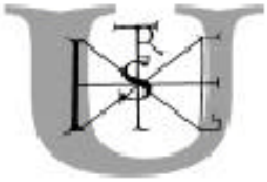

SZENT ISTVÁN EGYETEM

MICROSPORE CULTURE AND GENETIC TRANSFORMATION STUDIES IN BARLEY AND TRITICALE

DOKTORI ÉRTEKEZÉS

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ABBREVIATIONS

α-LeA	linolenic acid
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ANOVA	analysis of variance
AOC	allene oxide cyclase
AOS	allene oxide synthase
ATP	adenosine triphosphate
BAP	6-benzylaminopurine
BSA	bovine serum albumin
CaMV	Cauliflower Mosaic Virus
cDNA	copy DNA
CIAP	Calf Intestine Alkaline Phosphatase
CV%	coefficient of variation
cv.	cultivar
DH	doubled-haploid
DMF	dimethyl-formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELS	embryo-like structure = embryoid
Exp.	experiment
FDA	fluorescein diacetate
Fig.	figure
GUS	β-glucuronidase
IAA	indoleacetic acid
JA	jasmonic acid
JAs	jasmonates
JIP	jasmonate-induced protein
JM	methyl jasmonate; jasmonic acid methyl ester
jrg	jasmonate-regulated gene
kDa	kilo Dalton
KIN	kinetin
LOX	lipoxygenase
LSD	Least Significant Difference
LUC	luciferase
mcs	multiple cloning site
MDE	microspore-derived embryo
ms	microspore
MS	Murashige and Skoog medium or mean square
NAA	naphthalene acetic acid
No.	number
OD	optical density
OPDA	12-oxo-phytodienoic acid
PAA	phenylacetic acid
PAGE	polyacrylamide-gelelectrophoresis
PAT	phosphinothricin-acetyltransferase

PCR	polymerase chain reaction
PDS	Particle Delivery System
PEG	polyethylene glycol
PIG	particle inflow gun
PPT	phosphinothricin
PTGS	post-transcriptional gene silencing
PVP	polyvinilpyrrolidone
RNA	ribonucleic acid
rpm	revolution per minute
RuBPCase	ribulose 1,5-bisphosphate carboxylase
s	standard deviation
SDS	sodium dodecyl sulfate
SMC	shoot meristematic culture
TGS	transcriptional gene silencing
U	unit
vs.	versus

1. INTRODUCTION

Triticale (x *Triticosecale* Wittmack) and barley (*Hordeum vulgare* L.) are important food crops grown all over the world. Triticale is a synthetic amphiploid cereal which has been considerably improved since its first description in 1891 (Rimpau, 1891). Intensive research on this intergeneric hybrid, however, started only in the early 1950s in some countries, including Hungary (Kiss, 1955, 1966). During the last decade the harvested triticale area increased from 1.8 million ha in 1990 to 3 million ha in 2001 (FAO, 2001). Its increasing importance, however, is more obvious in Hungary: while the area was merely 2,280 ha in 1990, in 2001 it already reached 119,000 ha. In the same period, extensive studies on genetic problems (Lelley and Gimbel, 1989) and on molecular genetics (Balatero *et al.*, 1995; Wang *et al.*, 1996) of triticale have been performed. Furthermore, the methods of somatic (Stolarz and Lörz, 1986; Immonen, 1996) as well as haploid (Lukjanjuk and Ignatova, 1986; Immonen and Robinson, 2000) tissue cultures have been established and transgenic plants have also been produced (Zimny *et al.*, 1995). Although recent reviews on breeding strategies of triticale focus mainly on traditional methods (Lelley, 1992; Baier and Gustafson, 1996), novel techniques of tissue culture can also contribute to the success of these programs.

In contrast to triticale, the importance of barley has been well-known for centuries. With a harvested area of 54 million hectares and a production of 141 million tons, it is among the five most important cereals in the world (FAO, 2001). In Hungary, barley is at the third place with an area of 368,000 ha and a yield of 1,300,000 t (FAO, 2001). During the last century every possible aspects of barley breeding were investigated in detail. In addition to traditional methods, the studies in the main fields of yield, quality and resistance involved recent developments of biotechnology as well (for reviews, see Mannonen *et al.*, 1994; Lemaux *et al.*, 1999). Besides its use by the applied science of agriculture, barley is a preferred model species of basic research such as plant physiology and biochemistry, due to its diploid genome and autogamous nature. Thus, barley became the model organism in studies on the role of jasmonates and the analysis of function of the most abundant jasmonate-induced protein, JIP23, in monocots (for review, see Wasternack and Hause, 2002).

In this thesis studies on two independent fields of research are reported and discussed:

1. *Induction of haploid embryogenesis in isolated microspore cultures of triticale and barley under hormone-free conditions.*
2. *Preparation of novel vector constructs to alter endogenous levels of jasmonates and JIP23 via genetic transformation of barley.*

These topics cover two important fields of current plant breeding research: (i) the involvement of doubled-haploid plants in the traditional breeding process and (ii) the improvement of agronomically useful traits via genetic transformation. The use of barley as a model organism in both studies represents a step towards the combination of androgenesis and genetic transformation in the same breeding program in the future. This can be performed through the introduction of foreign genes into microspores or microspore-derived embryoids. The preparation of novel vector constructs which are functionable in the genetic background of barley can broaden the choice of vectors currently available for barley transformation.

