphos. Resistant clones were transferred to fresh selection medium.

2.17 Detection of GUS-activity

Callus or shoot explants were transferred with forceps to 1.5 ml plastic tubes containing 100-200 µl of GUS staining solution (X-Gluc, Gallagher, 1992) and incubated at the room temperature for 15-60 min.

X-Gluc solution

Phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.0)	100 mM
EDTA	1 mM
Potassium hexacyanoferrate (II)	1 mM
Potassium hexacyanoferrate (III)	1 mM
Triton X-100	0.3%
X-Gluc (dissolved in DMF)	1 mM

2.18 Computer programmes for DNA analysis and image processing

Image processing:

All images were transferred to a Umax Pulsar (Umax Inc., Taipei, Taiwan), a Macintosh PC 604 compatible computer, through an ActionCam digital camera (AGFA, Munich, Germany) and were processed using Adobe Photoshop 5.0 software (Adobe Inc., California, USA). Additionally, IBM-PC compatible Pentium computers of different manufacturers and scanner GT-9000 (Epson) were used.

Programmes:

Gel documentation: MWG-Biotech, Ebersberg

Phosphoimager editor: Tina 2.0 (Raytest)

Searches in databanks and sequence comparison: BLAST (NCBI-NIH = "National Center for Biotechnology Information – National Library of Medicine", USA)

Primer design, restriction maps: Vector NTI Version 4.0.2 (Informax)

3. RESULTS

3.1 Model system: tobacco protoplast culture

3.1.1. Culture of donor plants

Tobacco is often used in plant cell biology as a model plant mainly due to its good regeneration response in tissue culture. To obtain a protoplast preparation with high regeneration capacity it is very important to isolate the cells from healthy, strong donor material. Thus our first aim was to improve the growth conditions of donor plants. In order to optimise shoot culture, several media and plants of two cultivars, “petite Havana” and “Wisconsin 38”, were tested. Tobacco plants that had been grown on standard hormone-free MS or B5 media were characterized by presence of pale green or yellowish patches on their leaves. The problem was overcome, when seedlings were germinated on a modified B5 medium with an increased content of Mg^{2+} (1233 mg/l).

3.1.2. Thin alginate layer (TAL) technique: a novel and efficient method for the manipulation of protoplasts from higher plants

Tobacco leaf protoplasts were used to establish a new procedure. Optimised isolation conditions as well as protoplast embedding in thin alginate layers in combination with improved culture and physical parameters resulted in very high plating efficiencies (>95%) and fast shoot regeneration (in less than two weeks) from protoplast derived colonies (Fig.3.1, Table 3.1). The TAL-technique is the basis for fast shoot regeneration from tobacco leaf protoplasts and has also a positive effect on protoplast cultures of higher plant species other than tobacco (chapters3.2 and 3.4). Additionally, the embedding of protoplasts in thin alginate layers using polypropylene grids facilitates experimental manipulations with protoplasts or protoplast derived colonies, like the transfer to other media, cell tracking etc., due to mechanical stabilization of the layers.

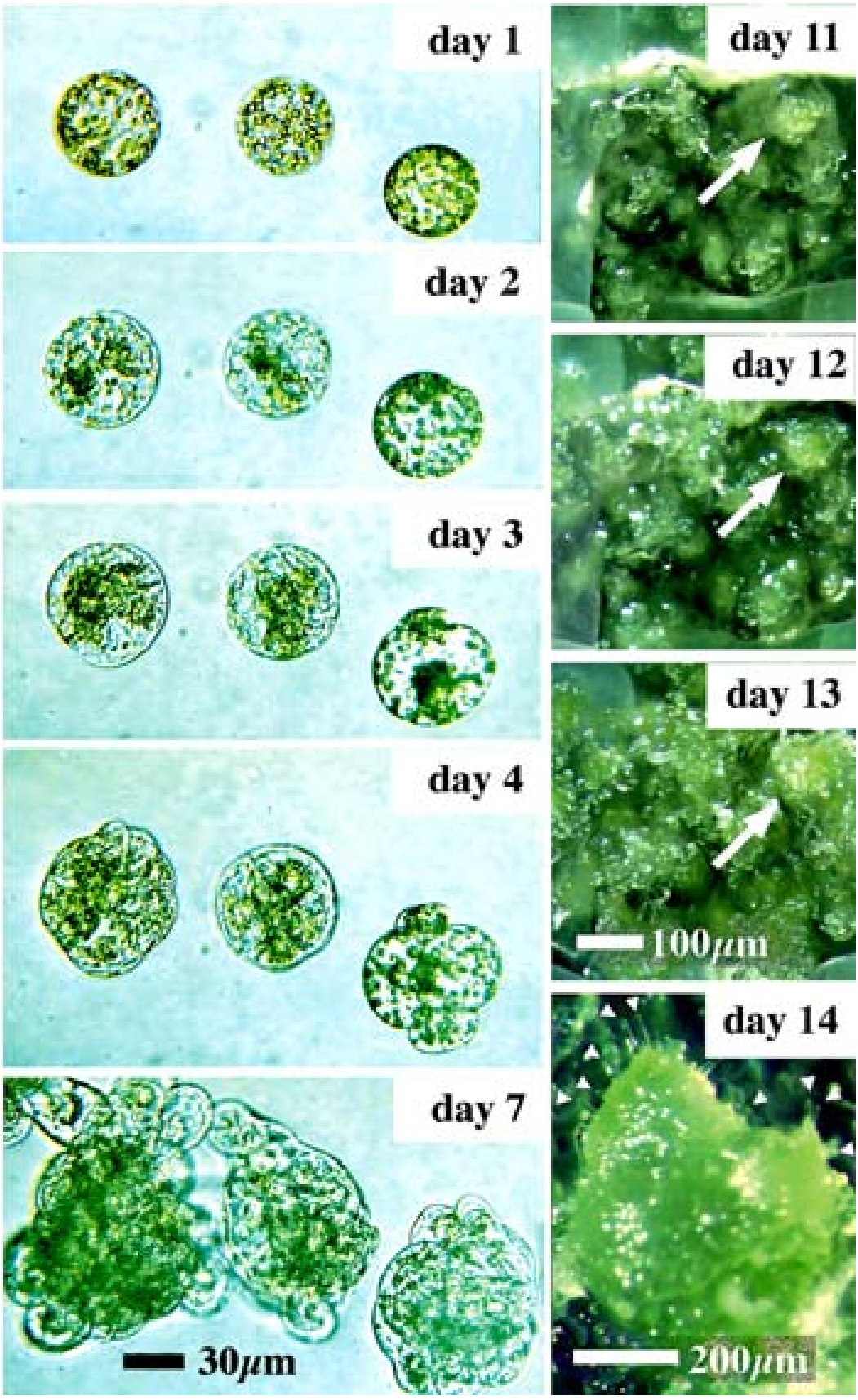


Fig. 3.1. Fast regeneration from tobacco leaf protoplasts: development of randomly selected protoplasts to colonies and shoot formation from a colony.

Table 3.1. Fast shoot regeneration from tobacco leaf protoplasts

	Culture vessel	Volume of medium	Medium	Day number	Efficiency *
Seed germination	720ml glass jar	120 ml	SCN	-21	95%
Shoot culture	720ml glass jar	120 ml	SCN	-18	-
Enzyme incubation	petri dish (10cm)	10 ml	F-PIN	-1	-
Protoplast isolation	12ml tubes	10 ml	MMM	0	$1.5 \cdot 10^6$ per leaf
Alginate embedding a	petri dish (10cm)	15 ml	Ca-A	0	-
Alginate embedding b	polypropylene grid	625 µl	MMM/AlgA	0	$4 \cdot 10^4$ per grid
Culture step 1	petri dish (6cm)	625µl + 2ml	F-PCN	0 - 7	
First divisions				2	6.5%
Second divisions				3	77.8%
Colony formation				7	95.8%
Culture step 2	Magenta vessel GA-7	70 ml	F-SRN	8 - 21	
First trichomes				13	<1%
Regenerated shoots				21	>80%
Transfer for rooting	petri dish (10 cm)	20 ml	MS	21	
First roots				30	14%

* Efficiencies were taken from selected experiments with cultivar 'petite Havana'; with 'Wisconsin 38' shoots appeared on day 14 of culture.

3.1.2.1 Improvements of conditions for protoplast isolation and culture

Isolation and embedding: Several factors, such as preplasmolysis, osmotic pressure and enzyme composition of the digestion medium are extremely important to obtain highest yields of uniform and healthy protoplasts. A specially designed preplasmolysis medium, F-PIN, allowed to minimize the stress of the treatment for freshly isolated protoplasts. Following standard filtration, flotation and sedimentation procedures (Koop et al., 1996) protoplasts were collected, washed and resuspended in MMM, an MES-buffered medium containing mannitol for osmotic and Mg^{2+} for ionic stabilization. Different magnesium salts were tested. The best combination was a mixture of 10 mM $MgCl_2$ and 10 mM $MgSO_4$ (20mM final concentration of Mg^{2+}). To use only magnesium sulphate or magnesium chloride in combination with a 20 mM Mg^{2+} concentration was suboptimal. While it didn't affect plating efficiency, it influenced the further development of the protoplast derived colonies - they looked pale and shoot regeneration occurred later. Two different types of embedding with alginic acid were tested. Comparing

filter sterilized alginate solution with an autoclaved one, no significant loss in culture efficiency was observed. Different concentrations of alginic acid in autoclaved medium were tested: 20, 24, 28 and 32 g/l. The best results were obtained using alginic acid at a concentration of 28 g/l. While filter sterilized alginate solution produced the same gel consistency at lower concentrations (12 g/l), it was difficult to sterilize the solution and did not result in significant difference in plating efficiency in tobacco in contrast to other species reported in the literature (Hall et al., 1997). Alginate embedding was performed in thin layers. Thin alginate layers are one of the key steps in the whole procedure to obtain a good and fast regeneration.

Protoplast density: An important factor influencing the plating efficiency of embedded protoplasts and their further regeneration capacity is cell density. Different combinations of volumes of culture medium (1, 2 and 4 ml) with different protoplast densities ($1 \cdot 10^4$, $2 \cdot 10^4$, $4 \cdot 10^4$, $1 \cdot 10^5$ pps/grid) allowed to define the conditions at which the highest plating efficiency in concert with the fastest regeneration response of protoplast derived colonies was observed. While obtaining the best plating efficiencies when protoplasts were embedded at high densities, further development and regeneration from protoplast derived colonies was inhibited due to a high density of colonies. The best results were observed, when $4 \cdot 10^4$ pps/grid when cultured in 2 ml of PCN medium.

Culture media: In preliminary experiments the fastest cell divisions and shoot formations were observed within a 4-5 week period using PCN as culture medium (Koop et al., 1996). In a series of consecutive experiments a new culture medium F-PCN was designed. Dynamic of divisions in both media was compared (Fig. 3.2). While the first divisions were observed already on the second day after protoplast isolation and at higher frequency (10%) in PCN medium, one day later higher division efficiencies of protoplasts that had been cultured in F-PCN medium were observed.

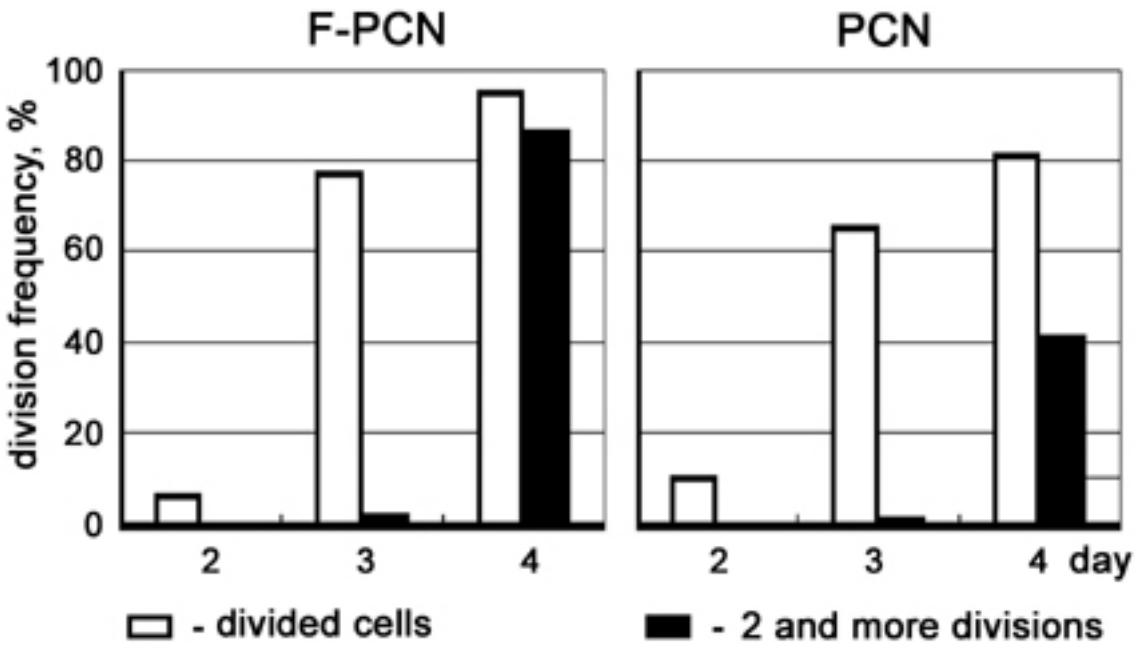


Fig. 3.2. Influence of F-PCN and PCN culture media on tobacco protoplast divisions.

A similar result was obtained with cells that had divided more than once. There was almost no difference in number of protoplasts that had divided twice after 3 days of culture in both media. Nevertheless, after one more day of culture (day 4), the number of aggregates with 4 or more-cells in F-PCN medium was more than twice as high if compared with the number of colonies which were formed in PCN medium. The most important factors of F-PCN (as well as F-PIN) are the reduced content of KNO_3, the absence of NH_4NO_3 and the addition of ammonium succinate as a source of nitrogen. F-PCN is the best medium for tobacco protoplast culture under our conditions. After the first division, protoplasts developed into microcolonies extremely fast. Already after 6-7 days of culture in F-PCN small visible microcolonies were formed (Fig. 3.1).

3.1.2.2 Fast shoot formation from protoplast derived colonies

Grids with microcolonies were transferred to different regeneration media one week after protoplast isolation. The fastest regeneration response (13 days after protoplast isolation) was observed on medium F-SRN-1 (F-SRN medium lacking 3% mannitol). However shoots were often vitrified and pale-green (Fig. 3.3c).

Therefore, different hormone concentrations (BAP 1 mg/l and NAA 0.1 mg/L or BAP 0.1 mg/l and NAA 0.01 mg/l) and addition of 3% mannitol were tested for their effect to improve the shoot morphology without delay in shoot regeneration (Fig. 3.3). It was found that colonies, which were cultured in medium containing mannitol and reduced hormones, regenerated strong dark green shoots at a high efficiency (Fig. 3.3d).

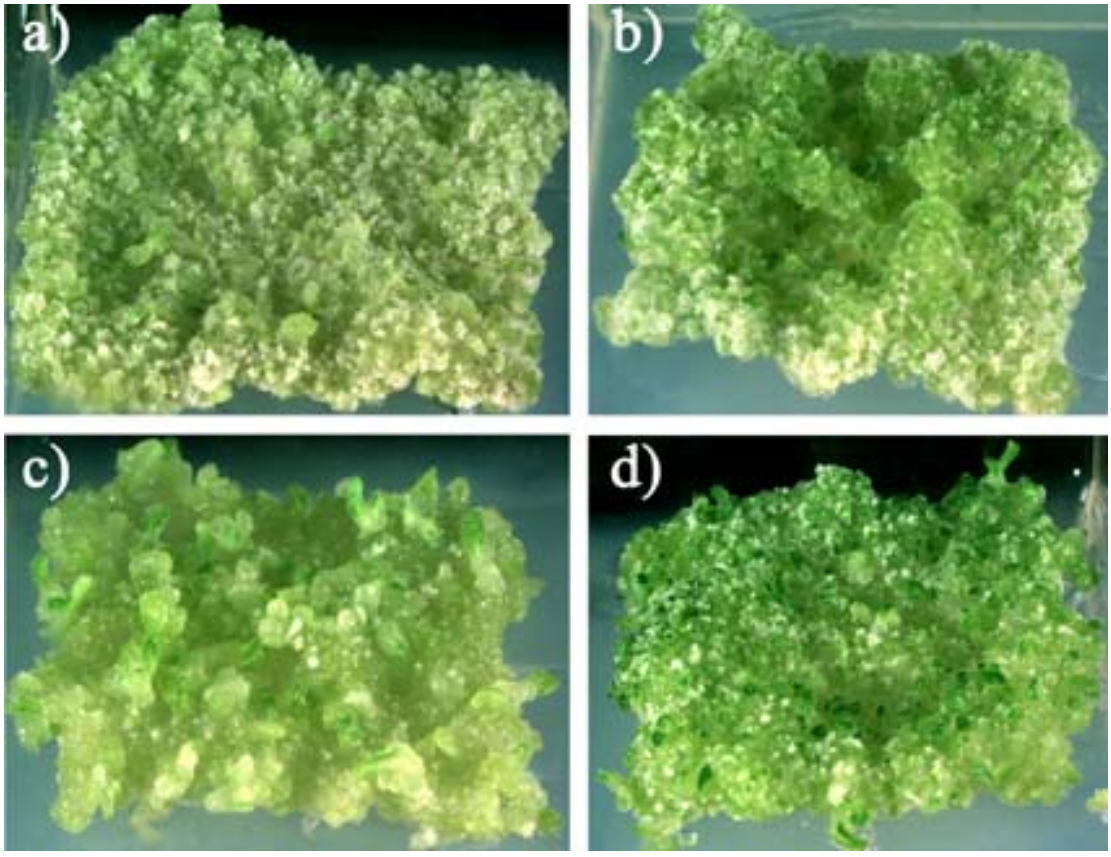


Fig. 3.3. Influence of phytohormones and 3% mannitol on shoot regeneration from protoplast derived colonies: regeneration of vitrified shoots on F-SRN medium with BAP 1 mg/l and NAA 0.1 mg/l without (a) or with addition 3% mannitol (b); increased shoot regeneration on F-SRN medium containing BAP 0.1 and NAA 0.01 without (c) or with addition 3% mannitol (d).

3.2 Rapeseed protoplast culture

3.2.1 Design of a new culture medium for rapeseed plants

It was not possible to prevent the formation of undesirable yellowish patches on the leaves indicative of nutrient deficiency, using the growth conditions (MS or

half strength hormone-free MS medium) described in the literature (Fig. 3.4, a). The optimisation of the mineral composition in the nutrient medium leads to plants with strong green leaves, without any patches (Fig. 3.4, b). The most important changes in the medium, RS, were the absence of ammonium ions and an increased content of Mg^{2+} and Na^{+}.

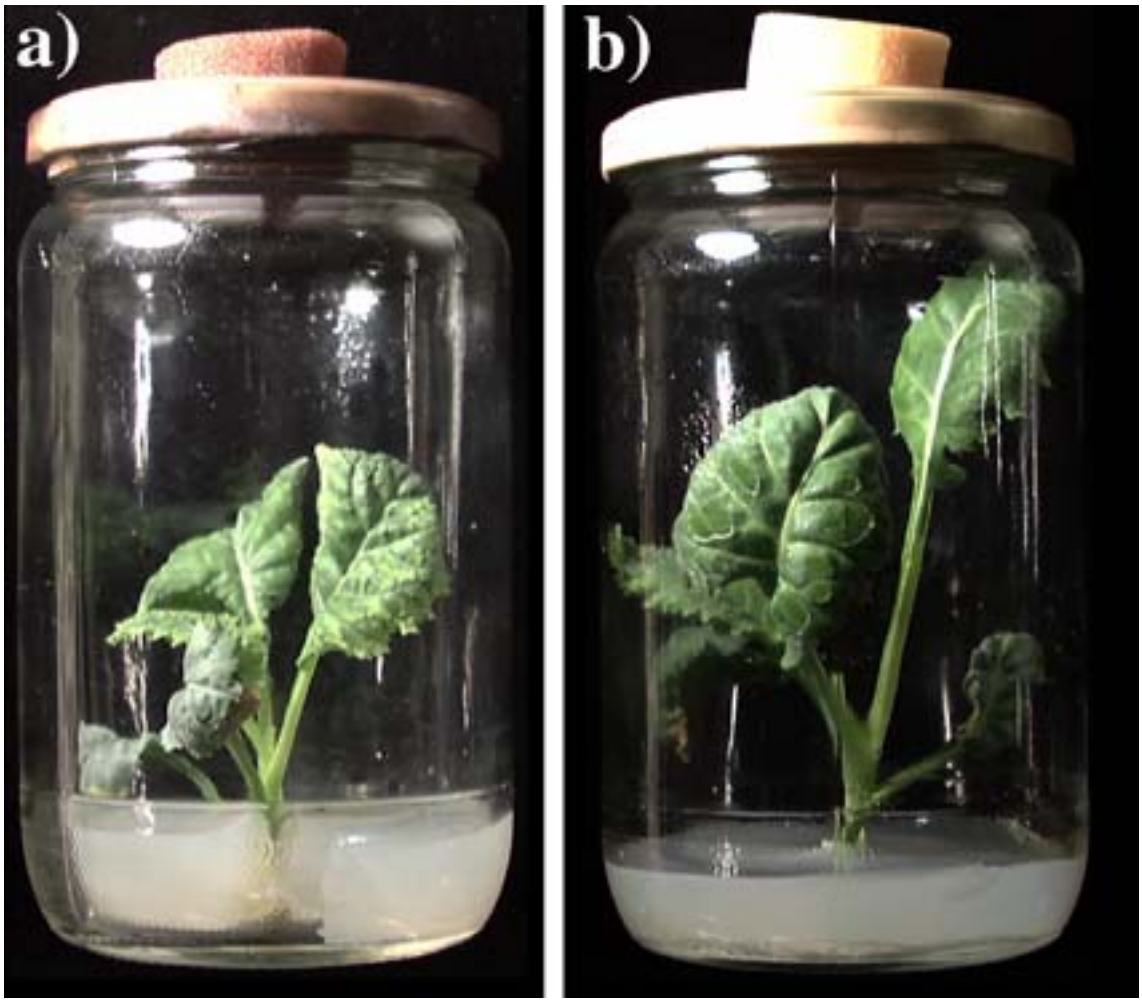


Fig. 3.4. Rapeseed plants after four weeks of culture in MS/2 (a) and RS (b) media, cultivar "Westar".

3.2.2 The TAL-technique and a new culture medium improved protoplast culture of rapeseed

The TAL-technique, which had been developed for tobacco protoplasts, was successfully applied for the culture of rapeseed leaf protoplasts. The use of isolation (CPW 13M, CPW 22S, W5) and culture media (PC medium) as described

in the literature (Glimelius, 1984; Glimelius et al., 1986; Tomzik and Hain, 1988, 1990) in combination with the TAL-technique gave stable results, but low efficiency. The first divisions, at an efficiency 3-5%, were observed on the third day after protoplast isolation. Plating efficiencies were 20-25%. This is only half of the efficiencies obtained by other researchers (Pelletier et al., 1983; Glimelius, 1984). In order to improve our culture conditions new isolation and culture media were designed. The final result is shown in Fig. 3.5.

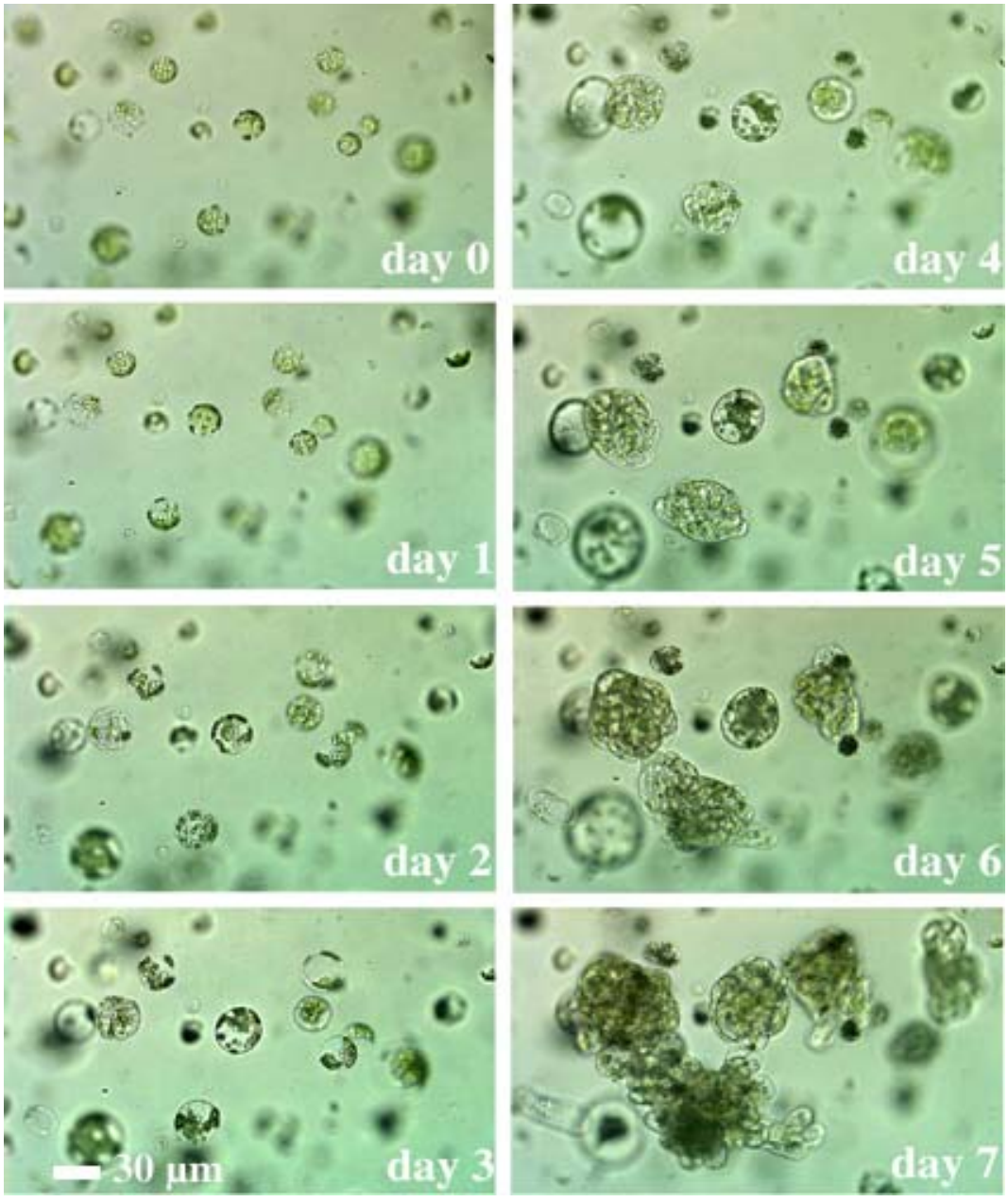


Fig. 3.5. Rapeseed leaf protoplast development in the first week of culture, cultivar "Drakkar".

The optimal density was 6-6.5·10^4 pps/ml of culture medium. Protoplast culture in the improved medium, PCBr, resulted in fast and efficient cell divisions. Moreover, PCBr medium prevented the formation of a brown exudate around microcolonies in comparison with PC medium. PCBr medium in contrast to PC medium, which is based on K8p medium (Kao and Michayluk, 1975), contains other macrosalts, hormone and vitamin composition. These modifications are significant for fast protoplast development at high efficiencies. The first divisions could be observed already on the second day of culture, increasing to up to 30% on the next day. By the 6-th day, colonies, visible without a microscope, were formed (Fig. 3.5). After 2-4 additional days of culture in liquid medium the grids with microcolonies could be transferred to solid medium (Fig. 6a). Plating efficiencies in the breeding lines tested were 36-42% for "Westar" and 45-55% for "Drakkar". Thus, combining the TAL-technique with new media (PIBr, MMM, MMS, PCBr) a reproducible and efficient rapeseed protoplast culture, suitable for PEG-mediated plastid transformation, was established.

3.2.3 Cotyledon protoplasts

Alternatively, protoplasts were isolated from etiolated and non-etiolated cotyledons. The combination of the TAL-technique with conditions that were established for leaf protoplasts allowed for an additional increase of the plating efficiency if cotyledon protoplasts were used as starting material. First divisions of cotyledon protoplasts were already observed after 24 hours in culture. The plating efficiencies of protoplasts, which had been derived from etiolated cotyledons, were 76-80% for both breeding lines tested, "Westar" and "Drakkar". Protoplasts from non-etiolated cotyledons formed colonies at the same frequency as those from leaf protoplasts for cultivar "Westar" or at a better frequency, 60%, for "Drakkar".

3.2.4. Shoot regeneration from protoplast derived colonies

It is a general problem that most media for the regeneration of rapeseed protoplasts are efficient only for certain genotypes. Several regeneration media (Glimelius,

1984; Pelletier et al., 1983; Thomzik and Hain, 1988) were tested for their plant regeneration capacity of protoplast derived colonies. Unfortunately, all these media are not suitable because of a very low efficiency of shoot formation, which was <1% and varying from one experiment to the other. An additional problem was vitrification of regenerates (Fig. 3.6b).

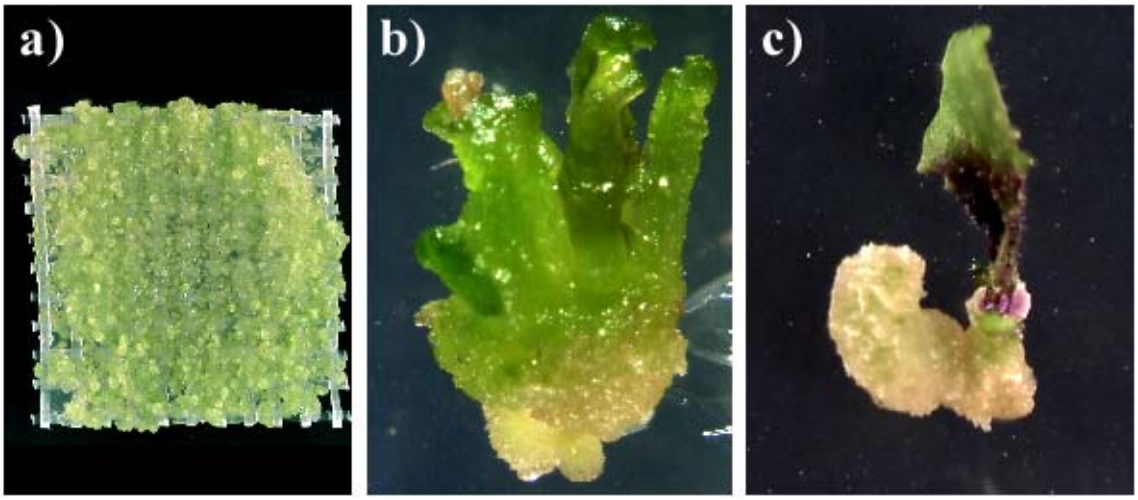


Fig. 3.6. Rapeseed protoplast culture: grid with protoplast derived colonies after two weeks of culture, cultivar "Drakkar" (a), shoot regeneration from protoplast derived colonies, cultivar "Westar" on SR medium (Thomzik and Hain, 1988) (b) and cultivar "Drakkar" on SRBr medium (c).

Different hormone combinations were tested in order to optimise the regeneration conditions. Giberellic acid was not important for regeneration, yet influenced the pigmentation of the colonies. Colonies became greener in the presence of the hormone. When auxin was given at low concentrations (up to 1 mg/l) better regeneration response was observed with media containing kinetin at 1-2 mg/l. With increasing auxin concentrations (2-3 mg/l), almost no regeneration was observed on media with kinetin. The best combination was when auxin concentrations of 2-3 mg/l were administered in combination with 2 mg/l BAP. From 1 to 2 of every 3 colonies tested formed shoots on medium with 2 mg/l BAP and 2 mg/l NAA, SRBr medium (Fig. 3.6c). The problem of vitrification was not observed. However, in 9-cm petri dishes the regeneration efficiency was 10-15%.

A possible reason could be the change of the gas volume. The fastest shoot formation was observed 28 days after protoplast isolation. Usually, shoots were formed only 1,5 months after protoplast isolation. Protoplast derived colonies from cotyledons did not have uniformity with respect to their regeneration response, and the regeneration efficiency under established conditions varied greatly - from 2 to 16%. Although some modifications of regeneration conditions are still required, regular shoot regeneration is obtained for both breeding lines tested and shoot induction from single colonies in 6-well dishes might be preferable for experiments on plastid transformation in rapeseed.

3.3 Sugarbeet: shoot regeneration from explants and callus

3.3.1 Seed germination

In contrast to the germination of tobacco and canola seeds, the germination of sugarbeet seeds took longer and required a cold treatment for high efficiency (Fig. 3.7).

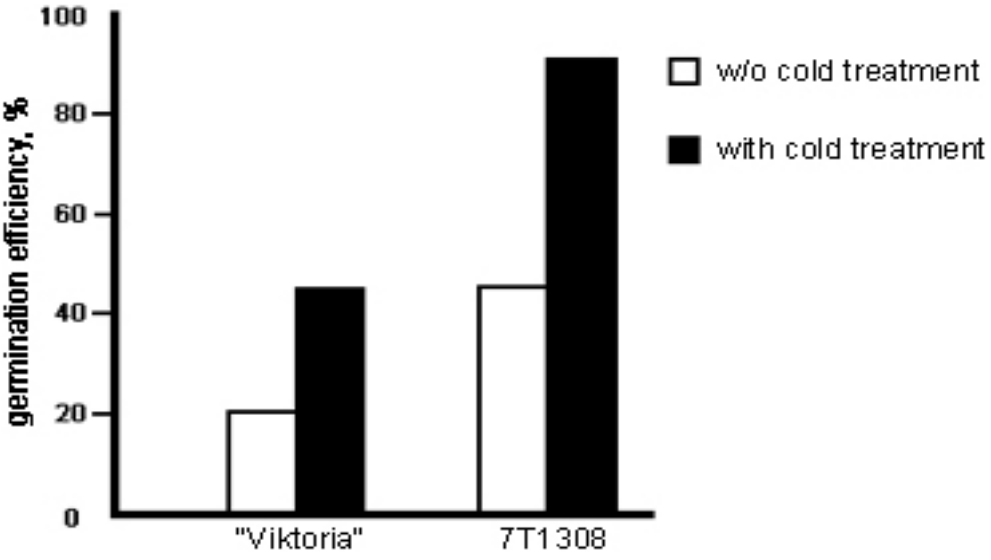


Fig. 3.7. Effect of a cold treatment on the germination efficiency of two sugarbeet cultivars. Results represent the average values obtained from 3 independent experiments (300 seeds were tested in each).

While germination efficiencies of untreated seeds were 22% for “Viktoria” and 48% for 7T1308, those for treated seeds were 47% and 95% accordingly. MSB2 medium was used as the germination medium.

No differences were observed in germination efficiency when testing different sugar concentrations and the hormone composition of the germination medium. These parameters are important, however, for further callus induction from seedling explants. Seedlings that were growing on medium containing 2 mg/l BAP in the dark were characterized by elongated hypocotyls. The average length of etiolated hypocotyls was 28 mm and it was only 12 mm for seedlings that were germinated in light (Fig. 3.8 a, b). Germination on MSB2 medium supplemented with 2 mg/l NAA (MSB2N2) in the dark resulted in the reduced length of hypocotyls as well (14 mm in average) (Fig. 3.8c).

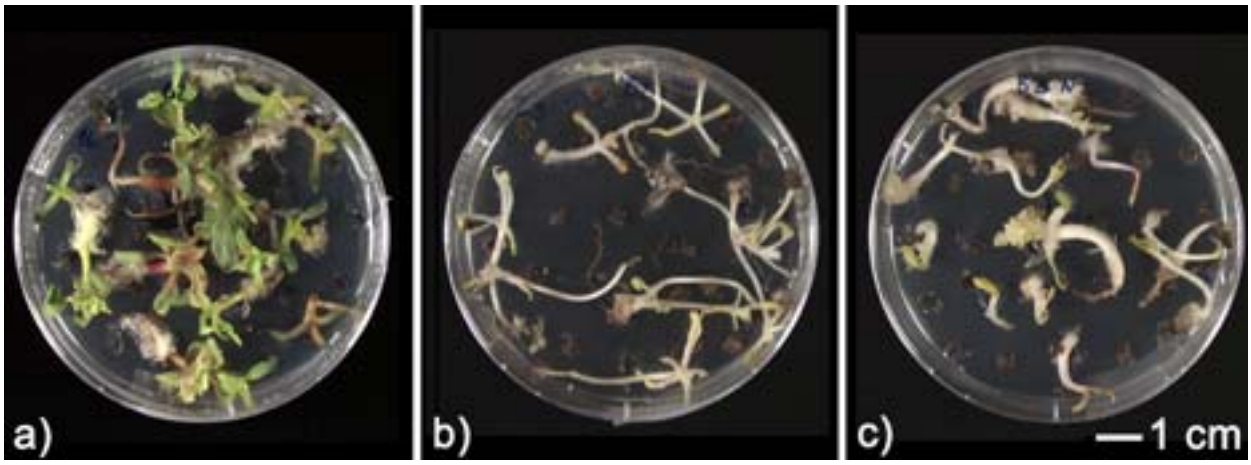


Fig. 3.8. Seed germination on MSB2 medium in the light (a), in the darkness (b) and on MSB2N2 in the darkness (c), breeding line “Viktoria”.

For all further experiments, a cold treatment and germination on medium MS15B2 in the darkness were used as standard conditions.

3.3.2 Direct shoot regeneration

Shoot regeneration through direct organogenesis is the most effective way to produce true-to-type regenerates in sugarbeet (Toldi et al., 1996). Direct

organogenesis is less genotype dependent (Detrez et al., 1989; Jacq et al., 1992) and regenerates are genetically stable. Explants of different origin of 2 sugarbeet breeding lines were tested for their capacity to regenerate shoots directly. Shoot formation was observed from hypocotyls, cotyledons, petioles, leaves, basal tissue and even roots. Till now, there was no information about direct shoot regeneration from the roots of sugarbeet. Here, we document for the first time direct shoot organogenesis from root tissue (Fig. 3.9). This type of shoot formation was, however, observed only once. The regenerate was formed from the root of an established 1-year old shoot culture on MS medium supplemented with BAP 0.25 mg/l. Although the efficiency of regeneration from root explants seems extremely low, the fact and possibility to obtain shoots from roots in sugarbeet might be regarded as an important observation for our understanding of differentiation and regeneration processes.

We tested various explants for their regeneration capacity. The data are summarized in Table 3.2.

Table 3.2. Efficiencies of shoot formation from explants of different origin

Explant source	Viktoria	7T1308
Roots	single event	-
Cotyledons	5%	2%
Hypocotyls	13%	9%
Basal tissue	21%	19%
Leaves	21%	14%
Petioles	42%	32%

Usually, shoot explants started to form regenerates 7-10 day after induction, while the first shoots from seedling explants were observed not earlier than two weeks. Cotyledon, hypocotyl and basal tissue explants regenerated normally only one or rarely a few shoots on the surface of the explants (Fig. 3.10 a). Leaf and petiole explants formed shoots along the middle rib (both, Fig. 3.10 c). When the capacity to form friable callus from leaf explants of other breeding lines was investigated, shoot formation has been observed on the explant surface but not at the rib

(Fig. 3.10 b). However, such type of shoot regeneration was rare and very genotype dependent.

Fig. 3. 9. Shoot formation from a root of breeding line "Viktoria".

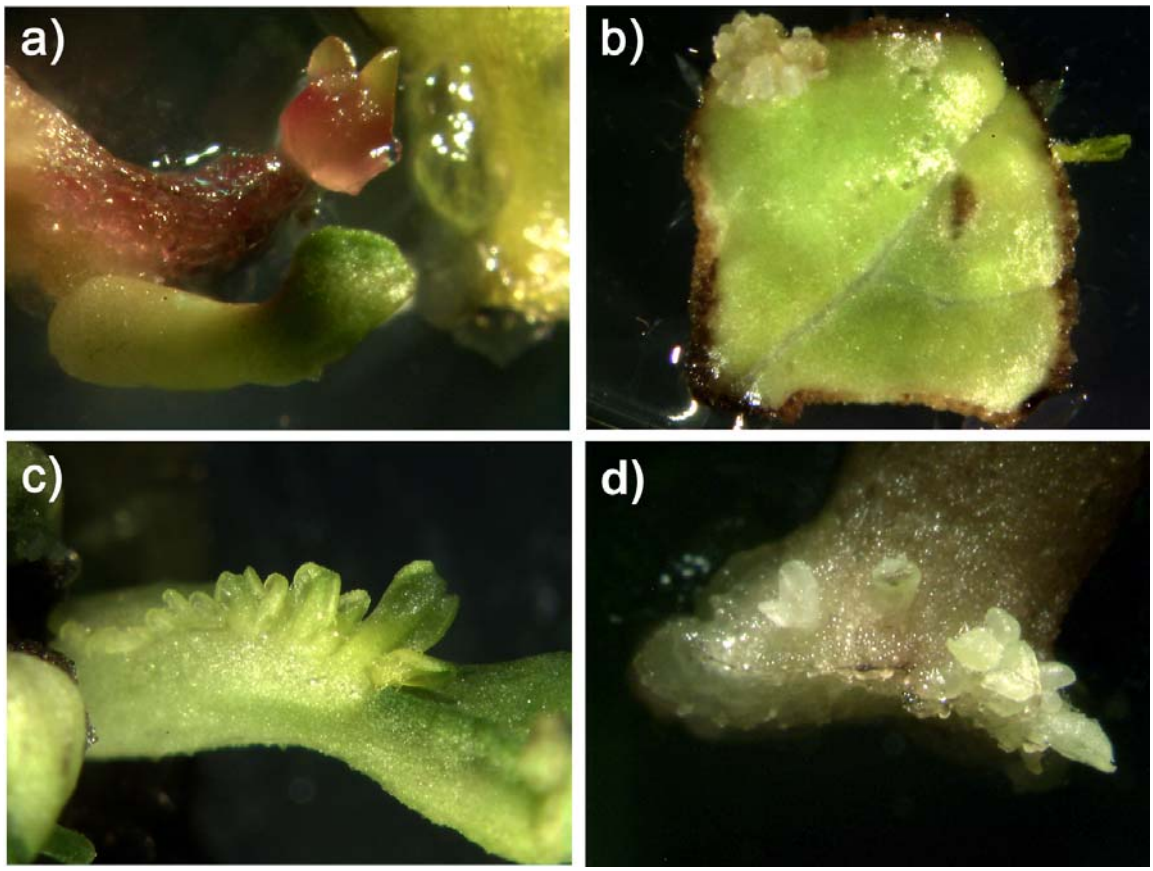


Fig. 3.10. Direct shoot organogenesis from sugarbeet explants of different tissue origin: cotyledon (a), leaf explant (b), petiole (c) and hypocotyl (d). a, c, d –cultivar "Viktoria", b – cultivar 6K0020.

Additionally, the influence of two different cytokinins, zeatin and BAP, on a regeneration activity of sugarbeet petiole explants was investigated. While no significant differences in the regeneration efficiency were observed (43% for 1 mg/l BAP and 41% for 2 mg/l zeatin, cultivar "Viktoria"), regenerates that were obtained from explants cultured on medium containing BAP were vitrified.

It is necessary to remark, that explants demonstrating higher regeneration efficiency (petioles and leaf explants), regenerated shoots from deeply buried cells or cell layers. Although regeneration from seedling explants generally was observed from the upper cell layers, the efficiency of the process is low. Very few regenerable cells per explant and difficulties to deliver DNA in the lower cell layers render those systems less useful for gene transfer experiments. Thus, an alternative system for shoot regeneration from callus was required and developed.

3.3.3 Screening of genotypes for regeneration capacity

Only friable (soft, nodular) callus of sugarbeet has regeneration activity (Krens et al., 1990). Ben-Tahar et al. (1991) proposed the method of friable callus induction with subsequent successful genetic transformation. Here, 47 breeding lines of sugarbeet, including the control cultivar Rel1, were tested for their capacity to form friable regenerable callus and for its further regeneration activity under the conditions described by Ben-Tahar et al. (1991).

The first estimation of callus formation efficiency was done after 30 days of explant culture in the dark. 24 breeding lines out of 47 genotypes tested formed regenerable callus 1 month of explant culture in the darkness. There was no significant difference in callus formation efficiency for leaf explants and middle rib explants. Also, no essential influence on callus formation efficiency was found with which side (abaxial or adaxial side down) explants were in the contact to medium. While all genotypes demonstrated a high response to produce non-regenerable callus (except of cultivar 1F0076, where less then 50% of explants

formed this callus), efficiency of regenerable callus formation was not so uniform. Only explants from seven genotypes (1F0076, 2B0017, 6B0064, 6B2838, 6B3907, 6K0020 and 8K0034), exhibited multiple callus formation at a high efficiency. No correlation between friable and non-regenerable callus formation was observed. Friable callus appeared either on the explant surface (Fig. 3.11 b) or in contact with the medium (Fig. 3.11 c), either on explants that were green, (Fig. 3.11 a,b) but also on explants that looked partly or completely brown (Fig. 3.11 d).

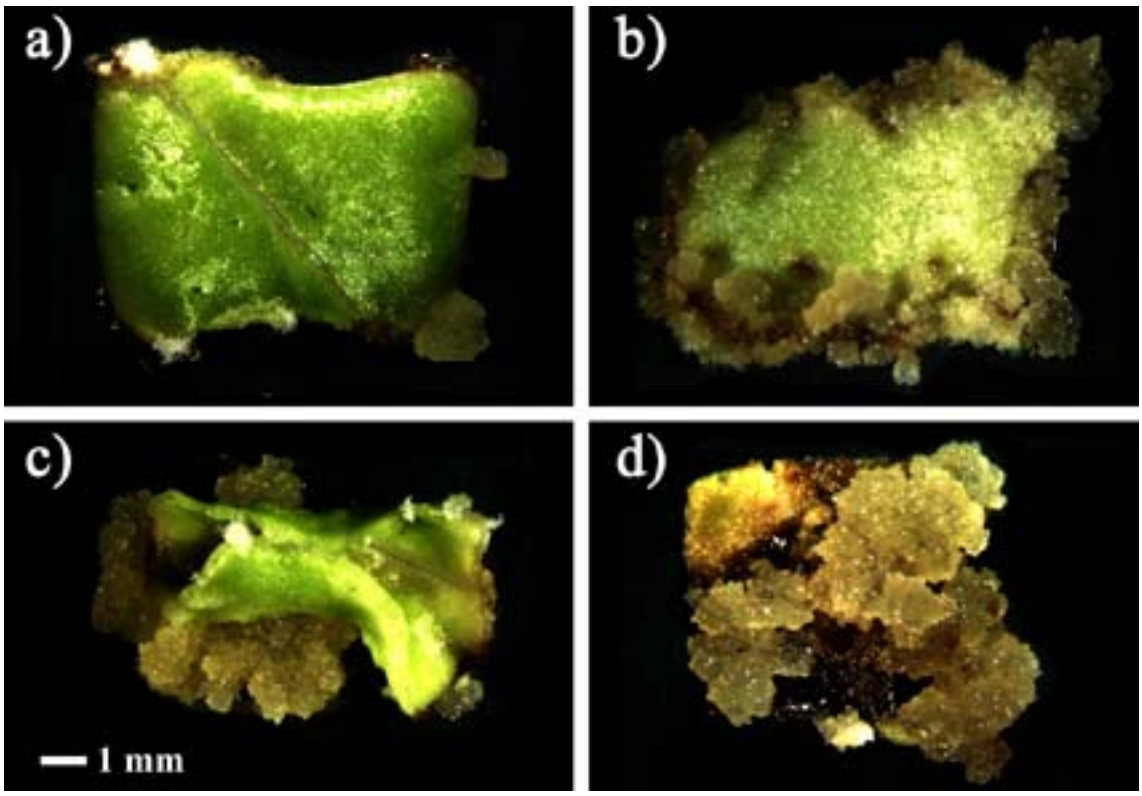


Fig. 3.11. Callus formation from leaf explants in sugarbeet: the control Rel1 (a); multiple callus formation, explant alive, line 6B2838 (b); multiple callus formation in the contact with the medium, line 3B0064 (c); multiple callus formation on died explant, line 1F0076 (d).

Moreover, explants of some genotypes could form both types of callus, regenerable and non-regenerable, on the same explant. Non-regenerable callus displayed different morphologies: white, or brown, or colourless soft callus consisting of enlarged elongated cells and compact white, or brown, or colourless callus (Fig. 3.12). Root formation from the explants and shoot formation either directly

from the explants, or, mainly, from regenerable callus were observed as well. No correlation between root and shoot organogenesis was detected.