Anther culture was early established in triticale (Wang *et al.*, 1973) and improvements of its protocol have been usually studied parallel with wheat. As reports from other species such as rapeseed, barley and wheat suggest, an established method of microspore culture can provide further opportunities for the improvement of this alternative cereal crop through biotechnology. Effects of media conditioning on the *in vitro* development of isolated triticale microspores and pollen grains have already been described but plant regeneration has not been reported (Keller,

1991). An efficient method for the isolation and culture of isolated triticale microspores has been developed only in our laboratory to date (Monostori *et al.*, 1998; Pauk *et al.*, 2000). The published results are integrated in this work as well. Our primary aim was to establish the method of isolated microspore culture for triticale. Here, our experiencies gained with barley and wheat microspore cultures were utilized (Puolimatka *et al.*, 1996; Monostori and Pauk, unpublished). Thus, the objectives for this part of the thesis were:

- to establish the method of isolation and culture of triticale microspores and to regenerate fertile, green dihaploid plants,
- to describe *in vitro* development of isolated triticale microspores on the sporophytic pathway,
- to study the effects of one hormone-free and two media of various hormone composition on microspore embryogenesis, and
- to evaluate the ploidy level of regenerants from different genotypes.

The results of the triticale experiments and our preliminary results from barley microspore culture raise the question, whether hormone-supplementation of induction media is essential for the induction of androgenesis and plant regeneration in microspore culture. In haploid tissue cultures of barley and other cereals induction media are routinely supplemented with hormones in order to promote embryogenesis. Induction of androgenesis in hormone-free media may confirm the proposed decisive role of stress signals in switching microspores from gametophytic to sporophytic development (Touraev *et al.*, 1996a,b, 1997). On the other hand, the evaluation of regeneration capacity in cultures induced with or without exogenous growth regulators can elucidate the promoting role of hormones in haploid embryogenesis. In addition, the independence of embryogenesis and regeneration can be studied in terms of hormone-requirement. Information about species-specific hormone-requirement can be obtained if the hormone-free induction medium of triticale microspore cultures is tested in barley as well. Thus, the additional objectives of our work were:

- to study the induction of androgenesis and plant regeneration in barley microspore cultures without exogenous hormone supply, and
- to compare the effects of the hormone-free medium successfully used in triticale microspore cultures with those of a medium of optimized nitrogen-composition previously established exclusively for barley (Mordhorst and Lörz, 1993).

Transgenic plants offer new possibilities to manipulate the biosynthetic pathways and to analyze the mode of action in most plant hormone classes. Recent advances which have made this new approach possible are (i) the cloning of genes/cDNAs coding for enzymes involved in the biosynthesis of plant hormones and (ii) the gene-transfer methods established for a number of plant species (for reviews, see Hedden and Phillips, 2000). Depending on the orientation of the DNA fragment in the transformation vector (sense or antisense), the genes coding for the biosynthetic enzymes in the transgenic plants are overexpressed or down-regulated, respectively. These changes in the regulation of a biosynthetic pathway can lead to increased or reduced levels of the corresponding hormone. Moreover, modulated hormone levels may cause phenotypic changes in the transgenic plants. This way, data have been provided to understand better the mode of action of auxins (Ficcadenti *et al.*, 1999), cytokinins (Hewelt *et al.*, 1994), gibberellins (Coles *et al.*, 1999) and ethylene (Hamilton *et al.*, 1990).

The endogenous level of jasmonates has been modified via homologous and heterologous transformations with *AOS*, coding for the key-enzyme of JA-biosynthesis, in dicotyledonous species (Harms *et al.*, 1995; Wang *et al.*, 1999a; Laudert *et al.*, 2000). In monocots, however, roles of

jasmonates have been studied via the exogenous application of jasmonates or in response to various stresses only. Alterations in endogeneous JA-levels of barley plants upon transformation with *AOS* in sense or antisense orientation can cause changes, among others, in tissue differentiation and in the process of senescence. Moreover, alterations in stress-response and in the expression of JA-responsive genes could help to get an insight into the function of jasmonates in barley.

JIP23, the most abundant JA-inducible protein in barley, accumulates in tissues osmotically stressed by solute transport as well as in mature leaves exposed to osmotic stress. For this protein, however, no putative function could be drawn from data base searches. In barley, its role as stress-protective protein has been proposed (Hause *et al.*, 1996, 1999). Furthermore, JIP23 may attribute to the well-known JA-induced down-regulation of photosynthetic genes as shown by heterologous overexpression in tobacco (Görschen *et al.,* 1997b). The homologous overexpression or the antisense repression of JIP23 can allow us to elucidate its role in tissue-differentiation, in stress-response and in the mediation of JA-functions in different tissues and developmental stages of the barley plant.

The second part of the current thesis represents studies performed in the frame of a two-year project on the role of jasmonates in the development of barley as well as on the analysis of function of JIP23 in this species. In these studies, a transgenic approach requires stable transformed plants – transient expression systems are not suitable to examine changes in gene-expression, hormone-level and other phenotypic traits in the course of development. For the given period of time, the primary goal of our work was to prepare the prerequisites for further stable transformation programs as well as for the biochemical and molecular biological studies in the future. Therefore, the objectives were:

- to prepare novel plasmid vectors carrying a resistance marker gene and one of barley AOS1 and JIP23 cDNAs in sense or antisense orientation, respectively,
- to test the functionality of the new constructs in the genetic background of barley via transient expression analysis of both the resistance and the important transgenes in PEG-transformed mesophyll protoplasts, and
- to establish a transformation protocol to be used in further stable transformation experiments (i) via optimization of bombardment parameters for a particle inflow gun and (ii) via preliminary particle bombardment studies using the new vectors and scutella of the cultivar ‘Salome’, the model genotype of jasmonate studies.