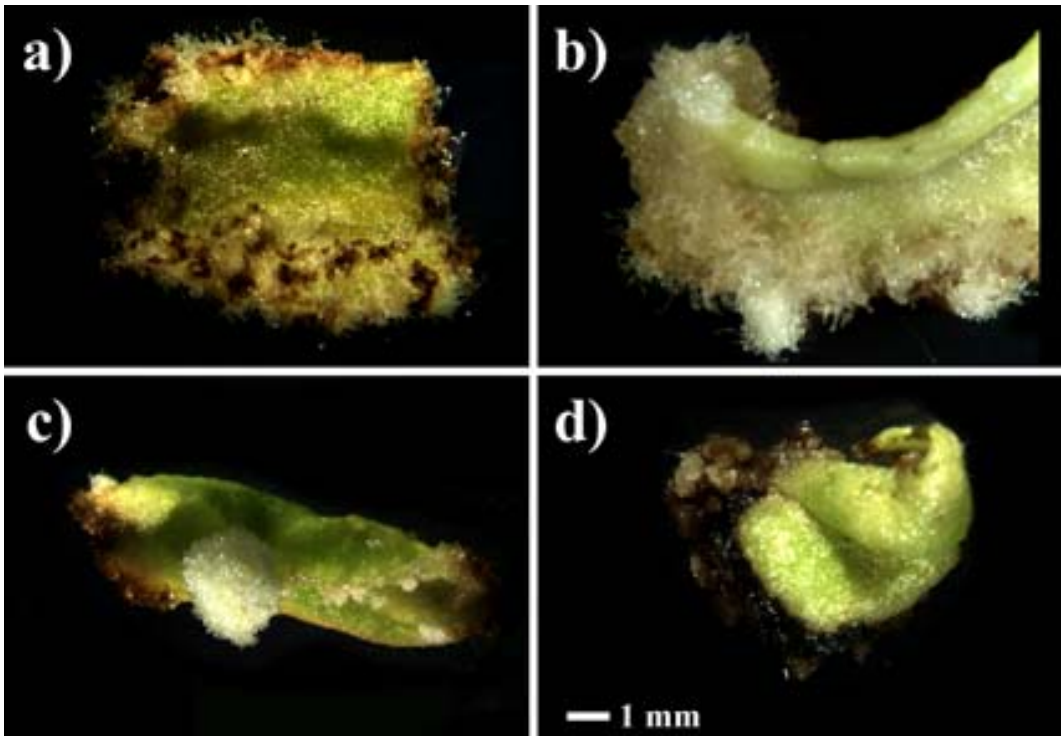
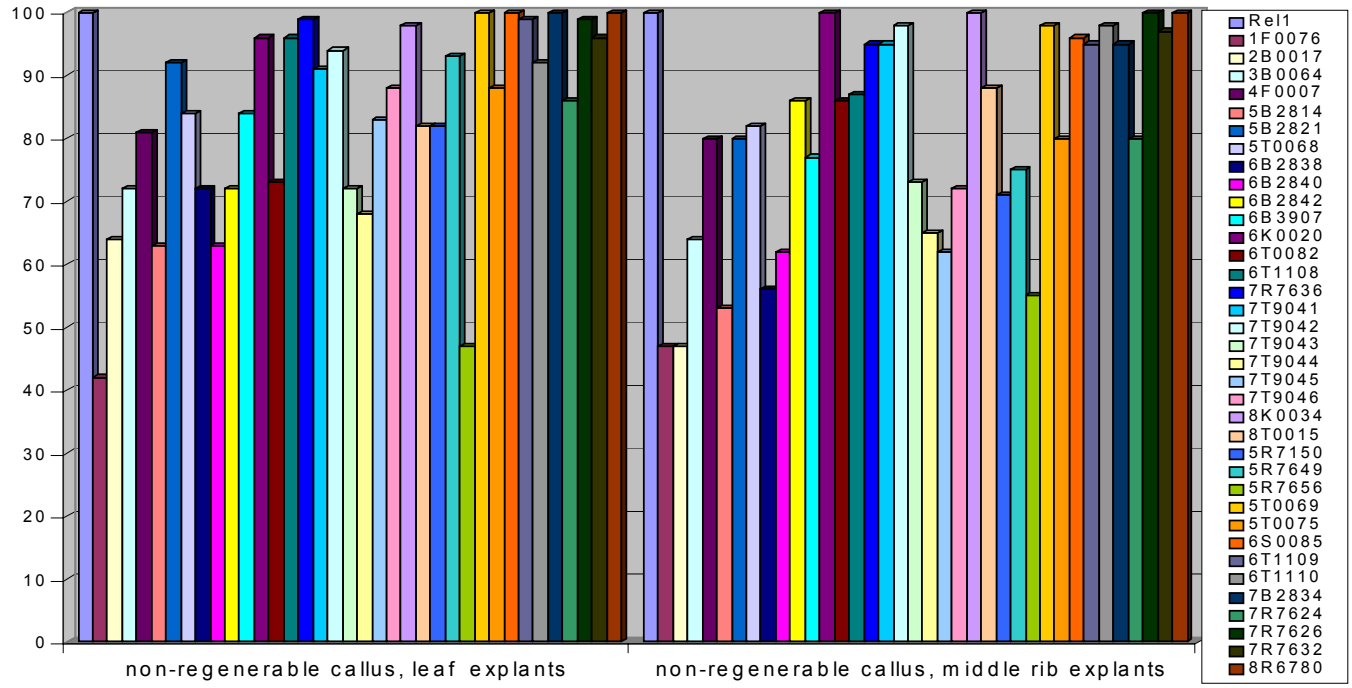


Fig. 3.12. Callus formation from leaf explants in sugarbeet: explant without callus formation, line 4F0021 (a); white non-regenerable callus from enlarged cells, line 2B0035 (b); white compact non-regenerable callus, line 7T9041 (c); colourless non-regenerable callus on the dying part of the explant, line 6S0086 (d).

After transfer to the light, efficiencies of compact callus formation were practically unchanged, thus demonstrating that compact callus in general was formed during the first weeks of explant culture (Fig. 3.13). However, efficiencies of regenerable callus formation were increased and 13 new genotypes responded with formation of friable callus (Fig. 3.14). Eight genotypes, including the control cultivar Rel1, regenerated shoots without additional transfer of the callus to fresh medium.

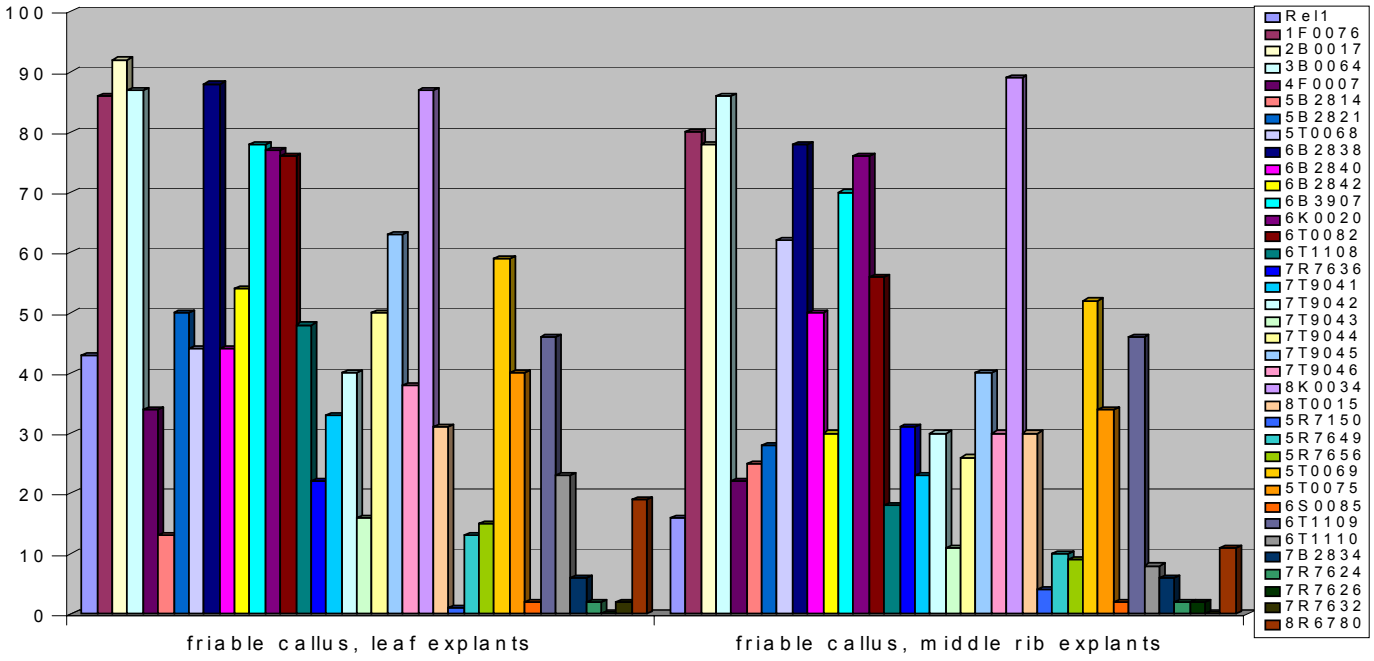
Following the procedure described by Ben-Tahar et al. (1991), calli from 5 breeding lines, 1F0076, 6B2838, 6B3907, 6K0020 and 8K0034 were tested for their regeneration activity. However, even after two subculture periods on solid medium no regeneration could be observed.

Fig. 3.13. Genotypes with friable callus formation: efficiency of non-regenerable callus formation from explants of different sugarbeet genotypes after transfer to the light (53 days of culture), in % of explants with response.



* Results represent summary obtained from 3 independent experiments

Fig. 3.14. Efficiency of regenerable callus formation from explants of different sugarbeet genotypes after 53 days of culture, in % of explants with response.



* Results represent summary obtained from 3 independent experiments

Thus, the differences in callus formation frequencies clearly demonstrate that using the protocol from Ben-Tahar et al. (1991) callus formation is a genotype-dependent process. Explants from 37 out of 47 genotypes tested formed friable callus formation, which corresponded to 83%. Shoot regeneration from formed friable callus was also genotype-dependent and it was observed for very few genotypes.

3.3.4 Friable callus formation from other sources

Shoot bases: Up to 20% of shoots formed friable callus after their transfer to medium MSB1 from hormone-free MS medium, however it was impossible to regulate the regeneration activity of that callus. Shoot organogenesis varied greatly from one experiment to the other.

Petioles: Friable callus was induced on MSB2 or MSB1 media in the light. Callus formation efficiencies were 22-27%, but shoot regeneration was unstable and at the range between 5 and 30%.

Roots: In the literature, there is no report on formation of regenerable callus from the roots of sugarbeet. Spontaneous friable root callus was observed on roots of 2-months-old etiolated hypocotyl explants, "Viktoria" (Fig. 3.15a). After transfer of this callus to fresh medium MSB2 only callus proliferation occurred and no regeneration. However, shoot organogenesis was induced after transfer of such callus to media with different hormone compositions. Shoot formation was observed on medium SRB (Fig. 3.15b). Regenerable friable callus was also induced from root explants, but the frequency of callus formation was extremely low, <0.001%. No friable root callus was obtained for breeding line 7T1308.

Thus, such systems of shoot regeneration from callus are dependent on the parameters of the particular experiment and they are not useful for further experiments, like gene transfer.

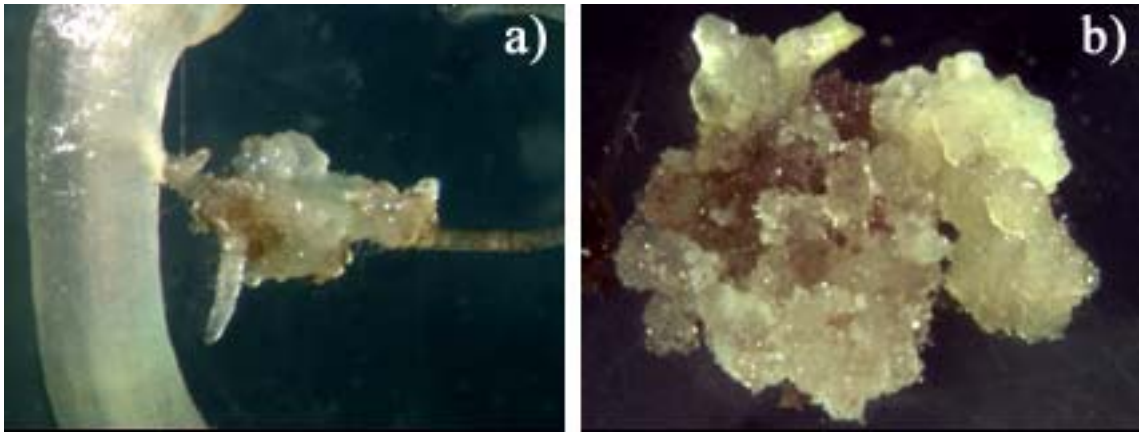


Fig. 3.15. Friable, regenerable root callus of sugarbeet: callus induction (a) and shoot formation (b).

3.3.5 Callus induction from etiolated hypocotyl and cotyledon explants

Since the regeneration capacity of hypocotyl or cotyledon derived friable callus was reported earlier (Catlin, 1990; Jacq, 1992), our efforts were concentrated on the development of a reproducible and highly efficient system of regenerable callus induction from seedling explants. Recently, Snyder et al. obtained similar results (1999), although they used different media for seedling germination and callus induction and without any explanation why those culture conditions were preferred to others. The establishment of our system that allows to obtain regenerable callus at high efficiency is described below step by step. All experiments were done using plant material of cultivar "Viktoria". Using the established conditions for explants from another breeding line, 7T1308, allowed to obtained even higher efficiencies.

Cytokinin: BAP was tested in different concentrations (0, 0.25, 1, 2 and 4 mg/l). The efficiency of friable callus formation on hormone-free MS medium was 7-8%. During the culture on medium supplemented with BAP at different concentrations, 11-14% of hypocotyl explants formed callus in the darkness. Supplement of 2 mg/l BAP was important to obtain the highest efficiencies of shoot regeneration from callus – up to 50% for "Viktoria and 95% for 7T1308. BAP at a concentration 4 mg/l already had an inhibiting effect.

Auxin: Different concentrations of NAA (0, 0.1, 0.5, 1 and 2 mg/l) were tested in combination with BAP. No positive effect was observed.

Light conditions and seedling age: Hypocotyl and cotyledon explants from seedlings of different age (two-, three-, four- and five-weeks old) that were germinated either in the darkness or in the light were tested for their callus induction on MSB2 medium either in the darkness or in the light. The best callus formation efficiencies (12-14%) were obtained when hypocotyl explants from five-weeks-old etiolated seedlings were cultured in the dark. While for "Viktoria" there were no significant differences in callus formation efficiencies from etiolated hypocotyl explants during callus induction either in the darkness or in the light (12 and 11% respectively), the influence of light on callus formation efficiency for 7T1308 was significant (Fig. 3.16). When hypocotyl explants from 7T1308 were cultured in the dark, the callus formation efficiency was even higher (21%) than for that from "Viktoria", however, the callus induction was strongly inhibited by explant culture in the light (< 1%).

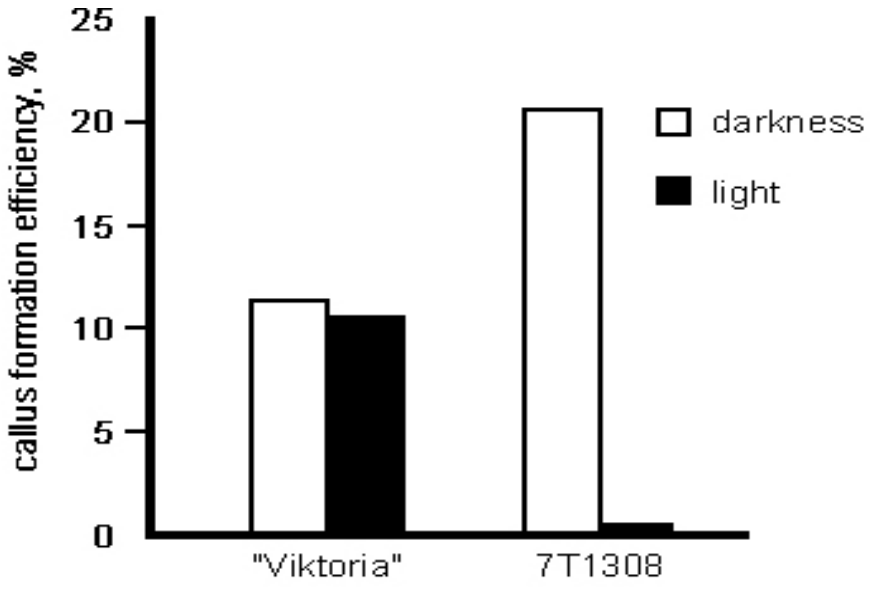


Fig. 3.16. Callus formation from etiolated hypocotyl explants under different light conditions.

Sucrose concentration: A prominent effect of a reduced sugar content in the culture medium was observed (Fig.3.17). Reducing the sucrose concentration to

one half resulted in almost twice the callus formation efficiency for both tested lines (21% for "Viktoria" and 43% for 7T1308). Sugar reduction to a concentration of 5g/l did not improve the efficiency further, but even with this low sugar content callus formation efficiency was good for both genotypes (12% for "Viktoria" and and 31% for 7T1308).

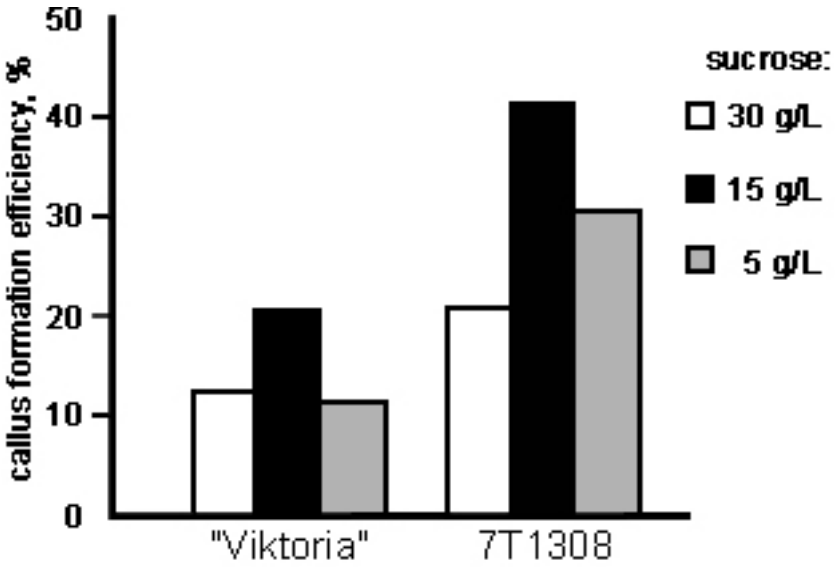


Fig. 3.17. Influence of sucrose on the callus formation efficiency from hypocotyl explants of sugarbeet.

3.3.5.1 Regeneration from hypocotyl callus

After transfer of hypocotyl callus to fresh medium (MSB2 or MS15B2) in the light or in the darkness, it showed a very high regeneration activity via shoot organogenesis, and rarely via somatic embryogenesis (Fig. 3.18). Both cultivars are characterized by stable and high regeneration frequencies under these conditions (40-50% for "Viktoria" and 85-95% for 7T1308).

An important factor is the age of the callus. The best regeneration frequency was obtained, when callus was used after a 4-5 week period of induction. Transfer of callus to fresh medium even only one week later reduced the regeneration activity drastically, by almost 50% for both genotypes. Therefore, 4-5-weeks old callus can be used for protoplast isolation and transformation experiments. Regenerates were

often vitrified, but an increase of the agarose concentration up to 1% (or agar-agar up to 1.4%) allowed normal shoot formation. Rooting of regenerates occurred after transfer to hormone-free MS medium.

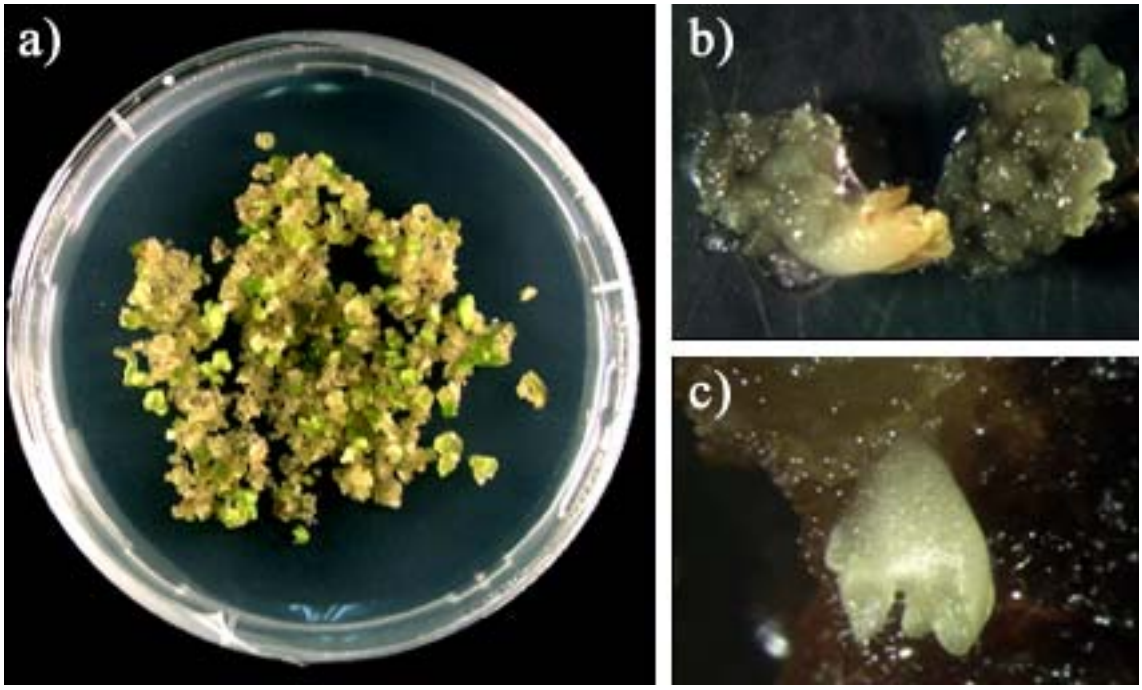


Fig. 3.18. Regeneration of sugarbeet from hypocotyl callus: shoot regeneration in the light, breeding line 7T1308 (a), shoot (b) and embryo (c) formation in the dark, breeding line "Viktoria".

Thus, our method of friable callus formation and shoot regeneration from such callus is reproducible and high efficient for both genotypes tested, requiring only one medium. Rooting of regenerates occurs on a second medium. Hypocotyl derived callus is the optimal source for experiments on protoplast culture and gene transfer.

3.4 Sugarbeet protoplast culture

3.4.1 Leaf protoplasts

So far reported, regeneration of sugarbeet shoots is possible only from leaf protoplasts (Krens et al., 1990; Lenzner et al., 1995). Hall et al. (1996a) discovered that regenerable protoplast derived callus originates from stomatal guard cells. To

obtain high yields of guard cell protoplasts, it is necessary to isolate large amounts of epidermis. Epidermis pieces can be obtained either by manual peeling or by using the blender method (Hall et al., 1996b). Here, an alternative method is described.

3.4.1.1 Combination of growth conditions for donor plants with strict digestion procedure results in a high yield of guard cell protoplasts

Established sugarbeet cultures, genotypes "Viktoria", "VRB" and "31-188", were grown on MS medium. PG_0B medium (De Greef and Jacobs, 1979), often used by other researches (Krens et al. 1990; Lenzner et al., 1995; Hall et al., 1996b) was tested. Unfortunately, after the transfer of plants to PG_0B medium their growth was almost inhibited. Thus, in our experiments all solid media were based on MS medium. During the culture on hormone-free MS medium new leaves grew only from the apex of shoots and laminas developed maximally. An addition of BAP even at a concentration of 0.25 mg/l induced germination of adventitious buds. Increased BAP concentrations (1-2 mg/l) resulted in strong reduction of lamina size, while leaves were pale green and vascular elements developed intensively. Leaves of shoot cultures that were grown on MSB1 or MSB2 were used for protoplast isolation.

Epidermal peelings: Isolation of epidermis from leaves of plants growing on hormone-free MS medium was performable (Fig. 3.19a). However, it was extremely difficult to peel significant amounts of epidermal stripes from leaves of sugarbeet, which was culturing on MSB2 medium. Isolated epidermis from these plants contained divided stomatal guard cells, what is demonstrated in the Fig. 3.19b. Nevertheless, all attempts to isolate a high number of intact protoplasts failed. The peeling of epidermis stripes was not useful due to low productivity and it was a very time-consuming procedure. Moreover, very often epidermal cells started to die immediately after the peeling.

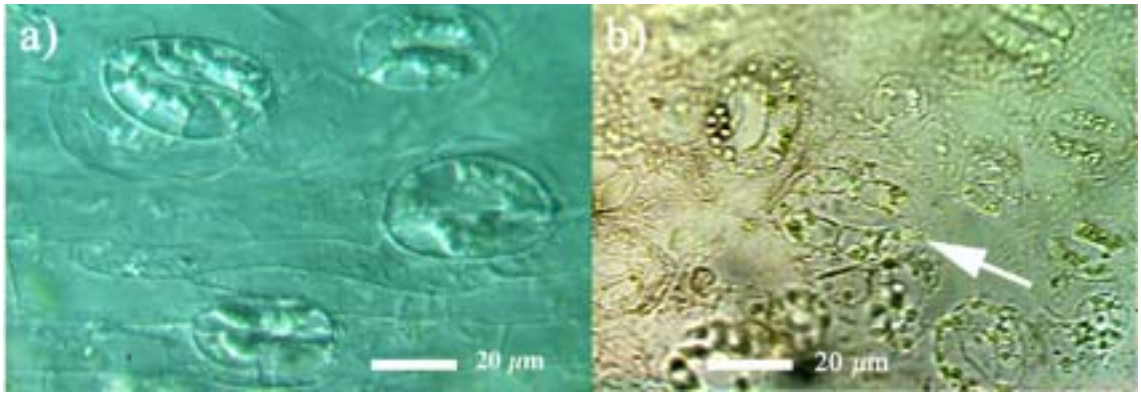


Fig. 3.19. Stomatal guard cells: stomata from the epidermis of plant that was growing on hormone-free MS medium (a) and divided stomata of the epidermis from plant, which was growing on MSB2 medium (b). Cultivar "Viktoria".

A new method of enrichment of protoplasts from stomatal guard cells: When protoplasts were isolated from leaves of plants growing on hormone-free MS medium, a high protoplast yield was obtained – up to 10^7 for digestion in the mixture containing cellulase Onozuka R-10 at 1% and macerozyme Onozuka R-10 at 1.5-2%. However, amounts of guard cell protoplasts were low (1-5% of total number of intact protoplasts). When using enzyme mixtures with high concentrations of enzymes and digesting leaves from plants cultured on MSB1 or MSB2, efficient amounts of guard cell protoplast could be obtained. Digestion in a mixture containing 1% cellulase Onozuka R-10, 2% macerozyme Onozuka R-10 and 0.4% driselase, resulted normally in 40-70% of guard cell protoplasts (Fig. 3.21a), achieving in some experiments up to 90%. Also, time of digestion in such mixture was reduced from 14-16 h to 6-8 h. Mesophyll protoplasts were about 40-60 μm in size and contained many chloroplasts. Therefore, stomatal guard cell protoplasts could be easily distinguished due to their size (about 20 μm) and small number of plastids (usually 8-12).

3.4.1.2 Protoplast culture and regeneration

Protoplast culture: An inclusion of 0.1 mM nPG in all isolation and culture media and protoplast culture in the dark are both important for sustained cell divisions,

microcolony formation and regeneration (Krens et al., 1990, Hall et al., 1993, Lenzner et al., 1995). In the absence any of these factors, no formation of protoplast derived colonies was observed in our experiments as well. After digestion and purification, protoplasts were embedded into thin alginate layers and cultured in the modified K8p medium or in the PCB medium (Table 2.3). The differences between K8p and PCB media are reduced organic contents and a different hormone composition in PCB. Protoplasts were also cultured in PCB0 medium with sugars and other organic compounds from PCB and mineral salts from F-PCN medium. No significant differences in division frequencies and plating efficiencies were found. In all culture media first divisions were observed after 4-6 days of culture. After about three weeks of culture small microcolonies were formed (Fig. 3.20a, b). Nevertheless, protoplast derived colonies, large enough for transfer to solid medium, were formed only 6-7 weeks after protoplast isolation (Fig. 3.21c). The plating efficiencies varied from >0.001 to 1% for all genotypes tested. Even if the proportion of embedded guard cell protoplasts was over 50%, plating efficiencies were the same. Surprisingly, only friable colonies were obtained, while formation of compact colonies, reported in the literature (Krens et al., 1990; Pedersen et al., 1993; Lezner et al., 1995), was never observed. Mesophyll protoplasts either did not divide at all, or their development was blocked after the first division. Typically, mesophyll protoplasts just enlarged in size, and they had a clearly visible nucleus (Fig. 3.20c).

Alginate embedding: Solutions with alginic acid were prepared in a different way: by autoclaving or with filter sterilization (Hall et al. 1997). Protoplast embedding in filter sterilized alginate medium resulted in at least 10 times better plating efficiencies in comparison with the experiments, where autoclaved medium was used.

Influence of casein hydrolysate: Szabados and Gaggero (1985) demonstrated a positive effect of casein hydrolysate on development of callus protoplasts in

sugarbeet. Different concentrations of casein hydrolysate were tested: 100, 200, and 500 mg/l. An addition even 100 mg/l of casein hydrolysate inhibited protoplast divisions.

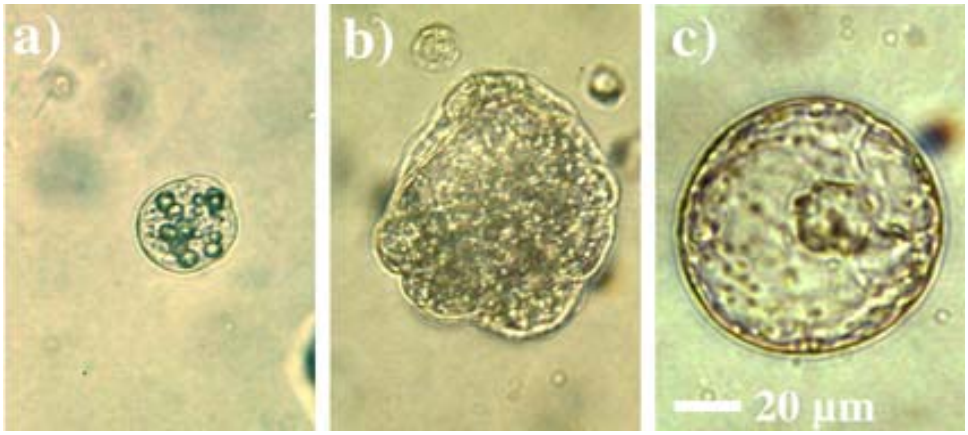


Fig. 3.20. Sugarbeet leaf protoplasts: first divisions of guard cell protoplast after 5 days of culture (a), microcolony after two weeks of culture (b) and one week old mesophyll protoplast (c). Cultivar "Viktoria".

Organogenesis: Shoot regeneration from protoplast derived callus was usually obtained on solid media containing 1 µm BAP (about 0.22 mg/l, Krens et al. 1990; Lenzner et al., 1995; Hall et al., 1996b). As a negative effect of PG_0B medium on donor plant growth was observed earlier, MS medium supplemented with 0.25 mg/l of BAP was used. Regeneration was not observed on this medium. Two cytokinins, BAP and zeatin, at different concentrations, 0.25, 0.5, 1 and 2 mg/l, were tested in agar- or phytagel-solidified media. Callus was cultured either in the dark or in the light. Nevertheless no shoot formation occurred.

System based on guard cell protoplasts was not successful in our hands. In order to improve sugarbeet protoplast culture an alternative system was developed.

3.4.2 Callus protoplasts

Until now there are no reports on successful plant regeneration from callus protoplasts in sugarbeet. All attempts to regenerate sugarbeet plants from callus protoplasts failed. Here, a new, reproducible and efficient method for protoplast

isolation, culture and further successful shoot formation is described for the first time. Protoplasts were isolated from friable regenerable callus of different origin: root callus, shoot base callus and hypocotyl callus (see chapter 3.3). While protoplast derived callus was obtained in each case, shoot organogenesis was achieved for protoplasts from hypocotyl callus only. The method was established on genotype "Viktoria" and optimal conditions were determined. Improved isolation and culture conditions together with the TAL-technique resulted in a very fast protoplast development and high plating efficiencies.

3.4.2.1 Protoplast culture

Protoplast culture: Freshly isolated protoplasts were characterized by a low number of plastids (normally about 20 organelles per cell). Cell size differed from 15 till 40 µm (Fig. 3.21).

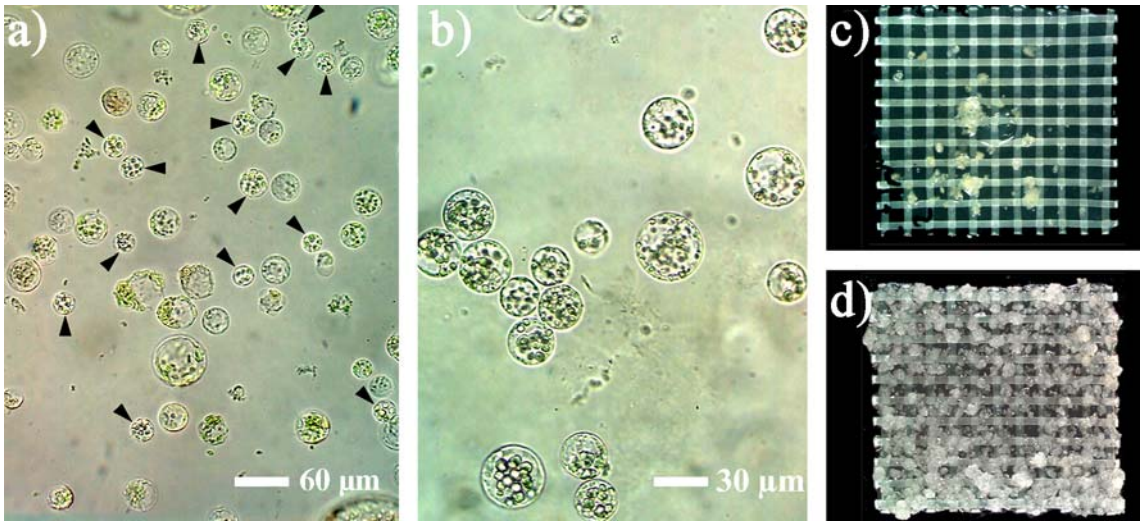


Fig. 3.21. Comparison of leaf and callus protoplast culture: leaf protoplasts, enriched for stomatal guard cell protoplasts (marked with arrowheads) (a), freshly isolated callus protoplasts (b), protoplast derived colonies from leaf (c) and callus (d) protoplasts after six and three weeks of culture respectively. Cultivar "Viktoria".

Culture of sugarbeet callus protoplasts in thin alginate layers in PCB medium resulted in very fast cell divisions and high plating efficiencies. Callus protoplasts from friable shoot base callus or from friable root callus started to divide after 1-2

days of the protoplast culture. Protoplasts from hypocotyl callus also rarely divided after 24 hours after isolation (Fig. 3.22a), but mainly first divisions were observed 2-4 days after protoplast isolation. After 2 additional days of culture, small cell aggregates, of about 10-12 cells, had formed and 9-10 days after protoplast isolation visible microcolonies were observed (Fig. 3.22c). It was possible to transfer grids with two-weeks old microcolonies to the agarose-solidified MS medium containing cytokinin BAP (0.25, 1 or 2 mg/l). Colonies increased in size up to 1-2 mm one week later after the transfer. Only friable callus was obtained. About 1-2% of dividing cells differed morphologically from the majority. They were larger in size (for comparison look Fig. 3.22 b and d) and after 4-5 divisions such cells stopped to grow and never developed into protoplast derived callus.

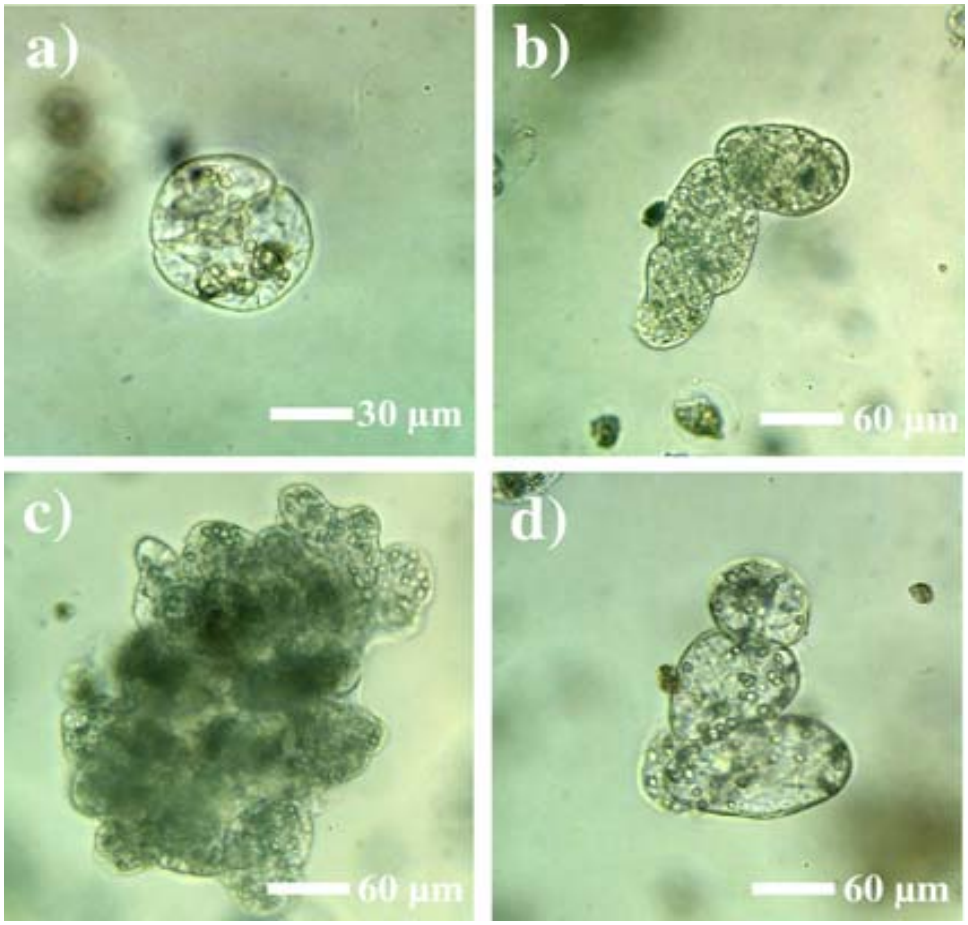


Fig. 3.22. Callus protoplast culture: first division, 24 hours after isolation (a), 5-days old microcolony (b), two-weeks old microcallus (c) and another type of microcolony formation (d). Genotype "Viktoria".

Protoplast density: Several protoplast densities were tested, i.e. $3.2 \cdot 10^4$, $6.4 \cdot 10^4$, $1 \cdot 10^5$ or $1.25 \cdot 10^4$ pps/ml. The highest plating efficiencies (up to 35%) were obtained, when protoplasts were embedded and cultured at a density of $6.4 \cdot 10^4$ pps/ml. The lowest cell density, at which sustained protoplast divisions were observed and visible colonies were formed, was $2 \cdot 10^3$ pps/ml. However, plating efficiencies under such conditions were about 1%.

Influence of nPG and light conditions: While leaf protoplasts could not form colonies without nPG, callus protoplasts, cultured at the same conditions, were able to divide with further formation of microcolonies. However, the plating efficiency of protoplasts, which were developing in medium containing nPG, was up to 35% and thus 30-100 times higher in comparison with culture in nPG-free medium (0.3-1,5%). Culture in the dark was absolutely required for sustained cell divisions and further colony formation. Rare divisions were observed during culture in the light, but no protoplast derived colonies were formed.

Influence of phytohormones: Different combinations of NAA and BAP in PCB medium were tested. Unexpectedly, protoplast derived colonies were obtained in all tested culture media except of hormone-free PCB (Table 3.3). In further experiments PCB medium with 2 mg/l NAA and 1 mg/l BAP was used, as the standard medium.

Table 3.3. Plating efficiency of sugarbeet protoplasts from hypocotyl callus in PCB medium with different hormone compositions, cultivar "Viktoria"

NAA, mg/l	BAP, mg/l	Plating efficiency,%
0	0	0
2	0	13
2	1	28
2	2	27
1	2	23
0	2	8

The division frequency of callus protoplasts from breeding line 7T1308 was similar to that of genotype "Viktoria" (23-28%). Unfortunately, the plating

efficiency was drastically lower, 0.1-0.3%. Such significant difference could be explained by deterioration of water quality we experienced that time; a few weeks later experiments on tobacco protoplasts were also inhibited due to this factor. Nevertheless, protoplast derived callus was successfully obtained for both breeding lines and proved its regeneration activity afterwards.

3.4.2.2 Shoot regeneration from protoplast derived callus

Different hormone combinations were tested. No shoot regeneration was observed on any culture medium containing thidiazuron. Only white compact callus, sometimes containing greenish compact structures, was formed. Additionally, on media supplemented with thidiazuron friable callus became more vitrified in comparison with the starting material. Shoot regeneration was observed only on media with BAP as the cytokinin. In contrast to leaf protoplast culture, where shoot formation usually was observed in the dark (Krens et al., 1990; Lezner et al., 1995), regeneration activity of protoplast derived callus cultured in the dark was very low. Regeneration was observed on MS medium containing 0.25 mg/l BAP, 0.25 mg/l TIBA and 1 mg/l NAA (Fig. 3.25c). When callus was cultured in light better results could be obtained. The highest regeneration frequency, 10%, was observed on MS medium supplemented with 2 mg/l BAP, 1 mg/l NAA and 1 or 2 mg/1 TIBA. Regenerates from medium with TIBA 2 mg/l were always vitrified and it was extremely difficult to obtain morphologically normal shoots from the primary regenerates. Typically, protoplast derived colonies started to synthesize anthocyanins. This preceded either shoot formation or compact structures that were able to form shoots afterwards (Fig. 3.23a, b and 3.24b, d). These compact regenerable structures were often formed at the bases of compact callus (Fig. 3.24b). Primary regenerates were characterised by high regeneration activity (Fig. 2.24c, d).

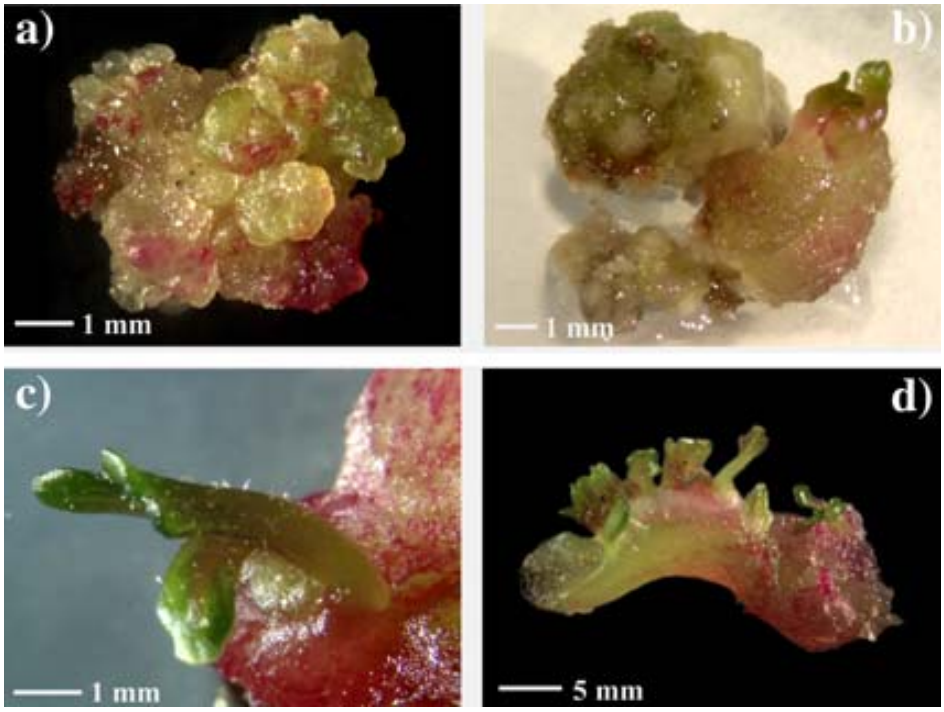


Fig. 3.23. Regeneration from protoplast derived callus and regeneration activity of primary regenerates: shoot induction from nodular callus (a), shoot formation from a compact structure (b), regeneration from primary regenerates (c and d). Cultivar "Viktoria".

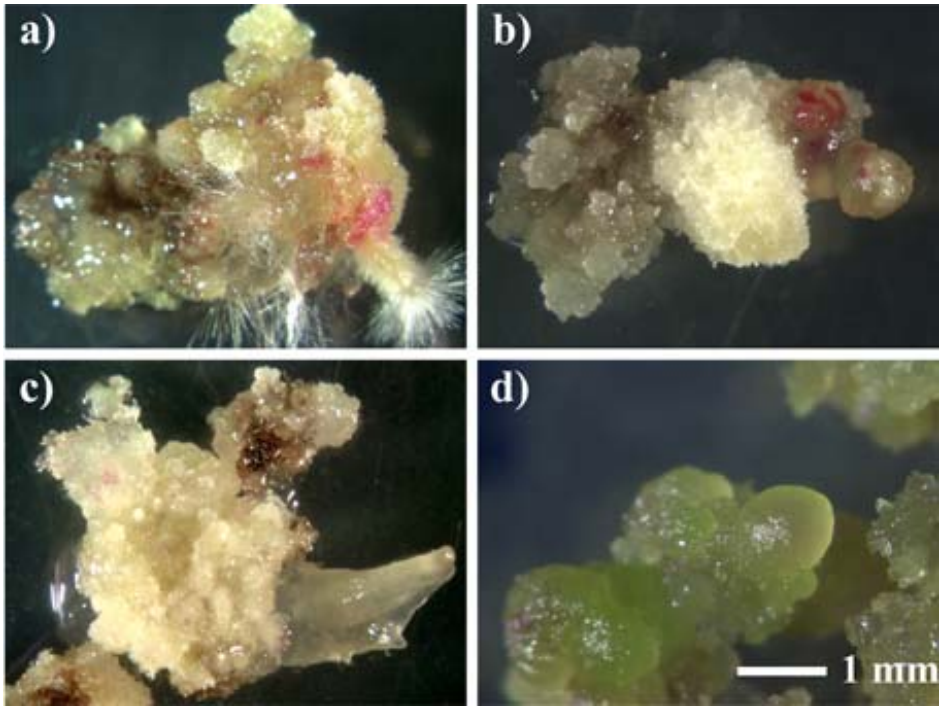


Fig. 3.24. Organogenesis from protoplast derived callus: root formation (a), formation of a compact regenerable structure (b), shoot regeneration in the darkness (c) and shoot induction in the light (d). a – genotype VRB; b and c – genotype "Viktoria"; d – genotype 7T1308.

Shoot formation was observed only from protoplasts isolated from hypocotyl-derived callus. Just rhizogenesis (Fig. 3.24a) and compact callus formation were obtained from callus protoplasts of other origin. Regeneration efficiency of protoplasts from hypocotyl callus in cultivar 7T1308 under our conditions was even higher than for cultivar "Viktoria"– up to 30%. However, regenerates were always vitrified.

Influence of callus age on regeneration activity of protoplast derived colonies: Protoplasts were isolated from hypocotyl callus (cultivar "Viktoria") of different age - directly after callus induction and after one or two or three months of culture. While there was no observations of reduction in plating efficiencies, shoot regeneration from protoplasts that had been isolated from 3-months old callus was drastically reduced - less than 1%. Regeneration efficiencies of one- and two-months old callus were 7-10%.

Rooting of regenerates: All our attempts to root regenerates on a medium containing auxin NAA failed. Shoots were rooted after their transfer to hormone-free MS medium. Usually formation of roots was observed after 2-3 subcultures. Regenerates that were cultured on the auxin-containing medium often became brown at the base and even died.

Despite of some difficulties during regeneration and rooting steps, which still can be improved, a reproducible and efficient protoplast system was developed. Sugarbeet regenerates from protoplasts isolated from friable, hypocotyl-derived callus were obtained successfully for the first time.

3.5 Nuclear transformation in sugarbeet

Until recently, nuclear transformation in sugarbeet was a very difficult, inefficient and hardly reproducible procedure. Only during the last years the situation turned to the better side. Before starting experiments on plastid transformation in sugarbeet, both methods, which are also used in plastid transformation, were tested

for nuclear transformation.