2. REVIEW OF LITERATURE

2.1. INDUCTION OF HAPLOID EMBRYOGENESIS IN MICROSPORE CULTURE

Since the description of the first haploid mutant in higher plants (*Datura stramonium*; Blakeslee *et al.*, 1922) practice has proven the advantages of using haploids in plant breeding. Spontaneous or induced reduplication of the haploid genome results in homozygous lines in a single generation. Thus, the selection efficiency will increase, which leads to an accelerated breeding process (Snape, 1987; Morrison and Evans, 1988). Furthermore, recessive traits can be selected at plant level and gametoclonal variability can be utilized this way (for review, see Heszky, 2000). The first haploid plants of *in vitro* origin have been reported in *Datura inoxia* (Guha and Maheswari, 1964). During the last decades, however, hundreds of species have been successfully involved in anther culture and several varieties produced using this method have been released (for reviews, see Heszky, 1979; 2000; Foroughi-Wehr and Wenzel, 1989; Bajaj, 1990; Kush and Virmani, 1996; Forster, 2002).

2.1.1. Haploid breeding in cereals

During the last 15 years, doubled-haploidy has been extensively used for the production of novel cultivars in cereals: 116 barley, 21 wheat, 8 rice and 3 triticale cultivars/lines originate from various techniques of haploid production (for review, see Forster, 2002). These procedures are based on chromosome-elimination ('*bulbosum*-method') and *in vitro* androgenesis (anther and microspore cultures). Gynogenesis through ovary culture could not become widespread practice due to its low efficiency (Castillo and Cistué, 1993). '*Bulbosum*-method' is the traditional technique for haploid production in barley (Kasha and Kao, 1970; Devaux *et al.*, 1990). Two-third of the doubled-haploid (DH) barley cultivars have been produced by this procedure (Forster, 2002). Advances in anther culture, however, have recently made this fully *in vitro* approach the most widely used method for the production of haploids both in barley (Kuhlmann and Foroughi-Wehr, 1989; Luckett and Smithard, 1995) and wheat (for review, see Barnabás *et al.*, 2000).

While anther culture is an established method for plant breeding, isolated microspores have remarkable features to be utilized in plant biotechnology. They offer a unicellular system of haploid cells which can be isolated in large quantities and synchronized in development. Thus, microspores are excellent targets for transformation methods and *in vitro* selection (for review, see Jähne and Lörz, 1995; Dunwell, 1996, for application in barley, see Table 1). Moreover, they can be used in studies on the biochemical and molecular background of embryogenesis (for reviews, see Reynolds, 1997; Touraev *et al.*, 1997; in barley and wheat, see Reynolds and Kitto, 1992; Mordhorst *et al.*, 1994; Vrinten *et al.*, 1999). Microspore culture, however, also has an established role in the breeding of both mono- and dicotyledonous species: it has recently contributed to the production of five DH barley cultivars, and it is the method exclusively used for the production of DH rapeseed (for review, see Forster, 2002).

The comparison of the efficiency of anther and microspore culture of barley shows a 100-to 200-fold higher regeneration rate in microspore culture (1170-2040 vs. 10.9 green plants/100 anthers) (Davies and Morton, 1998). In barley anther culture, the highest regeneration rate was 1300 green plants/100 anthers (Kao *et al.*, 1991), while in microspore culture the maximum was 5000 green plants/100 anthers (Hoekstra *et al.*, 1993).

2.1.1.1. Main factors of isolated microspore culture in cereals

Among cereals, plant regeneration from isolated microspore culture has been first reported for barley (Köhler and Wenzel, 1985) followed by wheat some years later (Datta and Wenzel, 1987).

Microspore culture techniques in these species are thought to provide reliable information for establishing the protocol of microspore culture for triticale. A great number of factors influencing the efficiency of the methods have been determined to date.

Table 1. Microspore culture of barley

Genotype	Pretreatment	Hormones (mg/l)	Induction medium	Green plants/ 100 anthers	References
Shed culture					
Sabarlis	cold	1.5 2,4-D 0.5 KIN	P-9H	-	Sunderland and Xu, 1982
Dissa Igri	no	2.0 2,4-D 0.5 KIN	N_6	0.08 0.0	Köhler and Wenzel, 1985
Igri; Dissa; F_1; doublecross	cold	1.0 2,4-D 0.5 KIN	N_6 1-2	17; 14; 4; 4	Datta and Wenzel, 1988
Igri	cold/mannitol	1.0 IAA 0.2 KIN	FHG	266	Ziauddin *et al.*, 1990
Maceration					
Sabarlis	no	0.5 KIN	C_1	0.18/callus	Wei *et al.*, 1986
Igri	cold	1.0 IAA 0.2 KIN	FHG	608	Hunter, 1987
Igri	mannitol	1.0 IAA 1.0 BAP	C_1	1000	Kuhlmann *et al.*, 1991
Igri	mannitol	1.0 PAA	FHG	1000	Ziauddin *et al.*, 1992
Igri	mannitol (+ ABA, 2,4-D)	1.0 BAP	L_1	1240-5000	Hoekstra *et al.*, 1992, 1993, 1996, 1997; van Bergen *et al.*, 1999
Kymppi	cold	1.75 2,4-D 0.25 KIN	108***	266	Salmenkallio-Marttila, 1994
Igri	mannitol	1.0 PAA 0.1 KIN	FW**	400	Harwood *et al.*, 1995
Igri	mannitol	1.0 BAP	FHG	1738	Cistué *et al.*, 1995
Igri; Reinette; Hop	mannitol	1.0 BAP	FHG	2.1-0.6-0.1/10^3 ms	Castillo *et al.*, 2000
Microblending					
Igri Gimpel	cold*	1.0 BAP	N24A2.7G3	480 146	Mordhorst and Lörz, 1993; Jähne-Gärtner and Lörz, 1999
Igri	cold*	1.0 BAP	FHG	-	Scott and Lyne, 1994a,b
Igri F_1	cold	1.0 IAA 1.0 BAP	KFWC**	2040 140	Davies and Morton, 1998
Igri	cold/mannitol	1.0 IAA 1.0 BAP	C_1	n.d.	Simon and Foroughi-Wehr, 2000
Celinka; Cooper; Cork; Douchka; Gotic; Nikel; Maeva	mann./medium	1.0 BAP	FW1B**	24.5; 16.2; 0.2; 0.0; 3.8; 8.0; 4.2	Li and Devaux, 2001
30 cultivars	cold/mannitol*	10.0 PAA 1.0 BAP	FHG	720-1940/10^5 ms	Kasha *et al.*, 2001a,b
Vortexing					
Igri	mannitol	1.0 BAP	FHG	-	Vrinten *et al.*, 1999
Miscallenous					
Igri	mannitol	1.0 BAP	LMS-60M**	282 (m), 940 (b)	Olsen, 1991
Chernigovski; Dneprovski	cold	2.0 2,4-D 0.5 KIN	N_6	62.7 (s) 49.3 (m)	Tiwari and Rahimbaev, 1992
Igri; Bonanza; Duke; F_1	cold	0.5 NAA 1.0 KIN	mod. 49	-	Kao, 1993
Kymppi	cold	1.75 2,4-D 0.25 KIN	108C***	300/spike (m/b*)	Ritala *et al.*, 2001

n.d. = no data; m = maceration; b = microblending; s = shed culture; ms = microspore; * microblending of spikes; ** modified FHG; *** modified N_6

Donor plants. Genotype has the greatest impact on the efficiency of cereal microspore culture (for reviews, see Jähne and Lörz, 1995). In barley, cultivar 'Igri' was found to be the most responsive cultivar exhibiting the highest regeneration capacity (5000 green plants/100 anthers; Hoekstra *et al.*, 1993). Among other genotypes, the highest yield (266 green plants/100 anthers) has been achieved

with 'Kymppi' (Salmenkallio-Marttila, 1994). A genotype independent microspore culture system of great efficiency has recently been described for barley, yielding up to 1940 green plants per 10^5 microspores (Kasha *et al.*, 2001). In wheat, cultivar 'Chris' is preferred but filial generations of cross combinations are also frequently used (Hu *et al.*, 1995; Hansen and Andersen, 1998). Due to the diversity in expressing results, however, regeneration rates are hard to compare here.

Donor plants of appropriate quality can be obtained under controlled conditions in greenhouse or growth-chamber only. Low temperatures (12-18 ºC) are of advantage because plants grow more slowly, resulting in a more homogenously developed population of microspores (Jähne-Gärtner and Lörz, 1999).

Developmental stage of microspores. The period around the first pollen mitosis was found to be the critical stage when microspores are the most susceptible to enter an alternative way of development (for reviews, see Reynolds, 1997). Microspores of the mid- to late-uninucleate (occasionally to early-binucleate) stages exhibit the greatest responsivity in isolated microspore cultures of both barley and wheat (Ziauddin *et al.*, 1990; Hoekstra *et al.*, 1992; Kao, 1993; Mejza *et al.*, 1993; Hu *et al.*, 1995; Kasha *et al.*, 2001b).

Pretreatments. To switch microspores from gametophytic to sporophytic development, a signal is necessary. This is provided by stress factors (N- or carbohydrate-deficiency, heat- or osmotic stress etc.) which microspores are subjected to during pretreatment (Touraev *et al.*, 1996a,b, 1997). Stress-induced abscisic acid (ABA) inhibits further gametophytic development and apoptosis, thus maintaining the rate of viable microspores during pretreatment (van Bergen *et al.*, 1996; Wang *et al.*, 1999b). Pretreatments routinely used in barley and wheat microspore cultures include cold pretreatment of donor spikes and the incubation of anthers in mannitol. During incubation at 4-8 °C for 14-28 days, tapetum degenerates and the proportion of free microspores inside anther increases. This resembles the status observed prior to dehiscence under natural conditions, thus facilitating the isolation of microspores (Sunderland *et al.*, 1984). During the incubation of anthers in 0.3 M mannitol for 3-5 days, the lack of metabolizable carbohydrates together with salt- and osmotic-stress act as signals (Touraev *et al.*, 1996a,b; Hoekstra *et al.*, 1997; van Bergen *et al.*, 1999; Wang *et al.*, 1999b). The combination of cold and starvation stresses is proposed to provide induction and suspension of nuclear division independent of genotype (Kasha *et al.*, 2001a).

Isolation method. In barley, various methods of microspore isolation can be successfully used (Table 1). Shed-cultures, however, are more closely related to anther cultures because anthers floating in the induction medium presumably release conditioning factors into the medium (Köhler and Wenzel, 1985; Jähne and Lörz, 1995). Pestle maceration of isolated anthers is the most widely used isolation method and the highest number of green plants have also been achieved using this technique (Hoekstra *et al.*, 1993). Upon homogenization in a microblendor, microspores are released from the anthers faster and are subjected to less mechanical stress, thus leading to an increase in the number of green regenerants (Olsen, 1991). Microblending of spikes offers a simple, less laboursome isolation method of great efficiency (Mejza *et al.*, 1993; Mordhorst and Lörz, 1993; Kasha *et al.*, 2001b). While wheat microspores are more sensitive to isolation procedure than barley microspores, maceration is not recommended there. The most effective methods are microblending and vortexing (Gustafson *et al.*, 1995; Hu *et al.*, 1995).

Comparing the efficiency of the isolation methods in terms of microspore yield, microblending of anthers seems to be the most efficient method, although the yield of shed cultures can reach that of maceration (Table 2). No such data are available, however, for microblending of spikes neither in barley nor in wheat. Although, the highest number of green regenerants can be achieved by pestle maceration of anthers, the lower regeneration rate got by microblending of spikes is compensated by the relative ease and quickness of this method (Table 2).