3.5.1 Bialaphos selection

Vector pSL-GUS-INT-PAT contains the *pat*-gene as a selection marker and the *β*-glucuronidase (GUS) gene with an introduced intron as a reporter gene. The vector was used in our experiments for both tested transformation methods (the PEG method and the biolistic method). Hypocotyl derived callus was used to define optimal selection concentration. Different concentrations of bialaphos were tested, i.e. 0.25, 0.5, 1 and 5 mg/l. Even 0.25 mg/l of bialaphos already efficiently eliminated callus development. Finally, 1 mg/l of bialaphos was chosen as the selection concentration, since no escapes were observed under such condition. The same concentration of bialaphos was used to eliminate growth of protoplast derived microcolonies and it proved efficient to select resistant colonies.

3.5.2 PEG-mediated transformation of callus protoplasts in sugarbeet

Successful PEG-mediated transformation of sugarbeet protoplasts with further shoot regeneration from transformed cell lines were reported by Hall et al. (1996b). In those experiments protoplasts from guard cells were used. The conditions described by Hall et al. (1996b) were applied for nuclear transformation of callus protoplasts and comparable results were obtained. Different amounts of protoplasts ($1 \cdot 10^5$ and $5 \cdot 10^5$ pps/experiment) from hypocotyl callus (genotype "Viktoria") were treated with PEG in the presence of plasmid DNA (25µg/$5 \cdot 10^5$ pps). The transformation efficiencies were in the $5 \cdot 10^{-5}$ to $4 \cdot 10^{-4}$ range (results of 3 independent experiments). A smaller amount of treated cells resulted in reduced transformation frequency. All colonies that were resistant to bialaphos stained blue after the histochemical GUS test. Unfortunately, no regenerates were obtained and only green smooth structures were formed from resistant callus lines.

3.5.3 Biolistic transformation of friable hypocotyl callus

A successful and efficient method of nuclear transformation in sugarbeet was

established. During callus incubation on selection medium containing 1 mg/l of bialaphos the majority of the treated callus became brown and died, but small surviving calli appeared (Fig. 3.25 a). The transformation efficiency (number or resistant clones per number of hypocotyl explants which formed callus) was 9-18%.

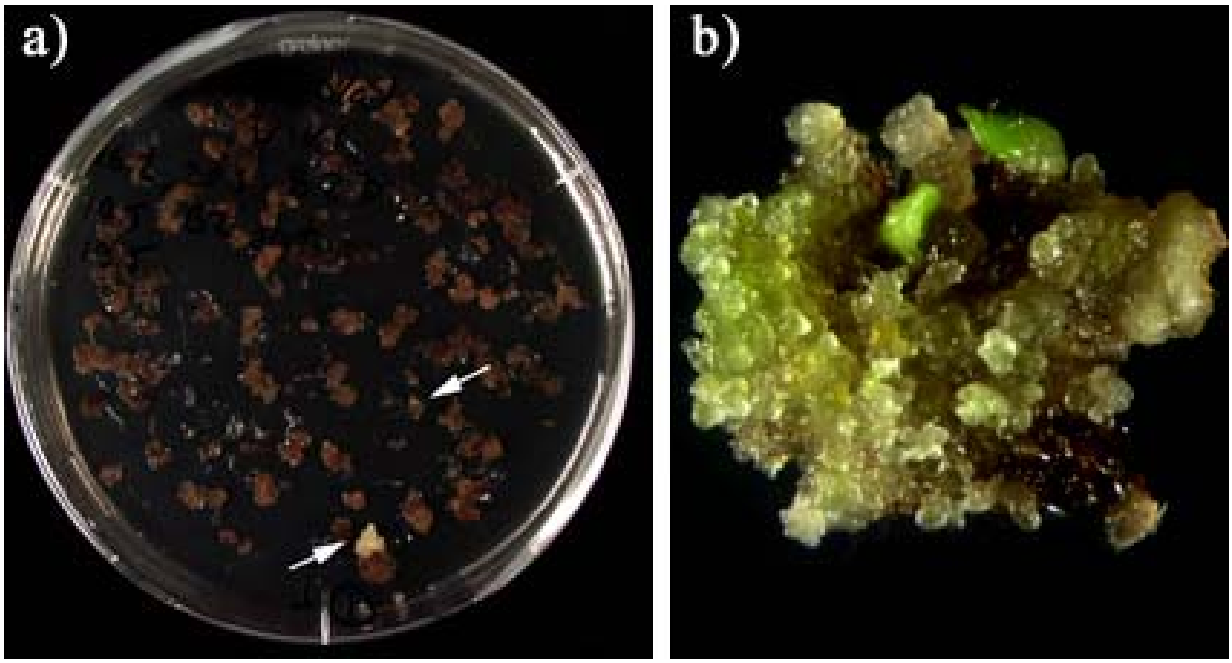


Fig. 3.25. Nuclear transformation in sugarbeet, cultivar "Viktoria": selection of bialaphos resistant colonies (a), regeneration from bialaphos resistant colony (b).

Resistant colonies were transferred to regeneration medium MSB2 with the same concentration of herbicide. After 3-6 additional weeks of culture regenerates were formed (Fig. 3.25b). The regeneration frequencies of selected lines were about two times lower (20-25%) in comparison with the regeneration efficiencies for control callus (40-50%), cultured on inhibitor free medium. Callus and regenerates were analysed for their GUS activity and subsequently by molecular methods. These results suggest that the problems of DNA uptake and shoot regeneration from transformed clones are successfully overcome and sugarbeet is no longer a recalcitrant species with respect to nuclear transformation.

3.5.4 Histochemical GUS analysis

Bialaphos resistant colonies were selected, and transferred for multiplication to fresh medium containing 1 mg/l bialaphos. After three weeks of culture pieces of growing callus lines were tested for their GUS activity (Fig. 3.26). Almost all lines except of line 1 expressed GUS-activity after 10-15 minutes of staining. It took more then 2 times longer until staining of line 1 started to be visible. Differences of the colour intensity could be explained by a position effect of DNA integration and also the number of gene copies per cell might be different. Cell lines 4 and 8 regenerated shoots containing GUS activity.

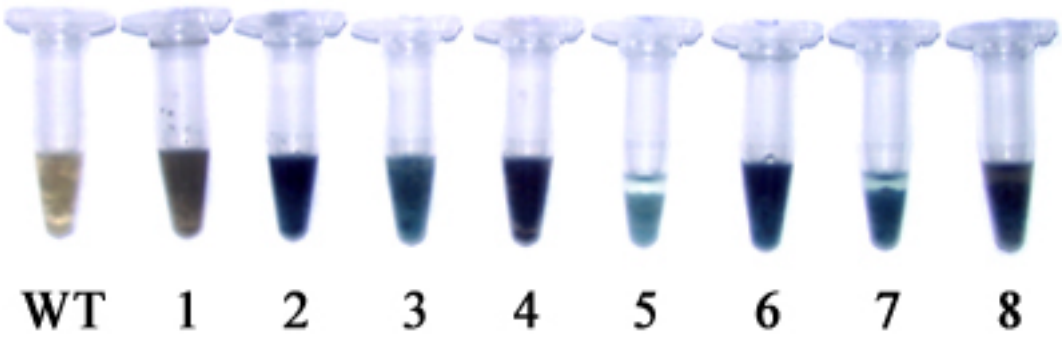


Fig. 3.26. GUS activity of bialaphos resistant colonies. Colonies were selected from three bombarded petri dishes with friable callus, cultivar "Viktoria".

3.5.5 Molecular analysis

PCR analysis: Total DNA, isolated from regenerates (line 4 and 8) was used for GUS gene detection by PCR-analysis. The expected size of the PCR fragment was 1577 bp. The result obtained was as expected: the size of PCR products for bialaphos resistant, GUS active regenerates was identical to the size of PCR-products of a positive control (plasmid DNA) (Fig. 3.27). Differences of band intensities were due to different DNA concentrations (DNA concentration for line 8 was about twice less) and also, possibly, different number of inserted gene copies per cell might be a reason.

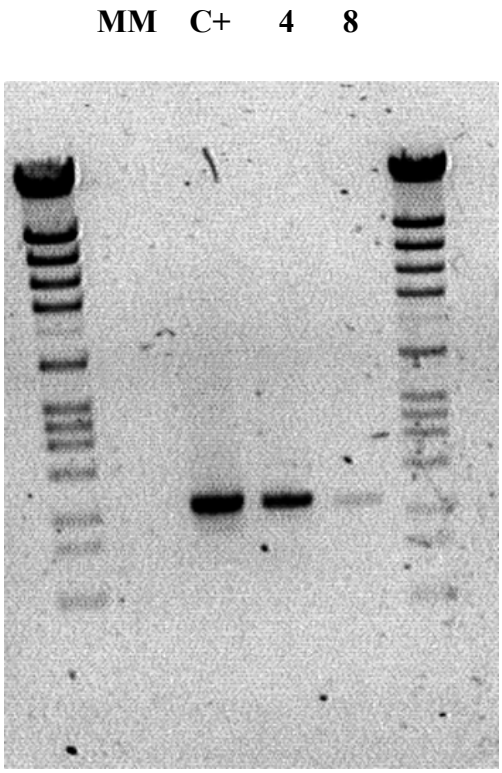


Fig. 3.27. Detecting of the *uid*A gene in total DNA of bialaphos-resistant sugarbeet regenerates by polymerase chain reaction (PCR) analysis: MM – master mix, C+ - vector pSL-GUS-INT-PAT, 4 – regenerate from cell line 4, 8 – regenerate from cell line 8.

Southern analysis: Southern blot analysis confirmed transformation and the presence of selectable marker and reporter genes. Total DNA from GUS-positive and bialaphos-resistant regenerates and a control (wild-type, WT) plant was tested. After DNA was loaded, *pat*- and GUS-probes were added for incubation. Plant DNA, digested either with *Eco*RI (Fig. 3.28 A gel), or with *Xba*I and *Hind*III (Fig. 3.28 B gel), produced the expected hybridisation signals of 2.5 kbp for the GUS-gene (A gel) and of 1.3 kbp for the *pat*-gene (B gel), whereas no signals were found with WT DNA. Weakness or absence of signals with DNA from regenerate 8 could be due to lower copy number of transgenes in total DNA and the concentration of total DNA was lower too. Bands of larger size might be produced due to a hybridisation of the pat-probe with digested DNA containing the *pat*-gene (A gel), or a hybridisation of the GUS-probe with DNA containing the GUS gene (B gel), since both probes were present during hybridisation.

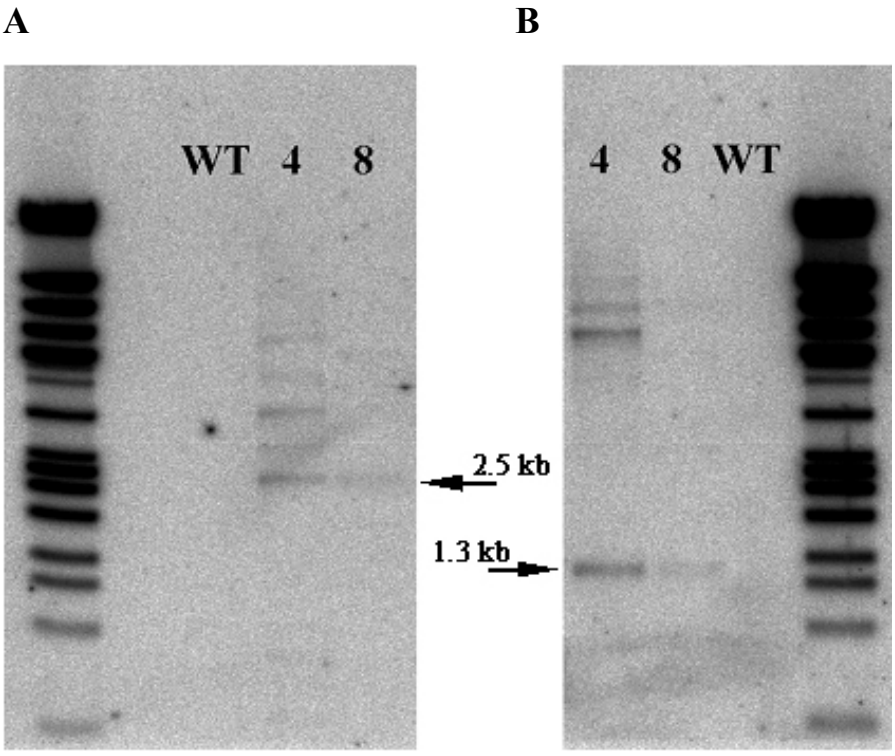


Fig. 3.28. Southern analysis of DNA from putative nuclear transformants. Total wild-type DNA (WT) and DNA from regenerates (4 and 8) was digested with *Eco*RI (A gel) and *Xba*I and *Hind*III (B gel). After electrophoresis on an agarose gel and transfer to nylon membrane a hybridisation was performed with probes for the *uid*A-gene and the *pat*-gene. Both probes were derived through restriction of DNA from vector pSL-GUS-INT-PAT.

3.6 Plastid transformation of rapeseed and sugarbeet

3.6.1 Construction of species-specific vectors

A vector, which is used in our laboratory for plastid transformation of tobacco and contains flanking sequences homologous to the plastome region between *trn*L and *rpl*32 (nt: 111515-116171 according to Shinozaki et al., 1986) is not suitable for other species. Comparison of this region with sequences from databases (http://www.ncbi.nlm.nih.gov) showed a low degree of homology between different plants (Appendix 3). Alternatively, the region *trn*V-*rps*7 (nt: 140126-142640) was used, since this plastome fragment is highly conserved in various plant species (Appendix 3). Using primers, designed for tobacco sequence, homologous fragments for species tested were amplified successfully (Fig. 3.29).

After the fragments were cloned into vector pGEM-T Easy, the orientation of inserts was confirmed by restriction analysis. Comparison of homologues is presented in Appendix 4. Primary vectors were named pSB and pRS for sugarbeet and rapeseed respectively (Fig. 3.30). Suitable restriction sites for further cloning of a selection marker, the *aad*A-cassette, were determined for both constructs. The *aad*A-cassette was inserted at unique restriction sites within the primary vectors. The PCR-amplified *aad*A-cassette (Koop et al., 1996) was successfully cloned into the rapeseed vector (pRS-*aad*A) at the *Bpu*1102I site, but all attempts to integrate the cassette at the *Bst*1107I restriction site of the sugarbeet vector failed. Another place that could be available for cloning was *Acc*I site. For this, *Acc*I restriction site was removed from multiple restriction site of vector pGEM-Teasy by double digestion with *Mlu*I and *Spe*I. Linearised vector (pSB-*Acc*I), containing unique *Acc*I site was religated. Cloning of the PCR-amplified *aad*A-cassette into pSB-*Acc*I vector was again not possible. Thus, to solve this problem the *aad*A-cassette was cut out from pRS-*aad*A vector with *Ksp*AI and *Sma*I and cloned into vector pSB-*Acc*I, linearised with *Acc*I. Insert orientation was confirmed by restriction analysis (Fig. 3.30). Functionality of the *aad*A-gene was confirmed by double selection of "Epicurian *coli* SURE 2" transformed with these vectors on LB-medium containing ampicillin and spectinomycin.

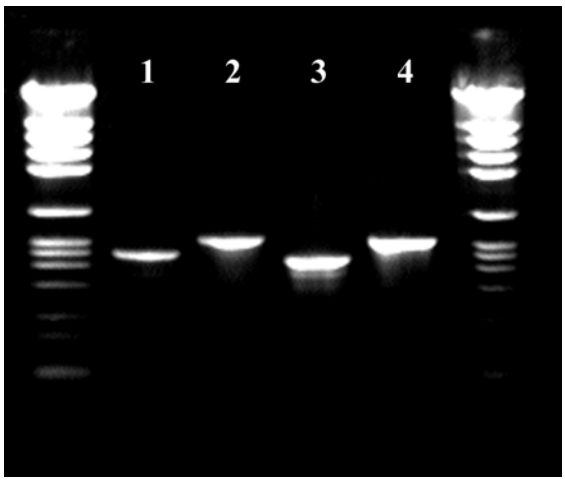


Fig. 3.29. PCR amplification of *trn*V-*rps*7 fragment from plastid chromosomes in different species: 1- *Nicotiana tabacum*, 2- *Arabidopsis thaliana*, 3- *Beta vulgaris*, 4- *Brassica napus*.

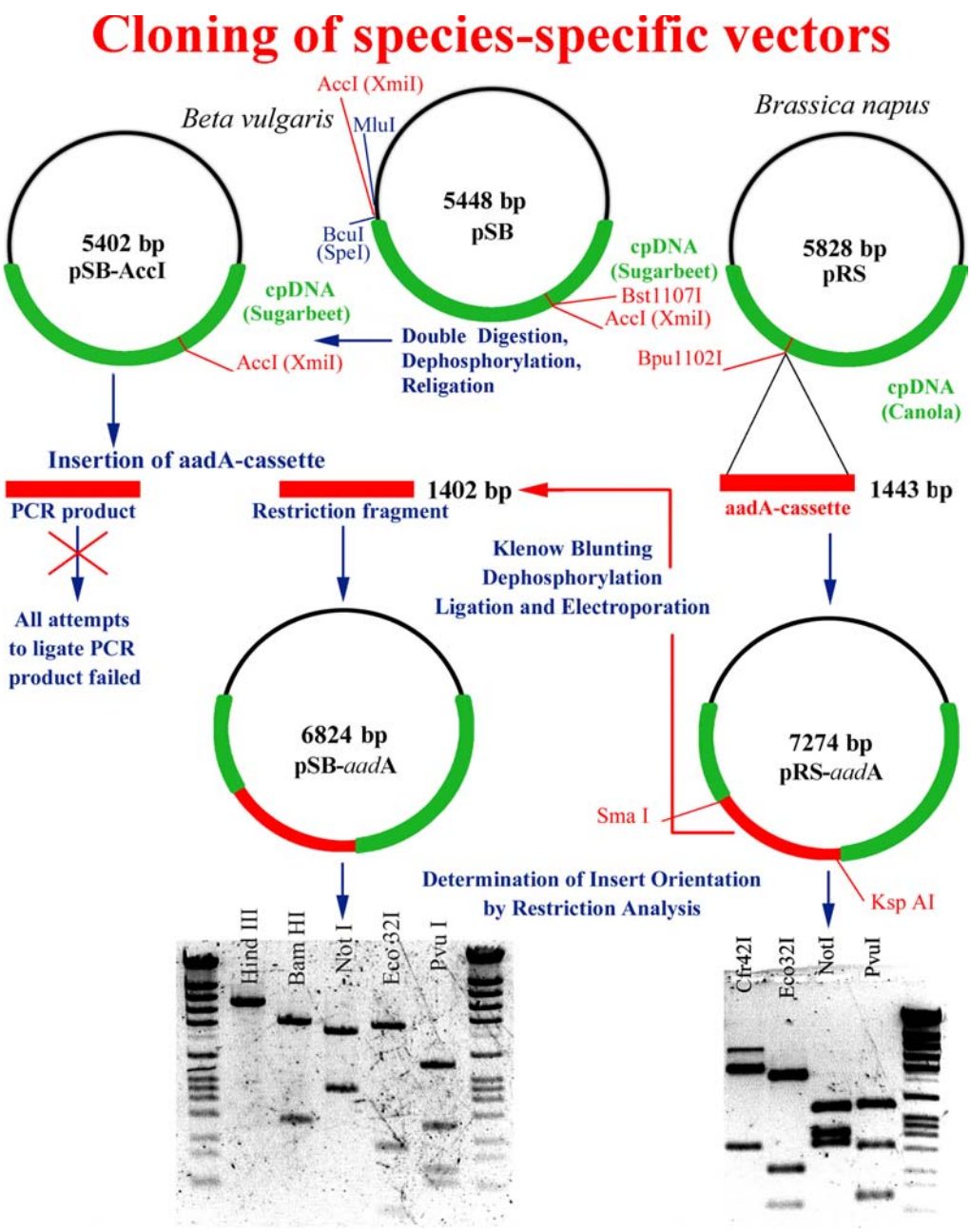


Fig. 3.30. Construction of species-specific vectors for plastid transformation in rapeseed and sugarbeet.

3.6.2 Determination of selection conditions

Protoplast derived colonies of rapeseed and hypocotyl callus of sugarbeet were tested to determine optimal selection concentrations. Rapeseed is insensitive to both antibiotics (spectinomycin and streptomycin). Protoplast derived colonies lost their green pigmentation already at the lowest antibiotic concentration tested (20 mg/l) as expected. However, their growth was not inhibited. Colonies were able to

grow even in the presence of antibiotics at the highest concentration (500 mg/l). No shoot regeneration was observed under selection conditions from protoplast derived colonies in rapeseed (Fig. 3.32). Only very few colonies started to form roots, but rhizogenesis was inhibited very soon after the initiation.

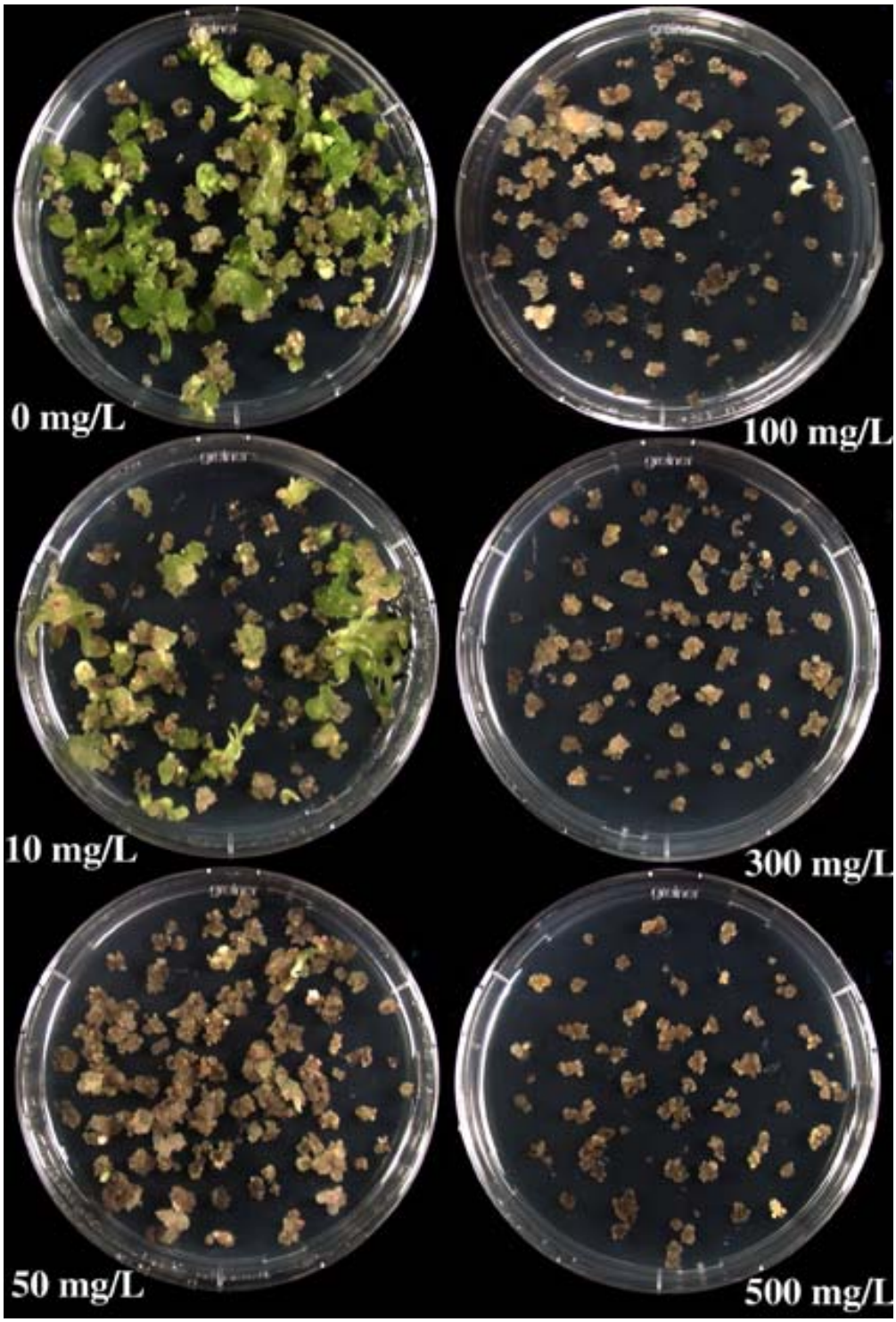


Fig. 3.31. Spectinomycin selection of sugarbeet callus, cultivar 7T1308.