Table 2. Efficiency of different microspore isolation methods in barley

Parameter	Shed-culture	Pestle maceration	Microblending of anthers	Microblending of spikes
Isolated microspores/ anther*	150-2770	2333-2708	3489	n.d.
Max. green plants/ 100 anthers**	266	5000	2040	2500

* Based on Sunderland and Xu (1982); Ziauddin *et al.* (1990); Olsen (1991); Hoekstra *et al.* (1992).
** Based on Table 1, cultivar 'Igri'.

In wheat microspore culture, maceration yields the highest number of microspores (2305 ms/anther). It is followed by vortexing of anthers (1886 ms/anther). Considering the viability of microspores, however, vortexing gives the better result (284 vs. 191 ms/anther) (Hu *et al.*, 1995). The highest yield of viable microspores per anther achieved by vortexing is 1525 (cultivar 'Chris') at a total number of 2720 microspores per anther (Hu and Kasha, 1997). Similarly to vortexing, microblending of anthers also has a positive effect on the viability of wheat microspores compared to maceration (53-80% vs. 27-32%) (Gustafson *et al.*, 1995).

Induction media. Besides nourishing microspores, components of induction media (e.g. hormones, nitrogen compounds) determine embryogenic development of microspores, which was initiated by signals during pretreatment (Jähne-Gärtner and Lörz, 1999). In barley microspore cultures FHG medium and its modified versions are used most widely (Table 1). FHG is a modified MS medium of reduced NH_4 content (10%). Other media such as N_6 and C_1 also feature a reduced NH_4 content compared to MS. In wheat microspore cultures a variety of media are used, the efficiency being highly genotype dependent (Gustafson et al., 1995; Puolimatka *et al.*, 1996; Hu and Kasha, 1997). For the anther culture responsive genotype 'Hja 24201' 190-2 induction medium was found to be optimal in wheat microspore culture (Puolimatka *et al.*, 1996). The effects of different components of the induction medium on androgenesis have been investigated in detail in anther and microspore cultures.

Green plant regeneration was significantly improved by decreasing ammonium-nitrate concentration and using glutamine as organic nitrogen source in anther culture (Clapham, 1973; Olsen, 1987). As it was described above, these modifications were adapted for microspore culture media as well. On this basis, optimal nitrate:ammonium and organic:inorganic nitrogen ratios were determined and used to increase regeneration rate in microspore culture. The medium of optimized N-composition was named N24A2.7G3, designating nitrate, ammonium and glutamine content in mM, respectively (Mordhorst and Lörz, 1993).

The carbohydrate source also has a decisive role in the initiation of haploid embryogenesis. Quick metabolism of sucrose, glucose or fructose leads to hypoxia and the accumulation of ethanol, thus causing the death of microspores. In contrast, slower metabolism of maltose does not have such lethal consequences, thus enabling the induction of androgenesis (Scott and Lyne, 1994a,b; Scott *et al.*, 1995). Due to its beneficial effect, 0.15-0.175 mM (54-63 gL^{-1})maltose was introduced to replace sucrose in induction medium (Hunter, 1987). Maltose of such concentration is routinely applied in microspore cultures of both barley and wheat.

In microspore cultures of wheat the decisive role of ovary co-culture has been observed (Mejza *et al.*, 1993; Puolimatka *et al.*, 1996; Hu and Kasha, 1997). Ovary co-culture resulted in significantly higher numbers of embryos and green plants in comparison with a completely defined medium (Hu and Kasha, 1997). In barley microspore culture, co-culture of ovaries is not routinely used. Its

application, however, was supposed to contribute to a decreased genotype-dependence (Li and Devaux, 2001).

Microspore density in the induction medium. A minimal density of viable microspores (0.05 x 10^5 /ml) is essential for their further development (Hoekstra *et al.*, 1993). Optimal densities were determined on a wide range of 0.2-6.0 x 10^5/ml, depending on genotype and culture protocol (Hoekstra *et al.*, 1993; Gustafson et al., 1995; Davies and Morton, 1998; Castillo *et al.*, 2000). In practice, a medium density of 0.5-1.0 x 10^5 microspores/ml is used most frequently (Cistué *et al.*, 1995; Hu et al., 1995; Puolimatka et al., 1996; Davies and Morton, 1998; Li and Devaux, 2001). The highest number of green 'Igri' regenerants was achieved in cultures of 0.2 x 10^5/ml microspore density (Hoekstra *et al.*, 1993). By microblending of spikes, best results were achieved when microspores of 'Igri' were cultured at densities of 1.0-2.5 x 10^5/ml (Mordhorst and Lörz, 1993; Kasha *et al.*, 2001b). In these cases the higher number of microspores should compensate the heterogeneity of microspore population caused by homogenization of the less developed florets of spikes as well. Pestle maceration and microblending of anthers provides a more homogeneous microspore population due to the processing of selected anthers.

Regeneration media. Plants are usually regenerated on hormone-free MS-based (Murashige and Skoog, 1962) media using sucrose as carbohydrate source (Hunter, 1987, Hoekstra *et al.*, 1992, Mejza *et al.*, 1993, Hu *et al.*, 1995). IAA or NAA added to regeneration medium, however, were found to result in more vigorous barley regenerants (Castillo *et al.*, 2000).

2.1.2. *In vitro* androgenesis in triticale

For the production of haploid triticale plants both chromosome-elimination technique and *in vitro* androgenesis can be successfully used. Haploid/dihaploid plants were received from wide crosses with maize and pearl millet (Inagaki and Hash, 1998; Wedzony et al., 1998). Similarly to barley, however, anther culture is the established method for haploid production in triticale, resulting in three released DH cultivars to date (for reviews, see Lukjanjuk and Ignatova, 1986; Forster, 2002). Unlike in its two "ancestors", wheat (Mejza *et al.*, 1993; Puolimatka *et al.*, 1996) and rye (Guo and Pulli, 1999), plant regeneration from isolated microspore culture has not been reported in triticale prior to our publications (Monostori *et al.*, 1998; Pauk *et al.*, 2000) the results of which are detailed in this thesis. Since the induction of the first anther culture-derived haploid plantlets (Wang *et al.*, 1973), each step of the protocol has been investigated in detail. Triticale genotypes of various ploidy levels have been involved in this research. The majority of reports, however, are about hexaploid triticale, thus reflecting its importance in plant breeding. Besides triticale anther culture protocols, technical details of microspore cultures of other cereals, primarily wheat and barley, meant the starting point for establishing the protocol of microspore culture in triticale.

Stress has an important role in the induction of androgenesis in triticale, too. Donor spikes are usually pretreated at 4 ºC for 7-21 days (Lukjanjuk and ignatova, 1986; Marciniak et al., 1998; González and Jouve, 2000). Prolonged cold stress as well as the combination of cold pretreatment with heat shock or mannitol starvation can improve plant regeneration at certain genotypes (Immonen and Robinson, 2000). As it is common in the haploid tissue cultures of cereals, however, genotype has the greatest impact on efficiency (Hassawi *et al.*, 1990; Karsai and Bedő, 1997; Marciniak *et al.*, 1998; González and Jouve, 2000). Highest green plant induction frequencies achieved with cultivars grown in Hungary were 1.5 green plant ('Presto'), 10.1 plants ('Moniko') and 3.2 plants ('Tewo') per 100 anthers. 'Moniko' responded with strong changes to altered culture conditions, while regeneration rates of 'Presto' and 'Tewo' could not be improved by any changes in protocol (Karsai and Bedő, 1997). These three cultivars acted as crossing partners in the donor genotypes of our microspore culture experiments. Independent of genotype, anthers containing microspores at the mid- to late-uninucleate stages are the most suitable for culture.

A wide range of induction media have been found to be superior to others for different genotypes in different laboratories. These include B_5 (Lukjanjuk and Ignatova, 1986), 85D12 (Hassawi *et al.*, 1990), P_2 (Marciniak *et al.*, 1998), N_6 (González and Jouve, 2000), C_{17} (Ponitka *et al.*, 1999) and W_{14} (Immonen and Robinson, 2000). The best green plant regeneration results for 'Presto', 'Moniko' and 'Tewo'have been achieved by using N_6 medium supplemented with 0.26 M maltose and glutamine (Karsai and Bedő, 1997). This composition corresponds to the preferencies regarding nitrogen-composition and carbohydrate source of induction media detailed for barley (see 2.1.1.). The highest green plant regeneration frequency in triticale anther culture (15.36 green plants/100 anthers) has also been recorded with maltose-supplemented N_6 medium (González and Jouve, 2000). As growth regulator, 2,4-D alone or in combination with kinetin is used in the induction medium (Marciniak *et al.*, 1998; González and Jouve, 2000; Immonen and Robinson, 2000). Regeneration media are usually supplemented with hormones (IAA, NAA, kinetin), although plant regeneration of greatest efficiency has been reported on hormone-free medium (González and Jouve, 2000). MS-based media are usually used, however 190-2 regeneration medium has been found to be superior to MS for cultivar 'Moniko' (Karsai and Bedő, 1997).

Previous to our work, isolated pollen/microspore culture without plant regeneration has been reported for triticale (Keller, 1991). First steps of androgenesis have been observed only after anther preculture or in anther-conditioned media. Furthermore, the positive effect of cold pre-treatment of spikes on pollen development was observed there.

2.1.3. The role of plant hormones in the induction of microspore embryogenesis

The role of hormones, if any, in the induction of androgenesis is less understood, while their role during embryo development has been found to be more evident (Sangwan and Sangwan-Norreel, 1990). In dicotyledonous plants like *Nicotiana*, *Datura*, and *Brassica spp.*, exogenous hormone supply is not essential for the induction of pollen embryogenesis – the required signal is offered by stress factors (Nitsch, 1977; Sangwan and Sangwan-Norreel, 1990; Swanson, 1990; Touraev and Heberle-Bors, 1999). *Gramineae*, such as barley, triticale and wheat, are known to belong to the group of plants which require hormones in the induction medium of anther cultures. Anthers of this group, however, can also exhibit response if induced in hormone-free medium in the first stage of culture (for review, see Dunwell, 1985). In practice, however, auxins and/or cytokinins are routinely added to culture media in anther and microspore cultures of cereals to promote embryogenesis induced by stress signals. The role of these hormones, however, is still unknown (for reviews, see Clapham, 1977; Jähne and Lörz, 1995). In microspore cultures of barley, benzylaminopurine (BAP) is the most frequently used hormone (Table 1), while in microspore cultures of wheat auxins, occasionally in combination with cytokinins, are used the most widely (Datta and Wenzel, 1987; Gustafson *et al.*, 1995; Hu and Kasha, 1997).

Induction of androgenesis without exogenous hormone-supply has been reported only in anther cultures of barley (Cai *et al.*, 1992) and oat (Kiviharju *et al.*, 1997) as well as in isolated microspore cultures of wheat (Touraev *et al.*, 1996). Our preliminary results in barley microspore culture suggested that hormone-free media could be used to promote androgenesis with great efficiency. Plant regeneration, however, resulted in albinos only, except for the genotype 'Jokioinen 1490' where a low number of green plants could be regenerated exclusively on hormone-free medium (unpublished data).