Etiolated hypocotyl callus from sugarbeet demonstrated a high sensitivity to spectinomycin. Different concentrations of the antibiotic were tested, i.e. 0, 10, 50, 100, 300 and 500 mg/l. 100 mg/l spectinomycin was found to be efficient for selection. Callus (cultivar 7T1308) was cultured on medium MSB2 with different concentrations of spectinomycin for four weeks (Fig. 3.31). Shoot regeneration at a concentration of 50 mg/l was significantly inhibited, but green regenerates were still observed. Starting from a spectinomycin concentration of 100 mg/l and higher no green regenerates were found. Callus was transferred to antibiotic-free medium to check toxicity of different concentrations of spectinomycin. After a selection period for four weeks, colonies were transferred to fresh, inhibitor-free medium. About 90% of the colonies, which were transferred from MSB2 supplemented with 100 mg/l spectinomycin, were again able to grow and to regenerate green shoots. For callus from media with higher antibiotic concentrations (300 and 500 mg/l), only 30% and 11% of transferred colonies respectively continued to grow. Therefore, spectinomycin at a concentration of 100 mg/l was used for callus selection.

3.6.3 Plastid transformation in rapeseed

Since no efficient regeneration procedure was established from leaf explants and stem segments (data not shown), the PEG-method for rapeseed protoplasts and the biolistic method for protoplast derived colonies were tested.

The PEG-method: Ten independent experiments were carried out. $5 \cdot 10^5$ protoplasts were treated in every experiment. About 30-60% of protoplasts survived after PEG treatment. First divisions were observed one day later than in the control (untreated) protoplasts. Nevertheless, microcolonies of at least 20 cells were formed on 7-9-th day of culture. Both antibiotics were added to liquid (PCBr) and afterwards to solid (SRBr) media at a concentration of 100 mg/l for selection of resistant colonies. Despite of rather high plating efficiencies only 6 pale green colonies were detected (Fig. 3.32). However, after their transfer to fresh selection

medium, colonies turned to the wild type.

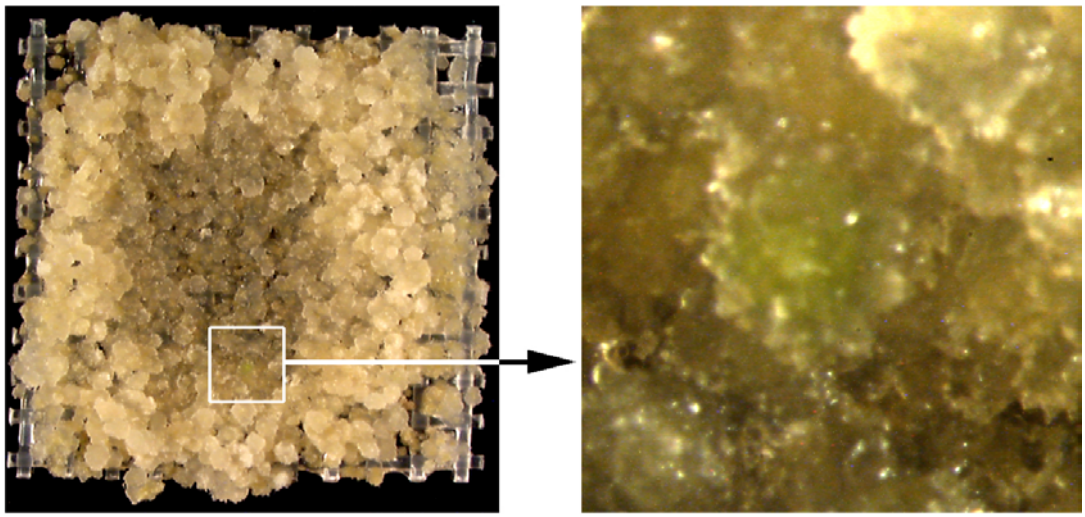


Fig. 3.32. Selection of protoplast derived colonies on SRBr medium supplemented with spectinomycin and streptomycin at a concentration of 100 mg/l each.

The biolistic method: Ten grids with protoplast derived colonies were bombarded. Unfortunately, after their transfer to SRBr medium supplemented with spectinomycin 100 mg/l no colonies with green pigmentation were detected.

The obtained data demonstrate that in rapeseed transformed cell lines can not be selected using spectinomycin and streptomycin.

3.6.4 Plastid transformation in sugarbeet by the biolistic method

Hypocotyl callus and hypocotyl explants were used for plastid transformation with the biolistic method. Unexpectedly, about 6% of bombarded hypocotyl explants formed friable callus during selection on MSB2 supplemented with 100 mg/l in the dark. However, after transfer of this callus to fresh MSB2 containing both inhibitors, spectinomycin and streptomycin, only two colonies survived. These cell lines are characterised by very slow growth and, therefore, a possibility that they are plastid transformants is low.

The data on callus bombardment are presented in Table 3.4.

Table 3.4. Sugarbeet plastid transformation: bombardment of hypocotyl callus

№ of exp.	Cultivar	Seeds, number	Seedlings, number	Explants, number	Callus formation, %	Bombarded, petri dishes	Resistant colonies, 22.03.01	Resistant colonies, 01.06.01
1	Viktoria	664	408	1180	19	6	1	0
2	7T1308	760	460	1400	36	8	3	2
3		685	394	1300	30	6	2	1
4		840	490	1450	32	5	5	2

Resistant colonies were selected on either MSB2 or MSB0.1 media supplemented with 100 mg/l streptomycin. Surviving calli appeared after 4-6 weeks of selection. Few colonies, which were growing during 2 subcultures on selection medium, suddenly lost the capacity to develop and finally died. However, at least two colonies without growth retardation and three other colonies with slower growth were selected. Moreover, green sectors and regenerable structures were formed and regenerates obtained. One of these colonies was tested for its capacity to grow in the presence of the second antibiotic, streptomycin (Fig. 3.33a).

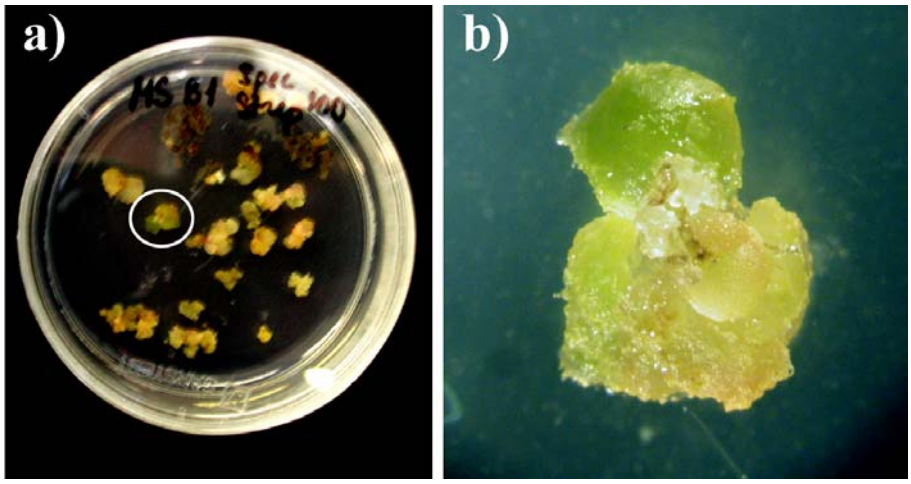


Fig. 3.33. Spectinomycin and streptomycin resistant cell line after the bombardment of sugarbeet callus with vector pSB-*aad*A (a). Selection was performed for four months on MSB1 medium supplemented with 100mg/l spectinomycin and 1 month with both inhibitors at the same, 100mg/l, concentration. Formation of green structure after four weeks selection with both antibiotics (b).

So far, no growth retardation is observed and new green structures are developing, thus confirming resistance to both inhibitors (Fig. 3.33b).

3.6.5 PCR analysis of resistant cell lines of sugarbeet

The presence of the *aad*A gene was detected by using total plant DNA and primers flanking a region within the gene. While no PCR products were obtained for wild type (WT) DNA, the fragment of the *aad*A gene of 528 bp was successfully amplified using the vector DNA as a control and total DNA from resistant callus lines (Fig. 3.34).

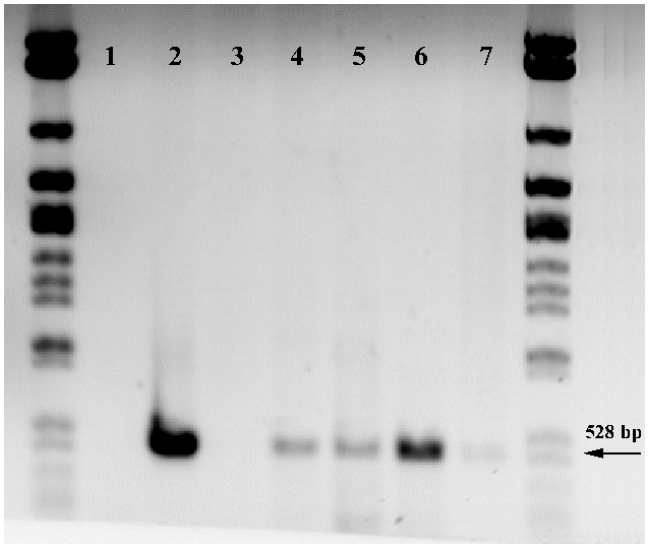


Fig. 3.34. PCR amplification of the *aadA* gene :1- master mix; 2- pSB-*aad*A; 3- WT; 4- line 1; 5- line 2, regenerate; 6- line 2, callus; 7- line 3.

Thus, resistance to antibiotics was due to expression of the *aad*A gene and not due to spontaneous mutations. First experiments to determine the correct integration of the marker gene into the plastome by PCR analysis did not result in amplification of fragments of the expected size. This result could be due to several reasons. For one, the construct may have inserted into the nucleus. Second, the integration could be in a location of the plastome, different from the one expected. Thirdly, there may be DNA regions identical to the targeted plastome sequence in the mitochondria genome. Although unlikely, the possibility cannot be excluded at this point, that the resistant gene was inserted into the chondriome. Further molecular analysis will distinguish between the three different possibilities. Nevertheless, all conditions are now established for the successful transformation of the plastome and subsequent regeneration of transformed sugarbeet plants.

4. DISCUSSION

4.1 A novel, highly efficient technique for protoplast culture

Plant regeneration from protoplasts of angiosperm plants was first reported for *Nicotiana tabacum* leaf protoplasts (Takebe et al. 1971). By now this method is applicable to more than 200 species. Protoplasts are generally cultured in liquid media (Takebe et al., 1971; Binding, 1974; Schieder, 1975; Partanen et al., 1980) or embedded in gels of proper osmotic pressure (Nagata and Takebe, 1971; Brodelius and Nilsson, 1980; Adaoha-Mbanaso and Roscoe, 1981; Shillito et al., 1983). Embedding of cells in alginate is one of the mildest cell immobilisation procedures (Brodelius and Nilsson, 1980) and has become popular in protoplast culture (Draget et al., 1988). Following protoplast development, osmotic pressure is gradually reduced, when microcolonies of 10-20 cells are formed (Evans and Bravo, 1983). Colonies are grown to calli, which afterwards can be triggered to form shoots and eventually roots. Any of these steps generally requires a different culture medium (Koop and Schweiger, 1985).

Alginate embedding. In previous reports on tobacco protoplast regeneration, a period of up to 5-6 weeks of culture was required before colonies reached a size of about 1 mm (Takebe and Nagata, 1984). The period for establishing rooted plants is given as three to four months (Gleba et al., 1984). Earlier, in our group, shoot regeneration was observed after a total culture period of four to five weeks (Koop and Schweiger, 1985). Using a novel culture procedure, which required embedding of protoplasts in thin alginate layers in combination with improved culture media, allowed us to obtain shoot formation from tobacco leaf protoplasts in less than two weeks (Dovzhenko et al., 1998a, b). In our laboratory we undertook experiments assessing the influence of alginate embedding using the “film layer technique” (Golds et al., 1992) and found that it improves cell viability and shortens the time period from protoplast to colony formation considerably. We further found that the

thickness of the gel layers plays an important role concerning the exchange of metabolites and influences the rate of cell division and development. Using "single-cell nurse culture", an alternative culture technique, a gel layer of only 0.5 mm between target and feeder cells was found to reduce the culture efficiency by 50% (Schäffler and Koop, 1990). The use of thin alginate layers is one of the key steps in the procedure. There are several advantages to this system. First, the polypropylene grids mechanically stabilise the gel layers and thus facilitate manipulations with the embedded protoplasts. Second, gel layers of uniform thickness are easily produced. Third, the grids are also convenient for defining the location of individual cells for tracking their development. Embedding of protoplasts in thin alginate layers resulted in high plating efficiencies and fast protoplast regeneration.

Improvement of the culture media. One of the prerequisites for successful protoplast isolation and culture are the growth conditions of the donor plants (Shepard and Totten, 1975; Kao and Michayluk, 1980; Masson and Paszkowski, 1992). The best results could be obtained, when the donor plants were grown from seedlings on B5 medium with an increased Mg^{2+} content, which reduces the appearance of yellowish or pale patches. Protoplast isolation from leaves of donor plants with the filtration-flotation-sedimentation procedure (Koop et al., 1996) resulted in a high yield of uniform and healthy protoplasts. The absence of NH_4NO_3 during preplasmolysis and in the culture medium seems to be significant, as ammonium from inorganic salts has been found to negatively influence protoplast survival (Upadhya, 1975; Zapata et al., 1981). Specially designed isolation and culture media allowed to reduce the time of culture in liquid medium of embedded protoplasts from 4-5 weeks to 1 week and to obtain very fast protoplast development with a high plating efficiency. Concerning the other mineral compounds in the culture media, we found it advantageous to increase the concentration of calcium, as it is important for membrane stability. Further, a beneficial effect has been observed when ammonium succinate was added.

Ammonium succinate has also been used successfully for the protoplast culture of barley (Tewes et al., 1991) and even for shoot culture of *Nicotiana plumbaginifolia* (Borisjuk et al., 1998).

The TAL-technique that was developed here for *Nicotiana tabacum* protoplasts has also proven successful for other species including *Brassica napus* (this study), the extremely recalcitrant *Beta vulgaris* (this study) and *Arabidopsis thaliana* (Luo, 1997), evening primrose (Kuchuk et al., 1998) and potato (in preparation). Therefore, the technique can be regarded as an important contribution to protoplast culture protocols in general. The combination of the novel culture technique with new culture media and optimised physical parameters resulted in extremely rapid shoot regeneration. It is a very simple and highly efficient method, and requires only two media for protoplast culture and shoot regeneration. Intermediate steps for adjustment of the osmotic pressure are no longer necessary. Factors, which are important for successful and fast protoplast culture in higher plants and which should be considered when designing a protocol, are presented in Fig. 4.1.

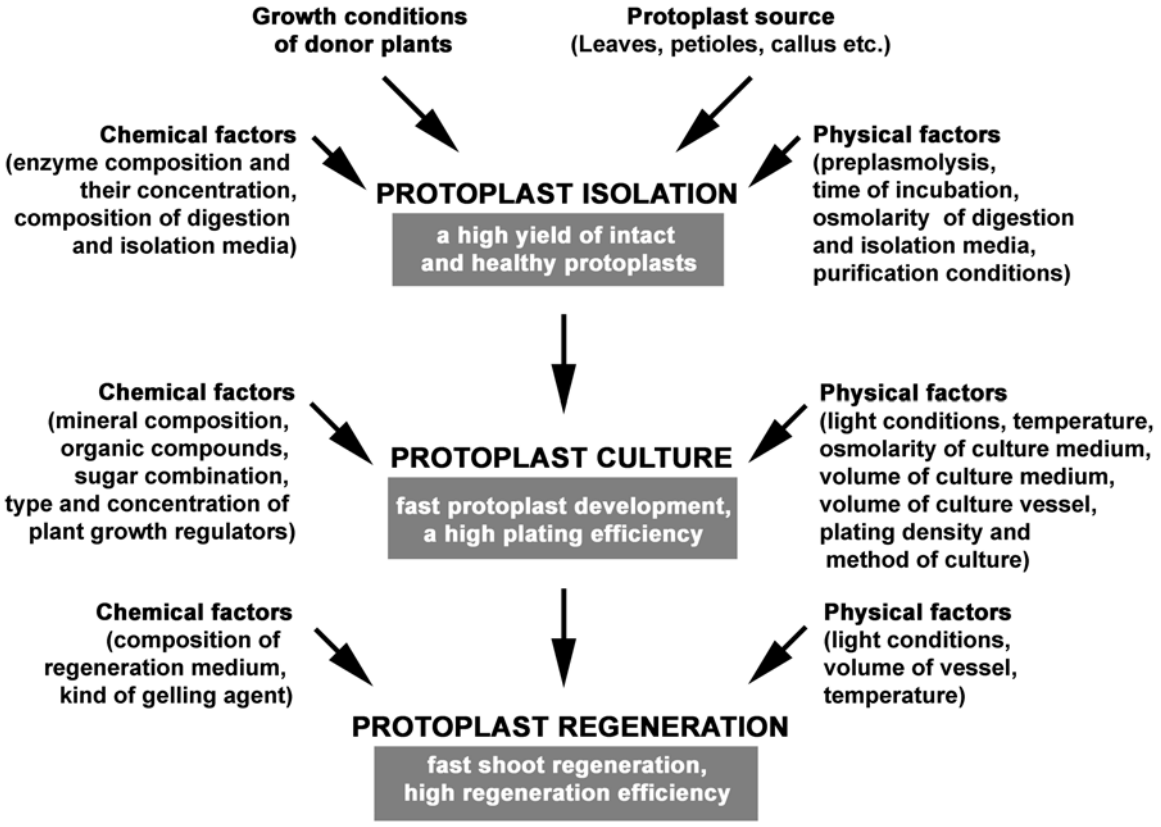


Fig. 4.1. Factors influencing protoplast culture and regeneration.

4.2 Protoplast culture in rapeseed

For successful PEG-mediated plastid transformation (Kofer et al., 1998) a reproducible and efficient protoplast system is required. So far, stable plastid transformation has been observed only when leaf protoplasts were used as a protoplast source (Golds et al., 1993, Koop et al., 1996). Thus, we established the protoplast culture of rapeseed from leaves and cotyledons, as cells from these plant organs contain a high number of chloroplasts. The novel protoplast technique, TAL-technique, was successfully used to improve the protoplast culture system in rapeseed.

4.2.1 Factors influencing plating efficiencies

Since the first successful shoot regeneration from rapeseed leaf protoplasts was reported (Kartha et al., 1974), many species of *Brassica* have been regenerated to whole plants (Schenk and Hoffman, 1979; Glimelius, 1984; Chatterjee et al., 1985, Gupta et al., 1990). Shoot or embryo formation was observed for protoplasts, which had been isolated from leaves (Kartha et al., 1974; Li and Kohlenbach, 1982; Pelletier et al., 1983; Glimelius, 1984), cotyledons (Lu et al., 1982), hypocotyls (Glimelius, 1984; Chuong et al., 1985, Barsby et al., 1986; Thomzik and Hain, 1988), and roots (Xu et al., 1982). However, despite intensive studies on rapeseed protoplasts, only the hypocotyl system proved rather efficient and only for a limited number of genotypes. Shoot regeneration from protoplast derived colonies still remains a problem, as regeneration media designed for some breeding lines are often not efficient for others (Thomzik and Hain, 1988). Here we describe the successful protoplast isolation and regeneration for two rapeseed cultivars, “Drakkar” and “Westar”.

Growth conditions of donor plants. The growth conditions of *Arabidopsis thaliana*, a species closely related to rapeseed, determine the response in protoplast culture (Masson and Paszkowski, 1992). Rapeseed plants were normally grown on

hormone-free MS (Glimelius et al., 1984) or half strength MS (Thomzik and Hain, 1988) media. However, applying the growth conditions described in the literature, in our experiments donor plants formed leaves containing yellow patches for both cultivars tested. A new medium, RS, was designed. The complete removal of ammonium nitrate and increased concentrations of Mg^{2+} and Na^{+} resulted in the development of dark green and healthy leaves. By using such leaves for digestion, high yields of protoplasts were obtained.

Culture conditions. In the literature there are several methods for the culture of rapeseed protoplasts. Protoplasts were cultured either in liquid culture media (Kartha et al., 1974; Li and Kohlenbach, 1982; Glimelius, 1984), also microcultures (Spangenberg et al., 1985), or they were embedded in agarose, either in agarose layers or droplets (Thomzik and Hain, 1990; Thomzik, 1993) or in "agarose islands" (Cheng et al., 1994). No information about the application of alginate embedding for rapeseed protoplasts was found. Culture medium PC (Glimelius et al., 1986) was often used resulting in high plating efficiencies. In this study plating efficiencies for leaf protoplast cultures in PC medium reached about 25%. Protoplast embedding in thin alginate layers in combination with an improved culture medium, PCBr, gave increased plating efficiencies of up to 50% for leaf protoplasts and up to 80% for cotyledon protoplasts. Moreover, the release of brown exudates by protoplast derived colonies, that causes a drastic decrease in protoplast divisions and development, which has been often reported in the literature (Schenck and Röbbelen, 1982; Glimelius, 1984; Thomzik and Hain, 1988), was almost eliminated. Although Glimelius (1984) suggested that rapid growth of hypocotyl protoplasts prevented formation of the brown precipitate, after embedding in thin alginate layers almost no brown precipitates were observed, even when protoplasts were cultured in PC medium (Glimelius et al., 1986) where cells divided and developed slower than in the new culture medium PCBr.

A new culture medium. PCBr medium in comparison with PC medium contains a

reduced concentration of mineral salts, a different phytohormone composition and 100 mg/l glutamine. A positive influence of glutamine on the development of suspension cultures of *Cardamine pratensis* and *Silene alba* was reported earlier (Bister-Miel et al., 1985) and was also observed in this study. In the literature all protoplast culture media for rapeseed were characterised by a high auxin/cytokinin ratio (Pelletier et al., 1983; Glimelius, 1984) and different auxins (2.4-D and NAA) were presented. BAP was used as source of cytokinin. Here, the highest plating efficiencies were obtained when protoplasts were cultured in PCBr medium containing the cytokinin kinetin at high concentrations (3 mg/l) and the auxin NAA (1 mg/l). First divisions were observed already within 24 hours after protoplast isolation for cotyledon protoplasts and already on the second day for leaf protoplasts. The protoplast derived microcolonies grew fast and could be transferred to solid medium already after 10-12 days of culture. This rate of growth is even faster than it could be reached for the efficient and fast hypocotyl protoplast system by Thomzik and Hain (1988), which required at least 14-18 days for the same state of colony development. Thus, for rapeseed the combination of a new culture medium with the TAL-method resulted in very fast protoplast development at high plating efficiencies of 50-80%.

4.2.2 Shoot regeneration from protoplast derived colonies

In addition to high plating efficiencies reproducible and efficient shoot regeneration system from protoplast derived colonies is also required for successful PEG-mediated plastid transformation. Plant regeneration from rapeseed protoplasts was reported for a number of rapeseed breeding lines (Kartha et al., 1974, Li and Kohlenbach, 1982; Pelletier et al., 1983; Glimelius, 1984; Thomzik and Hain, 1988; Cheng et al., 1994) and for other species of *Brassica* (Xu et al., 1982; Chatterjee et al., 1985; Gupta et al., 1990). However, despite of successful shoot formation from protoplasts of different origin the regeneration capacity was limited to specific genotypes. Regeneration media, which were designed for some breeding lines, proved less or not efficient for others (Thomzik and Hain, 1988).

When different regeneration media (Kartha et al., 1974; Pelletier et al., 1983; Glimelius, 1984; Thomzik and Hain, 1988) were tested on genotypes "Drakkar" and "Westar", shoot regeneration was infrequent, irregular and occurred at a very low efficiency (<1%). Therefore, the improvement of the regeneration conditions, on which protoplast derived colonies could form shoots with better efficiency was necessary. For this purpose 144 different phytohormone (NAA, BAP, kinetin and GA_3) compositions have been tested.

Gibberellins. While a stimulatory effect of GA_3 on shoot formation was reported by Kartha et al. (1974), no effect of GA_3 on the regeneration frequency was observed in our experiments.

Auxins. In contrast to Kartha et al. (1974) and Pelletier et al. (1983) who described shoot regeneration on media lacking auxins, in this study the presence of the auxin (NAA) was absolutely required to induce regeneration. No shoot formation was observed on media lacking the auxin.

Cytokinins. Some cytokinins demonstrated a stimulatory effect on shoot regeneration, while others were not efficient and even inhibited shoot regeneration from the same source (Tetu et al., 1987; Tegeder et al., 1995). Concerning the necessity of cytokinins, plant regeneration was observed on media containing either BAP or kinetin. Nevertheless the regeneration response was significantly better when BAP was used. If either BAP or kinetin were combined with auxin at low concentrations (up to 1 mg/l), shoots were formed mostly on media containing 1-2 mg/l kinetin. At higher auxin concentrations (2-3 mg/l) a better regeneration capacity was observed for colonies cultured on media containing BAP (2 mg/l).

Gas volume. The volume of the culture vessel played an important role in the formation of shoots by protoplast derived colonies. When callus was cultured on SRBr medium in 9 cm petri dishes, the regeneration efficiencies were 10-15%. However, 1-2 colonies from every 3 clones, which were transferred to a well in 6-

well dishes, regenerated shoots on the same medium. These data show that the regeneration efficiency can be improved further.

Thus, a reproducible and efficient isolation, culture and regeneration system for rapeseed protoplasts could be established. Now the system is efficient enough for the application of plastid transformation.

4.3 A recalcitrant species sugarbeet (*Beta vulgaris L.*)

Until recently sugarbeet represented a recalcitrant species with respect to techniques based on protoplast culture (Hall et al., 1996b; Hall et al., 1997) and on gene transfer (Snyder et al., 1999). A detailed study on sugarbeet was performed in order to overcome the problems. Different strategies were tested and efficient protocols for sugarbeet regeneration from protoplasts and for gene transfer were developed.

4.3.1 Direct shoot regeneration

Direct shoot organogenesis in sugarbeet was observed from various explants, including cotyledons (Krens et al., 1996; Joersbo et al., 1999; Snyder et al., 1999), hypocotyls (Krens and Jamar, 1989), petioles (Saunders and Doley, 1986; Freytag et al., 1988; Krens and Jamar, 1989), leaf cuttings (Miedema, 1982), shoot bases (Lindsey and Gallois, 1990) and epicotyl-originated thin layer explants (Toldi et al., 1996). There are several advantages of direct shoot regeneration in sugarbeet. For one, direct organogenesis is less genotype dependent (Detrez et al., 1989; Jacq et al., 1992; Toldi et al., 1996) and regenerates show more genetic stability (Detrez et al., 1989). In this study explants of different origin were compared concerning their regeneration capacity such as leaves, petioles, cotyledons, hypocotyls, base tissue and roots. The direct shoot regeneration from roots could be observed for the first time.

The best shoot organogenesis was observed from petiole explants in both breeding

lines tested (42% for “Viktoria” and 32% for 7T1308). Similar results were obtained by other research groups (Freytag et al., 1988; Krens et al., 1989). Concerning the phytohormones, the use of only BAP at concentrations from 1 to 2 mg/l was sufficient for shoot induction. No auxins were tested since an inhibitory effect on shoot regeneration was reported earlier (Krens et al., 1996). In contrast to tobacco explants, where regenerates are formed from any part of the explant, sugarbeet explants form shoots only in local areas. Thereby, direct shoot organogenesis has limitations to be used for DNA transfer due to local regeneration capacities regeneration efficiencies combined with low regeneration efficiency in general. Possible applications for this method could be for maintenance of important germplasms (McGrath et al., 1999), vegetative micropropagation (Freytag et al., 1988; Krens and Jamar, 1989) or an improvement of sugarbeet as a crop plant by screening for somaclonal variants (Wright and Penner, 1998).

4.3.2 Regenerable callus

Shoot organogenesis from callus, so called indirect regeneration, is an intensively studied area in plant cell biology of sugarbeet. Infrequent and genotype dependent shoot regeneration from callus was observed in earlier studies (Hooker and Nabor, 1977; De Greef and Jacobs, 1979; Van Geyt and Jacobs, 1985; Saunders and Doley, 1986; Krens and Jamar, 1989). The establishment of several efficient and reproducible systems was described by Ben-Tahar et al. (1991), Jacq et al. (1992), and Snyder et al. (1999). In their reports leaf (Ben-Tahar et al., 1991) or seedling explants (Catlin, 1990; Jacq et al., 1992; Snyder et al., 1999) were used to obtain regenerable callus at high efficiencies. So far, friable callus seems to be the only type of callus that leads to regeneration (Krens et al., 1990; Catlin, 1990). In this study we searched for an optimal explant type and thus tested different explants for their capacity to form regenerable callus.

The most important factor to obtain regenerable callus appeared to be the origin of

the explant (Krens et al., 1989, 1996; Jacq et al., 1992). The induction of regenerable callus from petioles, leaves and shoot bases was established, but a great variation in the regeneration efficiency (from 5 till 30%) made the use of callus from those sources for our aims unsuitable. The great variability in the regeneration efficiency might be due to differences either in the sensitivity to phytohormones between the organs (Krens and Jamar, 1989), or due to endogenous levels of hormone activity (Tetu et al., 1987). Genetic divergence leading to the difference in response within a breeding line can also not be excluded.

Friable callus could be obtained from root explants, however the efficiency was very low (<0.001%). Interestingly, this callus was morphologically identical to regenerable callus from other sources. Since totipotency of guard cells in sugarbeet was shown (Hall et al., 1996a), it was suggested that friable callus originated exclusively from stomatal guard cells. However, roots do not contain stomata and thus cells of other origin must be able to form friable callus. The root callus lines did regenerate shoots, however only after transfer to SRB medium, which was designed for plant regeneration from protoplast derived colonies and contained the antiauxin TIBA besides the phytohormones auxin and cytokinin. The beneficial effect of TIBA in sugarbeet was observed earlier by others (Hooker and Nabors, 1977; Tetu et al., 1987; Detrez et al., 1989; Roussy et al., 1996; Toldi et al., 1996). Tetu et al. (1987) suggested that shoot formation was not controlled by the auxin/cytokinin balance in sugarbeet, and that TIBA was required to decrease the level of the endogenous auxin. Nevertheless, the low efficiency of callus formation and/or the instability of shoot organogenesis are the main shortcuttings for this type of callus.

In searching for an efficient and reproducible system the patented method described by Ben-Tahar et al. (1991) was tested. In this method embryogenic callus from leaf explants was used for *Agrobacterium*-mediated transformation. Leaf explants from 47 breeding lines were tested. In this study several factors were

investigated, since some details of the method were unclear. Neither abaxial vs. adaxial side, on which explants were in contact with the culture medium, nor the presence or absence of the midrib in the explants had a significant effect on the efficiency of the formation of friable callus. Although the majority of breeding lines (37 out of 47) was able to form friable callus, that was morphologically identical to regenerable callus, the process was clearly genotype dependent. Eight cultivars, including the control genotype used by Ben-Tahar et al. (1991), could be induced to regenerate shoots without any additional manipulation of the callus. However, five breeding lines selected randomly did not produce any shoots following the standard protocol. Thus, the patented method is genotype dependent and is suitable only for certain breeding lines.

Seedling explants were used for the induction of regenerable callus (Catlin et al., 1990; D'Halluin et al., 1992; Jacq et al., 1992; Snyder et al., 1999). Thereby, our efforts were concentrated on the development of a reproducible and efficient method for callus formation and regeneration from this type of explant. Regenerable callus was successfully induced on cotyledon and hypocotyl explants. Hypocotyl explants showed higher efficiencies in callus formation and shoot regeneration. While different media for callus induction and shoot regeneration were used in other research groups (Jacq et al., 1992; Snyder et al., 1999), our method required only one medium, MS15B2, for all stages, starting with shoot germination and ending with seed regeneration from callus. In addition, we found it sufficient to use only one growth regulator, the cytokinin BAP (2mg/l). In contrast, Krens et al. (1989) observed severe inhibition of callus formation at BAP concentrations of 2 mg/l. We also tested an increasing temperature, since a positive influence of higher temperatures on callus induction was reported (Jacq et al., 1992). However, no beneficial effect of a raise in temperature was observed in our experiments (data not shown). In contrast, it was a big breakthrough for us that reducing the sucrose concentration by 50% increased the efficiency of callus formation by a factor of two. Three-weeks old seedlings were found to be

the preferable source for callus induction by Jacq et al. (1992), but in our system the best results were obtained using 1-month old material.

The method we developed is efficient, reproducible and simple, as it requires only one medium and results in a high regeneration efficiency of induced callus (40-50% for "Viktoria" and over 90% for 7T1308). Regeneration occurred in the dark or in the light, either by shoot formation, or by embryo formation. The callus was used in experiments on DNA transfer by the biolistic method and as an alternative source for protoplast isolation and regeneration, since so far protoplast regeneration had been only observed from leaf protoplasts (Krens et al., 1990; Hall et al., 1993; Lenzner et al., 1995).

4.3.3 Protoplasts from sugarbeet leaves

Despite of a great number of investigations on sugarbeet protoplast culture, only a few research groups succeeded in shoot regeneration from protoplasts (Steen et al., 1986; Krens et al., 1990; Weyens and Lathouwer, personal communication in Lenzner et al., 1995; Lenzner et al., 1995). Protoplast derived colonies have been successfully obtained from callus (Szabados and Gaggero, 1985; Bhat et al., 1985; Lindsey and Jones, 1989; Bannikova et al., 1994), petioles (Pedersen et al., 1993; Schlangstedt et al., 1994) and leaves (Krens et al., 1990; Schlangstedt et al., 1992; Hall et al., 1993; Lenzner et al., 1995). However, it was possible to obtain regenerates only from leaf protoplasts and, particularly, only from stomatal guard cell protoplasts (Hall et al., 1996b, 1997). Hall et al. (1996b, 1997) reported the development of an efficient regeneration system from guard cell protoplasts. Guard cells are highly differentiated cells and are unique considering their morphology and physiology. They are a relatively uniform population of leaf cells (Sack, 1987; Hall et al 1996a), lack plasmodesmata and are accustomed to regular fluctuations in osmotic potential (Willmer, 1993; Hedrich et al., 1994). The successful shoot regeneration from guard cell protoplasts of *Nicotiana glauca* has been reported Recently (Sahgal et al., 1994). An enrichment of protoplast preparations for guard

cell protoplasts could be achieved by increasing the amount of epidermal fragments (Hall et al., 1996a, 1997). In the present study attempts to use guard cells or leaf protoplasts were of limited success. Although protoplast derived colonies could be obtained in our experiments, the plating efficiency remained low (less than 1%), while Hall et al. (1997) reported division frequencies of >50%. In contrast to the protoplast system developed by Hall et al. (1996b, 1997), in our investigations shoot regeneration could not be observed at all. Modifications of the method described by Hall et al. (1997) explain the discrepancy in the results. First, leaves from sugarbeet plants maintained as shoot culture were used. Difficulties concerning the regeneration of shoots from protoplasts isolated from leaves of long-period shoot culture were observed earlier (Lenzner et al., 1995). Another difference was that we used the TAL-system. However, this could not have been the reason for discrepancies in the results as protoplast embedding in thin alginate layers gives faster protoplast divisions. First divisions were already observed after 4-5 days of culture, while first divisions in guard cell protoplasts generally occurred after 7-8 days (Hall et al., 1995). Also, in the literature the beneficial effect for protoplast embedding in alginate was demonstrated (Schlangschtedt et al., 1992; Hall et al., 1993). Modifications of isolation and culture media did not allow to obtain better results and plating efficiencies could not be improved.

An alternative method allowing to enrich the protoplast fraction to up to 90% of guard cell protoplasts was developed. Still, even for cultures, enriched for stomatal guard cell protoplasts, plating efficiencies were only about 1%. Protoplast embedding directly after digestion without a standard purification procedure was successfully demonstrated on tobacco leaf protoplast (Golds et al., 1994) and tested in our experiments. This procedure did also not result in an increase of the plating efficiency. Szabados and Gaggero (1985) reported a positive effect of casein hydrolysate on callus protoplasts in sugarbeet. However, in this study protoplast derived colonies were never formed during the culture in medium supplemented with casein hydrolysate.

Following the culture protocol for guard cell protoplasts (Hall et al., 1997) the antioxidant nPG, a lipoxygenase inhibitor, was added to all isolation and culture media. We also observed that protoplasts divided and formed colonies only in the presence of nPG and only in the dark. Both factors are critical and significant for successful protoplast culture (Krens et al., 1990; Lenzner et al., 1995). An interesting fact remains that only friable colonies were obtained in our experiments. Colony formation of the compact type, which had been reported in the literature (Krens et al., 1990; Hall et al., 1993; Lenzner et al., 1995), was never observed. Even if the number of guard cell protoplasts was low (<5%) only friable colonies were formed. So far, there is no explanation for this phenomenon.

Thus, our attempts to establish the protoplast system based on stomatal guard cell protoplasts failed. Problems to reproduce the guard cell system were also reported by Snyder et al. (1999). Searching for an alternative system, protoplasts were isolated from friable, regenerable, hypocotyl-derived callus.

4.3.4 Shoot regeneration from callus protoplasts

Significant progress concerning regeneration was achieved, when protoplasts were isolated from callus induced from etiolated hypocotyl explants. We established a simple and efficient method, which represents an alternative to the system based on the use of guard cell protoplasts (Hall et al., 1996b, 1997). While shoot regeneration from callus (suspension) protoplasts was often reported for other species (Binding and Nehls, 1980; Vasil et al., 1983; Ratushnyak et al., 1990), so far no regeneration of whole plants from callus protoplasts was achieved in sugarbeet (Szabados and Gaggero, 1985; Bhat el al., 1985; Lindsey and Jones, 1989; Bannikova et al., 1994). In these experiments protoplasts were cultured either in liquid medium (Szabados and Gaggero, 1985; Bhat et al., 1985; Bannikova et al., 1994) or embedded in agarose gels (Lindsey and Jones, 1989). One sugarbeet breeding line was tested in each investigation. Plating efficiencies from 8% (Bhat et al., 1985) to 35-38% (Szabados and Gaggero, 1985, Lindsey and

Jones, 1989) were obtained, which was similar to our results where the plating efficiency had been 30-35%, and in some experiments the division frequency was up to 64% (Lindsey and Jones, 1989). First divisions were observed after 1-3 days of protoplast culture.

As noted earlier, non of the groups mentioned observed shoot regeneration. Explanations could be the difference in starting material, as several groups used suspensions from hypocotyl-derived callus (Szabados and Gaggero, 1985; Bhat et al., 1985; Lindsey and Jones, 1989), while others used regenerable petiole callus (Bannikova et al., 1994). We have isolated protoplast from hypocotyl callus directly. In the literature there was no information about the age of cultures, but it seems that suspensions were at least 2 months old. In our investigations, we have found that the age of a callus played a significant role for successful protoplast regeneration. It was necessary to use hypocotyl callus no older than 2 months after induction. The regeneration efficiency decreased drastically (<1%) when protoplasts were isolated from callus of 3 months or older. One reason could be that a prolongated period for callus culture increases the number of chromosomal aberrations in sugarbeet as found by Jacq et al. (1992). The best results (10% the regeneration efficiency for breeding line “Viktoria” and 30% - for breeding line 7T1308) were obtained, when protoplasts were isolated from fresh callus material.

Another reason for the problems encountered by other groups, could be differences in culture conditions. Even when the light conditions for those were the same (i.e. protoplasts were kept in the dark), there was an important difference in the isolation and culture media, as we used the antioxidant nPG in either media. For leaf protoplasts it had been observed by several groups (Krens et al., 1990; Lenzner et al., 1995) that the addition of nPG to all media prior to colony formation was required for the successful shoot regeneration from protoplast derived colonies. Another significant difference is the application of the TAL-technique to the protoplast culture, as it promotes fast colony development.

Influence of phytohormones. 70 different variations of phytohormones were tested, since no regeneration was observed on MS medium supplemented with growth regulators according to Lenzner et al. (1995) and Hall et al. (1997). No positive effect of thidiazuron on the regeneration efficiency from protoplast derived colonies was observed. The best regeneration frequencies (10% for genotype "Viktoria" and up to 30% for genotype 7T1308) were obtained if all phytohormones were at high concentrations: 2 mg/l of BAP, 1 mg/L of NAA and 1 mg/l of TIBA. Shoot regenerates were successfully obtained for both breeding lines tested. Frequently vitrification of the regenerates was observed, which was stimulated by BAP and TIBA. Thus, both cytokinin and antiauxin could be responsible for vitrification as it was observed in other regeneration systems (Tetu et al., 1987; Toldi et al., 1996). The problem was overcome if buds from regenerates were transferred to fresh MS medium with an increased concentration of agar-agar (1.4%). In contrast to literature data (Krens et al., 1990; Hall et al., 1993; Lenzner et al., 1995) rooting of the regenerates on medium containing auxin failed, but it was successful on hormone-free MS medium. Regenerates were sent to Planta GmbH (Einbeck) in order to test for fertility of the regenerates and for seed set.

Our novel and efficient method for protoplast regeneration in sugarbeet is an alternative to the existing guard cell system by Hall et al. (1996b). The procedure of protoplast isolation and culture does not require special equipment, like a blender. It is simple by performance and the risk of contamination is low, in contrast to the method using guard cells (Hall et al., 1997). Our method is the first one that allows shoot regeneration from callus protoplasts with an efficient regeneration system, a prerequisite for the successful somatic hybridisation or genetic transformation experiments.

4.3.5 Nuclear transformation

For many years sugarbeet was recalcitrant for biotechnological manipulations. The main reason was the lack of reproducible and efficient gene transfer methods (Hall et al., 1996b; Snyder et al., 1999). Cells, with a good regeneration capacity, usually could be transformed only with low efficiency (Krens et al., 1989; D'Halluin et al., 1990) due to their location deep within a tissue. Transformation was often non-reproducible and genotype dependent (Lindsey and Gallois, 1990; D'Halluin et al., 1992; Krens et al., 1996). Cells that were easily transformable, normally did not show regeneration capacity (D'Halluin et al., 1992). To overcome the problem an increase in number for regenerable cells is required, since the attempts to regenerate the shoots from non-regenerable callus failed (D'Halluin et al., 1992; Krens et al., 1996).

For sugarbeet *Agrobacterium*-mediated transformation was often the method of choice for nuclear transformation (Lindsey and Gallouis, 1990; Ben-Tahar et al., 1991; D'Halluin et al., 1992; Krens et al., 1996). We concentrated our efforts to establish methods, which are also applicable to the transformation of plastids, i.e. the biolistic method as well as the PEG method. Using both transformation methods, we obtained transformed colonies that expressed bialaphos resistance and β-glucuronidase.

The PEG-method. Transformation efficiency for callus protoplasts was similar to the transformation efficiency of guard cell protoplasts and was from $5 \cdot 10^{-5}$ to $4 \cdot 10^{-4}$ (Hall et al., 1996b). Decreasing the number of treated protoplasts resulted in lower transformation efficiency. In contrast to data reported by Hall et al. (1997), where only <50% of the selected transformed calli stained blue with the histochemical GUS test, in our experiments all colonies that were resistant to bialaphos demonstrated the presence of *β*-glucuronidase. Unfortunately, no regenerates from resistant colonies could be obtained. We observed the formation of green smooth structures that could be regenerated into plantlets, however all attempts to induce

the regeneration of shoots failed. In the experiments on protoplasts isolated from leaves from shoots of established long-period *in vitro* culture difficulties to regenerate shoots from similar structures were observed as well (Lenzner et al. 1995). An inhibitory effect of the bialaphos selection could be an explanation for this and thus prolonged culture periods in the callus stage, which may lead to genetic abnormalities. The PEG method may be still applicable for nuclear or plastid transformation in sugarbeet, however the transformation efficiencies have to be improved and the difficulties in shoot regeneration need to be overcome.

The biolistic method. We developed a system for the successful transformation and subsequent shoot regeneration from hypocotyl derived callus. Bombardment of hypocotyl callus resulted in a high transformation efficiency, from 9 to 18% (number of resistant colonies per number of explants from which the callus was taken). Similar results were recently reported by Snyder et al. (1999), where the successful transformation of sugarbeet was achieved by the biolistic procedure also. The transformation efficiency was estimated by the number of transgenic plants obtained per plate of embryogenic callus treated. While 3-5g (fresh weight) of callus was plated in their experiments, in our experiments we did not use more than 1 g (fresh weight) of callus per petri dish. The regeneration efficiency for transformed callus was lower than for control callus (20-25% as opposed to 40-50%), nevertheless it was high enough to obtain shoots. All selected lines, that had been resistant to bialaphos, stained blue with the histochemical GUS test, but with different intensities. Such differences could be explained either by the position effect of DNA integration, or by the number of gene copies per nucleus. PCR analysis was used to confirm the presence of the transgene. The presence the *pat* and GUS genes, could also be confirmed by Southern blot analysis.

An efficient method for the genetic transformation and regeneration of sugarbeet was developed. High regeneration and transformation efficiencies for hypocotyl callus could be achieved and, thus, the system can be used to aim for plastid

transformation in sugarbeet.

4.4 Plastid transformation in rapeseed and sugarbeet

Both the biolistic and the PEG method are powerful tools for plastid transformation (Kofer et al., 1998). So far only few species from higher plants are reported for stable plastid transformation: tobacco (Svab et al., 1990), *Nicotiana plumbaginifolia* (O'Neil et al., 1993), *Arabidopsis thaliana* (Sikdar et al., 1998), rapeseed (Chaudhuri et al., 1998), potato (Sidorov et al., 1999) and rice (Khan and Maliga, 1999). Our laboratory was the first one in which the PEG method for stable plastid transformation was established (Golds et al., 1993). For the successful plastid transformation by the PEG method several prerequisites are required. First, an efficient protoplast culture system needs to be established and the species must be regenerable from protoplasts. Likewise, in the case of the biolistic method, target tissues/organs must be regenerable to plants at high efficiencies as well. Second, a vector for plastid transformation, containing homologous flanks that are routinely about 1 kb in size, a selectable marker and regulatory elements, must be available (Svab and Maliga, 1993; Zoubenko et al., 1994; Koop et al., 1996; Eibl et al., 1999). If, for example, a tobacco vector should be used for plastid transformation in another species, a very high homology to the corresponding sequences of the ptDNA of the plant of interest is required (Sidorov et al., 1999). Third, a good selectable marker is necessary (Kofer et al., 1998). As mentioned above, systems, which could be suitable for both methods, the PEG method and the particle gun method, were established. The PEG method could be used for rapeseed protoplasts, and the biolistic method – for protoplast derived colonies in rapeseed and for regenerable callus in sugarbeet.

Species specific vectors: In our laboratory we have commonly used the region between *rpl32* and *trnL* (nt: 111515-116171) for tobacco plastid transformation (Koop et al., 1996). This region appeared to be not highly homologous to the plastome chromosomes of other species (Appendix 2). After looking for an

alternative integration site, the region between *trn*V and *rps*7 (nt: 140126-142640 of the tobacco plastome, Shinozaki et al., 1986) was chosen. This region was used for the construction of species specific vectors, since the *trn*V-*rps*7 fragment had already been successfully used in constructs for plastid transformation in tobacco (Zoubenko et al., 1994; McBride et al., 1994, 1995), rapeseed (Chaudhuri et al., 1998), *Arabidopsis thaliana* (Sikdar et al., 1998), potato (Sidorov et al., 1999) and rice (Khan and Maliga, 1999). Sugarbeet and canola fragments were homologous to each other and to the tobacco region (Shinozaki et al., 1986). After sequence comparison of tobacco and rapeseed or sugarbeet fragments, it was found that non-homologous sequences were generally either in the area of open reading frames ORF131 and ORF70B or in non-coding regions (Appendix 4). We made a comparison of tobacco ORF131 and ORF70B with those from the same intergenic region of other species, for which the whole plastome sequence is presented (Table 4.1). Results of this comparison demonstrate that ORFs are reduced in size or/and fragmented in comparison with tobacco ORFs. This might reflect a functional relevance, e.g. in regulatory areas such as promoter or terminator sequences (Schmitz-Linneweber et al., 2001). Thus, species specific vectors were constructed. Both rapeseed and sugarbeet fragments were successfully cloned and the *aad*A-cassette was inserted.

Table 4.1. Comparison of non-conserved "open reading frames" encoded by arabidopsis, tobacco, evening primrose, rice, spinach and maize plastomes (*trn*V – 3´*rps*12 intergenic region)

Arabidopsis thaliana	*Nicotiana tabacum*	*Oenothera Elata*	*Oryza sativa*	*Spinacia oleracea*	*Zea mays*
—	ORF70B	ORF48	ORF72	ORF47	—
ORF36 ORF42 ORF49	ORF131	ORF25 ORF26b	ORF58 ORF85	ORF54a ORF54b	ORF58 ORF85

Selection: The *aad*A-gene (aminoglycoside-3´-adenyltransferase) confers resistance to spectinomycin and streptomycin (Svab and Maliga, 1993). Despite of the *aad*A-gene being a good plastome selectable marker for tobacco, it cannot be used for the selection for a number of species: barley and *Arabidopsis thaliana*

were immune to the antibiotic at high concentrations (Kofer et al., 1998). While clear sensitivity to spectinomycin was observed in sugarbeet, rapeseed protoplast derived colonies continued to grow vigorously, even if spectinomycin and streptomycin were both present at a high concentration (500 mg/l). Thus, it is not possible to use the *aad*A-gene as selectable marker for rapeseed.