2.2. PREPARATION OF NOVEL VECTOR CONSTRUCTS

2.2.1. Genetic transformation of barley

Modification of the endogenous JA-level via manipulation of the biosynthetic pathway in transgenic plants has recently offered new possibilities to study the role of jasmonates in dicotyledonous species (see 2.2.3.). The most effective transformation method for dicots, the indirect gene-transfer mediated by *Agrobacterium tumefaciens*, was applied in these cases as well. Over a long period of time this transformation method was considered to be inapplicable in monocots, which were known to be out of the host range of the bacterium. Recently, transgenic plants produced via *Agrobacterium*-mediated transformation have been reported in cereals, too (barley: Tingay *et al.*, 1997; maize: Ishida *et al.*, 1996; rice: Hiei *et al.*, 1994; wheat: Cheng *et al.*, 1997). The potential advantages of this system include the introduction of low copy number of the transgene, its preferential integration into transcriptionally active regions of the chromosome and high co-expression of the introduced genes. These features have been described in barley transformation as well (Tingay *et al.*, 1997; Horvath *et al.*, 2000; Wang *et al.*, 2001; Fang *et al.*, 2002). In spite of these remarkable characteristics of *Agrobacterium*-mediated transformation, however, direct gene-transfer is still the most widely applied strategy for the genetic engineering of monocotyledonous species. Among these methods the electroporation- and PEG-mediated transformation of protoplasts has become an established method in the production of transgenic rice (Zhang *et al.*, 1988). Its application for other species, however, was limited by the difficulties in plant regeneration as well as by the poor reproducibility of the protoplast-system. Among several promising attempts with various explants and techniques, the delivery of DNA-coated microprojectiles into regenerable cells or tissues through bombardment has recently proved the simplest and most effective transformation method in all cereal species (for reviews, see Datta, 1999; Gordon-Kamm *et al.*, 1999; Lemaux *et al.*, 1999; Vasil and Vasil, 1999b). Besides the commercially available and most widely used He-powered Particle Delivery System (Biolistic® PDS-1000/He by Bio-Rad; Kikkert, 1993) other particle bombardment devices are also in use (Iida *et al.*, 1990; Christou *et al.*, 1991; Jenes *et al.*, 1996). The usually home-made models of particle inflow gun (PIG) are driven through the system developed by Finer *et al.* (1992).

In barley also the biolistic gene-transfer was found to be the most effective method to perform transient expression studies (Mendel *et al.*, 1989; 1992; Schledzewski and Mendel, 1994; Hänsch *et al.*, 1995; Harwood *et al.*, 2000) as well as to produce transgenic plants. Particle bombardments are usually carried out with PDS-1000/He, but PIGs have also been successfully applied in the production of transgenic barley plants (Koprek *et al.*, 1996; Zhang *et al.*, 1999) (Table 3). The most widely used target tissue is provided by the scutellar surface of immature embryos. Due to its high responsivity in regeneration systems based on somatic embryogenesis, cultivar 'Golden Promise' is the genotype comonly used. The highest transformation frequency reported in barley (15.2%) was also achieved by particle bombardment of immature embryos of 'Golden Promise' (Nuutila *et al.*, 1999). Apart from this extra result, however, highest transformation frequency was approximately 4% (Wan and Lemaux, 1994; Jensen *et al.*, 1995; Zhang *et al.*, 2001). The highest transformation frequency achieved by *Agrobacterium*-mediated transformation was in the same range (4.2%), also with the same genotype and target tissue (Tingay *et al.*, 1997). Biolistic transformation of microspores as well as protoplast transformation usually results in transgenic plants at 10^{-6}-10^{-7} frequency (Table 3).

Table 3. Reports about transgenic barley plants

Target material	Genotype	Resistance	Transgene of	TF[j]	Reference

		gene	importance		
Microprojectile bombardment [a]					
microspore	Igri	*bar*		1 x 10^{-7}	Jähne *et al.*, 1994
embryo axis	Kymppi	*nptII*		0.4%	Ritala *et al.*, 1994
IE[c] callus MDE[d]	GP[g] Igri	*bar*		4.4% n.d. 0.1%	Wan and Lemaux, 1994
microspore	n.d.[h]	*bar*		n.d.	Jäger-Gussen, 1994
IE	GP Haruna Nijo Dissa	*hph*		0.6% 0.2% 0.7%	Hagio *et al.*, 1995
IE	GP	*bar*	*β-glucanase*	4.1%	Jensen *et al.*, 1995
IE[b]	GP Dera Corniche Salome Femina	*bar*		1.4% 1.3% 0.3-1-1% 0-1.6% 0-0.6%	Koprek, 1996 Koprek *et al.*, 1996
IE	Dera	*bar*	*cnx1* antisense	n.d.	Koprek, 1996
IE	GP	*bar*		3.9%	Lemaux *et al.*, 1996
IE	GP	*bar*	*lys*C, *dap*A	1.6%	Brinch-Pedersen *et al.*, 1996
microspore	Igri	*bar*		1 x 10^{-7}	Yao *et al.*, 1997
IE callus	GP Galena Harrington	*bar* *hpt*		n.d. 0.5% 1.2%	Cho *et al.*, 1998
microspore	Igri	*pat*	*Vst1*	n.d.	Leckband and Lörz, 1998
SMC[b, f]	Harrington	*bar/nptII*		0.8%	Zhang *et al.*, 1999
IE	GP	*bar*	hordein prom.	n.d.	Cho *et al.*, 1999a
IE	GP	*bar*	*Wtrxh*	n.d.	Cho *et al.*, 1999b
IE	GP	*cod*A/*P450*		n.d.	Koprek *et al.*, 1999
IE	GP Kymppi	*bar*	*Egl1*	15.2% 7.7 x 10^{-5}	Nuutila *et al.*, 1999
IE microspore	GP Igri	*pat*	*AcTPase*	1% 7 x 10^{-6}	Scholz *et al.*, 2001
IE	GP	*bar*	*Ds, AcTPase*	n.d.	Koprek *et al.*, 2000, 2001
microspore	Igri	-	*sgfp, pgfp*	3.3 x 10^{-7}	Carlson *et al.*, 2001
IE	GP	*bar*	S*nRK1* antisense	3.6%	Zhang *et al.*, 2001
PEG-mediated transformation					
suspension pp.	Igri	*nptII*		n.d.	Funatsuki *et al.*, 1995
callus pp.	GP	*nptII*		0.9 x 10^{-6} 1.5 x 10^{-6}	Kihara *et al.*, 1998
scutellum pp.	Clipper	*nptII*		n.d.	Nobre *et al.*, 2000
Electroporation-mediated transformation					
microspore pp.[e]	Kymppi	*nptII*		2.5 x 10^{-6}	Salmenkallio-Marttila, 1994
Agrobacterium-mediated transformation					
IE	GP	*bar*		4.2%	Tingay *et al.*, 1997
IE	GP	*bar*	*β-glucanase*	n.d.	Horvath *et al.*, 2000
callus	Schooner	*bar* *hpt*	*s-gfp* RPV, PAV PAV RPV, PAV	1.1-1.6% 1.8% 0-2.9% 0-1.2%	Wang *et al.*, 2001
IE	GP	*hpt*	*s-gfp*	3.4%	Fang *et al.*, 2002
Microinjection					
zygote pp.	Igri Alexis		*gus*A	1 plant 1 plant	Holm *et al.*, 2000