Rapeseed plastid transformation: Although there is a patent application for plastid transformation in rapeseed by the biolistic method (Chaudhuri et al., 1998), the efficiency of plastid transformation was not confirmed. Leaf explants of rapeseed were used as a target tissue. All our efforts to obtain plastid transformants by the PEG-method with leaf or cotyledon protoplasts or the biolistic method for protoplast derived colonies failed. Although some greenish colonies were selected, they all turned white after transfer to fresh selection medium. The differences between our conditions and those of the patent application were the transformation method, the target material, as well as higher concentrations of antibiotics (50 or 100 mg/l for both markers or for spectinomycin only). Chaudhuri et al. (1998) selected their transformants on medium containing only 20 mg/l of spectinomycin. Possibly, even a concentration of 50 mg/l was already significant to induce plastid ribosome deficiency (PRD) (Zubko and Day, 1998). Spectinomycin blocks plastid ribosomes and even in the case of a successful plastid transformation the number of transformed organelles might be too low to be identified by visual inspection of these cells. Moreover, transformed cells may have no clear advantage over wild type cells using spectinomycin selection, as non-transformed colonies also were able to grow on antibiotic containing medium. A possible solution could be a combination of the selection pressure with culture conditions at which cells/tissues should be dependent on autotrophy. Alternatively, other selectable markers are required. Except of the use of the *npt*II gene for kanamycin resistance (Carrer et al., 1993), the *aad*A gene is the only marker for plastid transformation so far reported (Kofer et al., 1998). Recently the betaine aldehyde dehydrogenase (BADH) gene from spinach has been used as a selectable marker (Daniell et al.,

2001), but for the successful application of the BADH-system transformed plants must be lacking endogenous BADH-enzyme activity.

Plastid transformation in sugarbeet: So far only plastids of mesophyll cells (Svab et al., 1990; Golds et al., 1993; Sikdar et al., 1998; Sidorov et al., 1999) or embryogenic cells (Khan and Maliga, 1999) were successfully transformed. Daniell et al. (1990) reported transient transgene expression in etioplasts isolated from cucumber cotyledons. In our experiments we applied the biolistic method to either etiolated hypocotyl explants or etiolated hypocotyl-derived callus. Cells from target material of sugarbeet contained either amyloplasts or etioplasts. The efficiency of resistant colony formation from bombarded tissues was rather low. In other species plastid transformation appeared to be less efficient in comparison with the tobacco system where the transformation frequency was at least 1 event per bombarded leaf (Svab and Maliga, 1993): 2 transformants for 201 bombarded leaf samples in arabidopsis (Sikdar et al., 1998) and 1 transformant per 15-30 bombarded leaf samples in potato (Sidorov et al., 1999). At least 3 colonies, that were resistant to spectinomycin, could be obtained after bombardment of 25 sugarbeet callus samples (see Table 3.4). They showed no growth retardation on selection medium supplemented with 100 mg/l spectinomycin, and some green sectors appeared. Potentially, they might be either mutants or nuclear transformants (Svab and Maliga, 1993; Kofer et al., 1998). There are specific point mutations in the 16S-rRNA gene that confer resistance to spectinomycin or streptomycin (Harris et al., 1989). Mutation in about three different sites can cause resistance to spectinomycin and about six sites result in spontaneous resistance to streptomycin. Nevertheless, after one colony was transferred to selection medium containing both spectinomycin and streptomycin at a concentration of 100 mg/l, there was no retardation of growth. Selection with both markers may be advantageous as chances of simultaneous point mutations at two different sites are very low (Svab et al., 1990). Preliminary molecular investigations show that all transformed lines contain the *aadA*-gene, thus resistance due to spontaneous DNA

mutations can be ruled out. Further molecular analysis will clarify whether it was in fact the plastome that was targeted by the transformation. If so, we are the first group to report successful plastid transformation in sugarbeet. In any case, all the preconditions have been established for genetic modifications.

4.5 Conclusions and perspectives

In this work a highly efficient protoplast regeneration system was established in *Nicotiana tabacum* using a novel protoplast culture technique, the TAL technique. The TAL technique can be regarded as an important contribution to the protoplast culture procedure in general, since the successful application of this method is efficient in other species, such as oilseed rape and also the extremely recalcitrant species sugarbeet. High plating efficiencies and reproducible protoplast regeneration were achieved for both species. Protoplast regeneration from callus protoplasts in sugarbeet was reported for the first time. Thus, both protoplast systems could be used for fundamental research, for example, studies on differentiation processes, such as the cell cycle and gene regulation. A good regeneration system is a prerequisite for manipulations in plant biotechnology, as for somatic hybridisation and nuclear transformation experiments. The main achievement is that our protocols make plastid transformation in both species investigated feasible. While new markers and systems are required for the selection of transplastomic clones derived from rapeseed protoplasts, the suitability of the PEG method for plastid transformation in other species was shown. For sugarbeet the biolistic method seems to be the most promising for successful plastid transformation. Our findings will facilitate the development of plastid transformation systems for other species in which there are problems to regenerate shoots from tissue explants. Fundamental research on plastid physiology and gene function of the plastome as well as further crop improvement are feasible using the newly established systems for plant regeneration from callus and protoplasts in both species tested.

5. SUMMARY

In the current study tissue cultures of rapeseed (cv. “Drakkar”, cv. “Westar”) and sugarbeet (cv. ”Viktoria”, cv. “VRB”, cv. ”31-188”, cv. ”7T1308” and 47 other breeding lines, Appendix 1) have been investigated for the establishment of conditions that make possible plastid transformation in both species. Tobacco leaf protoplasts (cv. ”petite Havana”, cv. ”Wisconsin 38”) were used to develop a novel technique – the TAL (thin-alginate-layers) technique. The TAL technique in combination with new culture media resulted in very rapid protoplast development and fast shoot regeneration (in less than two weeks). This method was also successfully applied to improve protoplast culture of rapeseed and of the extremely recalcitrant species sugarbeet. Factors, which included protoplast source, mineral and organic composition of isolation and culture media, influence of growth regulators etc. were investigated and conditions for protoplast culture and regeneration were established for both species.

According to reports in the literature, only protoplasts from guard cells could be regenerated into plants. Thus, an alternative and reproducible method of shoot regeneration from protoplasts isolated from hypocotyl derived callus was successfully developed. While no shoot regeneration was observed from guard cell protoplasts in our experiments, plant regeneration (efficiencies up to 30%) from callus protoplasts could be achieved for the first time in this study.

The influence of different parameters on the efficiency of callus formation from etiolated hypocotyl explants was investigated. Protoplasts from callus and hypocotyl derived callus were used for the experiments on nuclear transformation in sugarbeet. Both, the PEG method and the biolistic method were successfully applied to obtain nuclear transformants as confirmed by molecular methods (PCR analysis and Southern blot hybridisation). The biolistic method was applied for plastid transformation experiments in sugarbeet.

Species specific vectors containing the *aad*A cassette were constructed for plastid transformation in rapeseed and sugarbeet. However, difficulties to select plastid transformants were observed due to a high natural resistance to spectinomycin and streptomycin in rapeseed. In sugarbeet spectinomycin at a concentration of 100 mg/l was found efficient for selection and spectinomycin and streptomycin resistant colonies were obtained after callus bombardment. The presence of the *aad*A gene in antibiotic-resistant lines was proven by PCR analysis, but an integration of DNA into the plastome could not be verified so far. Efficient regeneration systems and methods of DNA transfer were established for rapeseed and sugarbeet and straightened the way for successful plastid transformation in either species.

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7. APPENDIXES

Appendix 1

A list of tested sugarbeet breeding lines for callus induction from leaf explants by Ben-Tahar et al. (1991)

Rel1	6B2838	7B2834
1F0076	6B2840	7R7624
2B0017	6B2842	7R7626
2B0035	6B2850	7R7632
3B0064	6B3907	7R7636
4B2712	6B3910	7T9041
4F0007	6B3911	7T9042
4F0021	6B3971	7T9043
5B2814	6K0020	7T9044
5B2821	6S0085	7T9045
5R7150	6S0086	7T9046
5R7649	6S0088	8B2753
5R7656	6T0082	8K0034
5T0068	6T1108	8R6780
5T0069	6T1109	8T0015
5T0075	6T1110	

Appendix 2

Rapeseed plastome fragment (2808 bp), homologous to tobacco fragment *trn*V-*rps*7 (nt: 140126-142640)

```
   1 ccacgtcaag gtgacactct accgctgagt tatatccctt cccccatcaa gaaatagaac
  61 tgactaatcc taagtcaaag ggtcgagaaa ctcaaggcca ctattcttga acaacttgga
 121 ttggagccgg gctttccttt cgcactatta cgggtatgaa atgaaaataa tggaaaaagt
 181 tggattcaat tgtcaactac tcctatcgga aataggattg actacggatt cgagccatag
 241 cacatggttt cataaaaccg tacgattctc ccgatctaaa tcaagccggt tttacatgaa
 301 gaagatttta ctcagcatgt tctattcgat acgggtagga gaaacggtat tcttttctta
 361 aacttcaaaa aatagagaaa tcagaaccaa gtcaagatga tacggattaa tcctttattc
 421 ttgcgccaaa gatcttccta tttccaaagg aactggagtt acatctcttt tccatttcca
 481 ttcaagagtt cttatgtgtt tccacgcccc tttaagaccc cgaaaaatta acaaattccc
 541 ttttcttagg aacacgtgcg agataaaaaa aaaaagagag aatggtaacc ccacgattaa
 601 ctatttcatt tatgaatttc atagtaatag aaatacatgt cctaccgaaa cagaatttgt
 661 aacttgctat cctataatct tgcctagcag gcaaagattt cactccgcga aaaagatgat
 721 tcattcggat caacatgaaa gcccaactac attgcattgc cagaattcat gttatctatt
 781 ggaaagaggt tgacctcctt gcttctatgg tacaatcctc ttcccgctga gcctcctttc
 841 ttccgtgatt aactgttggc accagtccta cattttgtct ctgtggaccg agaagaaagg
 901 actcactgcg ccaagatcac taactaacac taatctaata gaatagaaaa tcctaatata
 961 atagaaaaga actgtctttt ctgtatactt atgtatactt tccccggttc cgttgctact
1021 gcgggcttta cgcaatcgat cggatcatct agatatccct tcaacacaac ataggtcgtc
1081 gaaaggatct cggagacccg ccaaagcacg aaagccagga tctttcagaa aatgaattcc
1141 tattcgaaga gtgcataacc gcatggataa gctcacacta acccgtcaat ttgggatcca
1201 attcgggatt ttccttgagg gatattggta aggaattgga atgtaataat atcgattcat
1261 aatggattca tatcgataca gaagaaaagg ttctctatcg attcaacaag tgctgtactt
1321 atgggaaagc gatagagaaa gagaaaaaaa aaaacgaaga tttcacatag tgatttttt
1381 ttgatcaaaa aaaaatatga ttgaatttat ttcgtaccct tcgctcaatg agaacatggg
1441 tcagattcta taggatcaaa cctatgggac ttaagaatga tggaagggaa taaaatcaaa
1501 aaagaaatca aataaagaaa agagagggaa aataaagaaa taataagtaa ataaaaatga
1561 agtagaagaa cccagattac aaatgaacaa attcaaactt gaaaaagtct ctttctgatt
1621 ctcgaagaat gaggggcaaa gagattgatc gagaaagatc tcttgttctt attataagat
1681 cgtgtgattg gacccgcaga tgtttggtaa aaagaataat cttatccttt gagaataatc
1741 aaaaatagaa agtgttcaat tggaacatga aaacgtgacc gagtttatcc tagttactct
1801 tcgggacgga ggagattcgc gaacgaggaa agggacccaa tgacttcgaa agaattgaac
1861 gaggagccgt atgaggtgaa aatctcatgt ccggttctgt agagtggcag taagggtgac
1921 ttatctgtca acttttccac tatcaccccc aaaaaaccaa actctgcctt acgtaaagtt
1981 gccagagtac gattaacctc gggatttgaa atcactgctt atatacctgg tattggccat
2041 aatttacaag aacattctgt agtcttagta agaggggaa gggttaagga tttacccggt
2101 gtgagatatc acattgttcg aggaacccta gatgctgtcg gagtaaagga tcgtcaacaa
2161 gggcgttcta gtgcgttgta gattcttatc caagacttgt atcatttgat gatgccatgt
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2221 gaatcgctag aaacatgtga agtgtatggc taacccaata acgaaagttt cgtaagggga
2281 ctgaagcagg ctaccatgag acaaaagatc ttctttcaaa agagattcaa ttcggaactc
2341 ttatatgtcc aaggttcaat attgaaataa tttcagaggt tttccctgac tttgtccgtg
2401 tcaacaaaca attcgaaatg cctcgacttt tttagaacag gtccgggtca aatagcaatg
2461 attcgaagca cttattttta cactatttcg gaaacccaag gactcaatcg tatggatatg
2521 taaaatacag gatttccaat cctagcagga aaaggaggga aacggatact caatttaaaa
2581 gtgagtaaac agaattccat actcgatttc atagatacat atagaattct gtggaaagcc
2641 gtattcgatg aaagtcgtat gtacggtttg gagggagatc tttcatatct ttcgagatcc
2701 accctacaat atggggtcaa aaagccaaaa taaaagattt gagcccttat aaaaagaaaa
2761 cagattcttg aacccctttc acgctcatgt cacgtcgagg tactgcag
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Sugarbeet plastome fragment (2428 bp), homologous to tobacco fragment *trn*V-*rps*7 (nt: 140126-142640)

```
   1 ccacgtcaag gtgacactct accgctgagt tatatccctt ccctgccccc atcgagaaat
  61 agaactgact aatcctaagg caaagggtcg agaaactcaa cgccactatt ctactattct
 121 tgtcttgaac aacttggagc cgggacttct tttcgcacta ttacggatac gaaaataatg
 181 gggaaatttg gattcaattg tcaactgctc ctatcggaaa taggattgac tacggatttg
 241 agccatagca catgctttca taaaatcgta cgattttccc gatctaaatc aagcaggttt
 301 tacatgaaga agatttggct cggcatgttc tatttgatat aggtaggaga agaacccgac
 361 tcggtattca aaaaaaaaat agaggaagca gaaccaagtc aagatgatac ggatcaaccc
 421 cttcttcttg cgacaaagat cttacccttt ccaaaggaag ttccatctct tttccatttc
 481 cattcaagag ttcttatgtg tttccacgcc cccttgaaac cccgaaaaat ggacaaattc
 541 cttttcttag gaatacatac cgcactcgtc actccaaaaa ggataatggt aaccccacca
 601 ttaaccactt catttatgaa tttcatagta atagaaatac atgtcctacc gagacagaat
 661 ttggaacttg ctatcctctt gcctagcagg caaagactta cctccgtgga aaggatgatt
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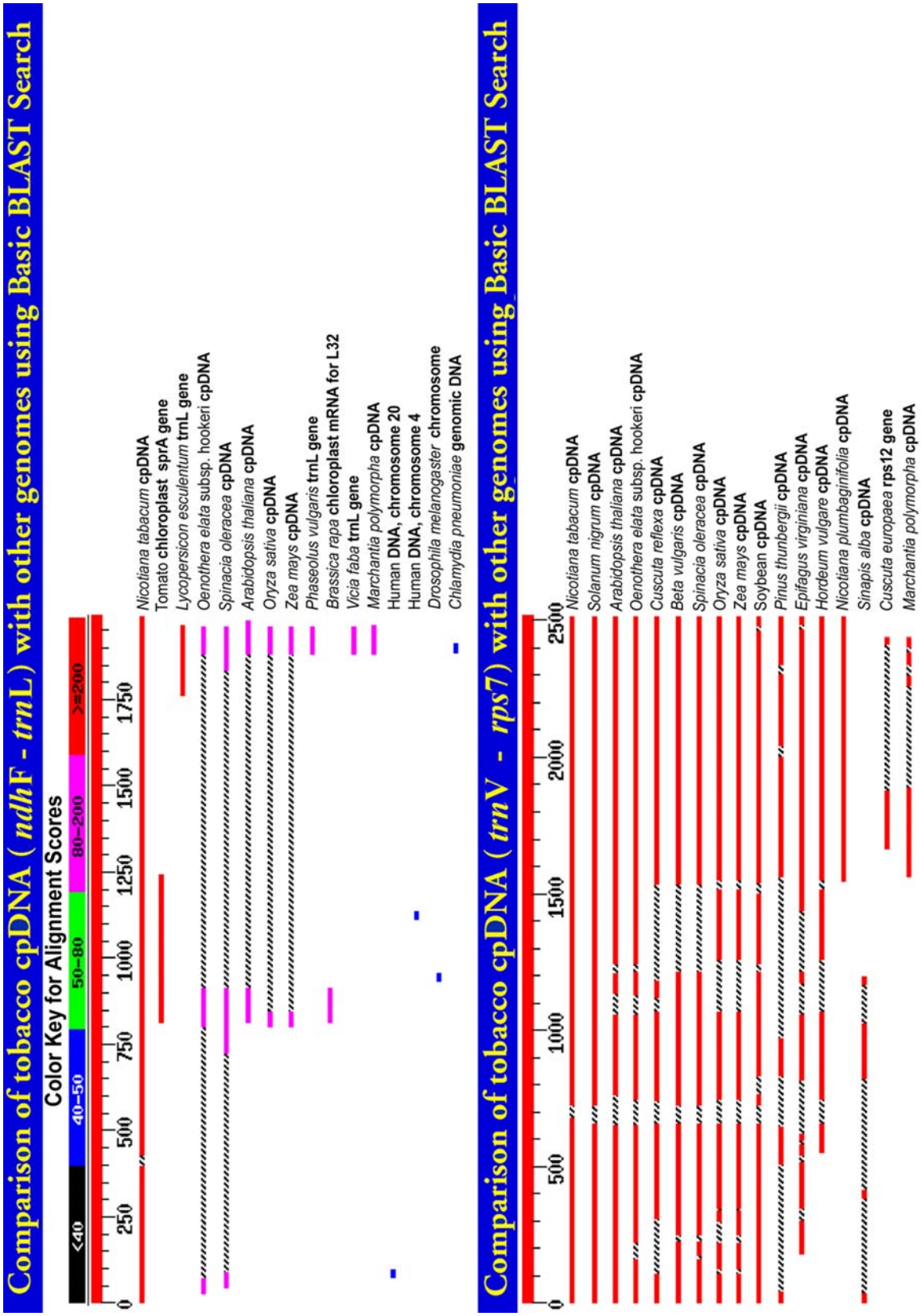
2281 gtacggtttg gagggagatc tttcatatct ttcgagatcc accctacaat atggggtcaa
2341 aaagccaaaa taagtgattt tagcccttat aaaaagaaaa ctgattcttg aacccctttt
2401 acgctcatgt cacgtcgagg tactgcag

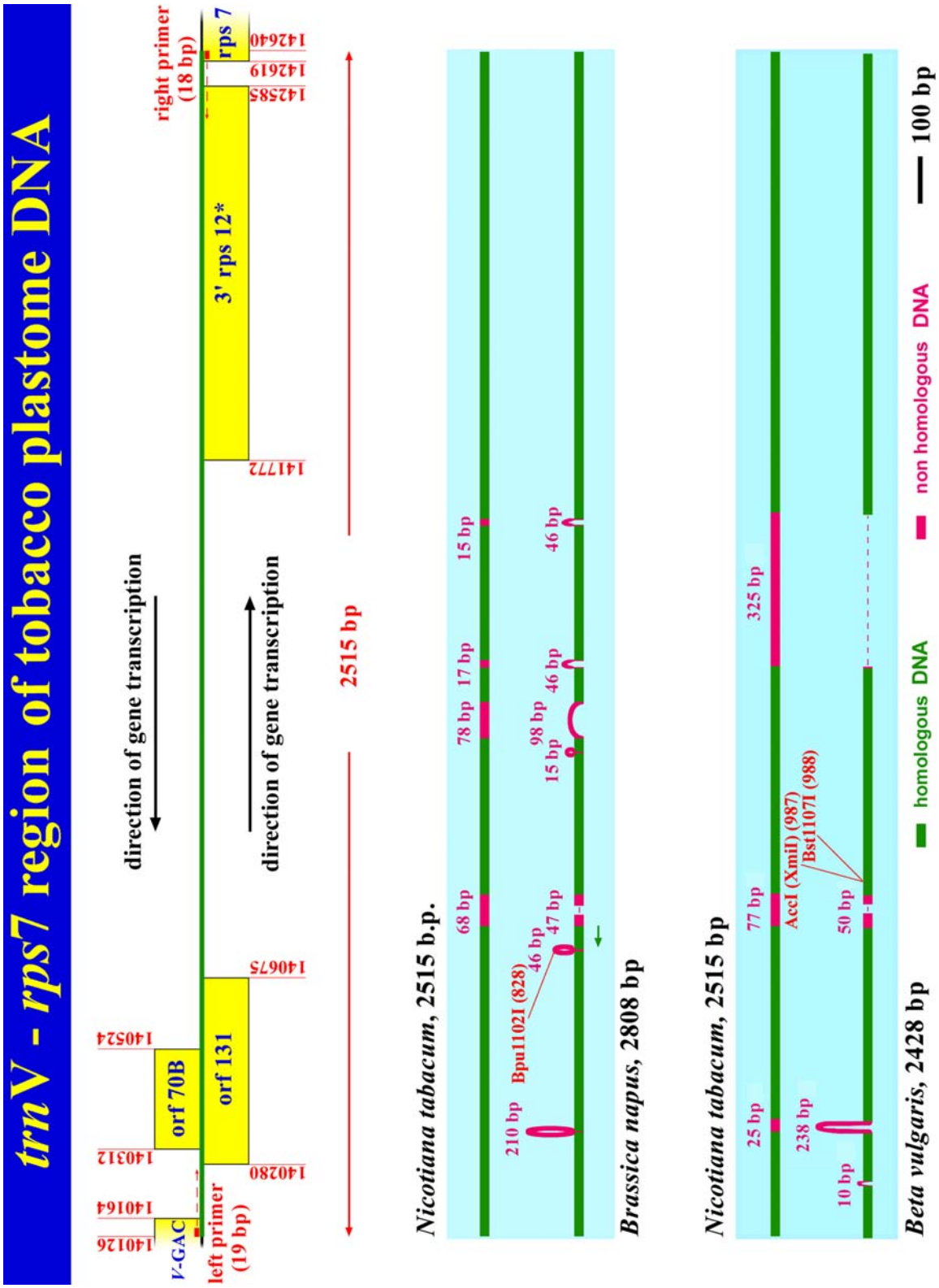
Appendix 3

Comparison of tobacco plastome fragments with sequences from the DNA Database (http://www.ncbi.nlm.nih.gov)



Appendix 4

Homologous plastome sequences (*trn*V-*rps*7, 140126-142640 for the tobacco plastid chromosome) from tobacco, rapeseed and sugarbeet



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CURRICULUM VITAE

FIRST NAME	Alexander
LAST NAME	Dovzhenko
DATE OF BIRTH	January 12, 1974
PLACE OF BIRTH	Kyiv, Ukraine
MARITAL STATUS	single
NATIONALITY	Ukrainian

SCHOOL EDUCATION

1981 - 1991	Secondary school № 3 in Kyiv Graduation: with a silver medal

UNIVERSITY EDUCATION

1991-1996	Study of biology at Kyiv University by name T. Shevchenko, Kyiv specialization: cell biology and genetic engineering (at the Department of Cell biology and Genetic engineering) Diploma thesis "Elaboration of biotechnological methods for ukrainian cultivars of sugarbeet (*Beta vulgaris* L.)" (in Institute of Cell biology and Genetic engineering, Kyiv) Graduation: Diploma with honours

PUBLICATIONS AND PRESENTATIONS

1. Dovzhenko A. et al. Thin-alginate-layer technique for protoplast culture of tobacco leaf protoplasts: shoot formation in less than two weeks. Protoplasma 204 (1-2): 114-118, 1998.

2. Dovzhenko A. et al. Tobacco leaf protoplasts: from cell to shoot in less than two weeks. Abstracts of II International Symposium on Plant Biotechnology, Kyiv, 4-8 October, 1998

3. Lössl A, Eibl C, Dovzhenko A, Winterholler P and Koop HU (2000) Production of polyhydroxybutyric acid (PHB) using chloroplast transformation. In: The 8th International Symposium on Biological Polyesters (ISBP 2000) Sept 11-15, 2000, Massachusetts Institute of Technology, Cambridge, MA, USA

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