[a] microprojectile bombardment using PDS; [b] particle bombardment with both PDS and PIG; [c]IE = immature embryo; [d]MDE = microspore-derived embryo; [e]pp. = protoplast; [f]SMC = shoot meristematic culture; [g]GP = Golden Promise; [h]n.d. = no data; n.d. at TF: no rate calculated; [i]TF = transformation frequency: fertile plants/ targets

The selection of transgenic tissues and plants in cereals is usually based on the introduction of transgenes coding for antibiotic or herbicide resistance. The neomycine-phosphotransferase gene (*npt-II*) encoding resistance to kanamycin and geneticin (G418) is primarily used in dicotyledonous plants, while the hygromycin resistance gene (*hpt* or *hph*) is the most frequently used selection marker in rice (Shimamoto *et al.*, 1989; Nandadeva *et al.*, 1999). Both *npt-II*- and *hph*/*hpt*-based

selection have contributed to the production of transgenic barley plants, although the most widely used selection system in barley is based on the expression of phosphinothricin-acetyltransferase (PAT) encoded by the *bar* and the *pat* marker genes (Table 3). Phosphinothricin (PPT) acts as a competitive inhibitor of glutamine synthetase. The resulting ammonia accumulation and glutamine deficiency lead to the death of the plant cells. The *pat* gene of *S. viridochromogenes* origin (Wohlleben *et al.*, 1988) and the *bar* gene from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) confer resistance to the herbicides phosphinothricin (PPT), Basta® and bialaphos (L-PPT; Glufosinate-ammonium). PAT inactivates PPT by acetylation (de Block *et al.*, 1987; Dröge *et al.*, 1992).

Besides the improvement of transformation efficiency, agronomically important traits such as malting and feeding quality (Brinch-Pedersen *et al.*, 1996; Jensen *et al.*, 1996; Nuutila *et al.*, 1999; Cho *et al.*, 1999b; Horvath *et al.*, 2000) as well as resistance against pathogens (Leckband and Lörz, 1998; Wang *et al.*, 2001) have also been improved via the transformation of barley. Further results were the alteration of nitrate-reductase activity (Koprek, 1996) and the introduction of transposable elements (Koprek *et al.*, 2000, 2001; Scholz *et al.*, 2001) (Table 3).

In spite of the above-mentioned reports on successful production of transgenic plants, however, there are problems which hinder genetic transformation in becoming a routine method for the improvement of barley (Lemaux *et al.*, 1999): (i) only few genotypes were found to be amenable to *in vitro* cultivation and to give high transformation frequency in the current transformation systems (Table 3), (ii) somatic mutation and stable epigenetic changes can arise during *in vitro* culture and may hinder various stages of the transformation process, (iii) using current transformation methods, transgene insertions occur randomly and their locations are not always optimal for gene expression; furthermore, the insertion of multiple copies, more common with direct gene4-transfer, can lead to both gene inactivation and genetic instability (for reviews, see Iyer *et al.*, 2000). Considering these problems, the establishment of an *in vitro* plant regeneration system for the given genotype and the optimization of the bombardment parameters are essential for achieving the highest transformation frequency in the stable transformation experiments.

2.2.2. Modification of biosynthetic pathways by genetic transformation

The two ways to manipulate plant hormone levels through a transgenic approach are the expression of genes coding for enzymes involved in hormone biosynthesis or hormone degradation (for review, see Hedden and Phillips, 2000). To date, the biosynthesis, degradation and function of auxins (Tinland *et al.*, 1991; Pandolfini *et al.*, 2002), cytokinins (Smigocki, 1991; Hewelt *et al.*, 1994; Faiss *et al.*, 1997), gibberellins (Huang *et al.*, 1998; Coles *et al.*, 1999), ethylene (Hamilton *et al.*, 1990; Oeller *et al.*, 1991) and jasmonates (Bell *et al.*, 1995; Harms *et al.*, 1995; Wang *et al.*, 1999a; Laudert *et al.,* 2000) have been studied in transgenic plants. In the experiments with the "classical" plant hormones several parameters have been determined which could interfere with the transgenic strategy. These include: (i) the site of overproduction (Smigocki, 1991; Hewelt *et al.*, 1994), (ii) metabolism of the hormone (Faiss *et al.*, 1997), (iii) gene dosis (Hewelt *et al.*, 1994), (iv) the type of the promoter controlling the transgene (Tinland *et al.*, 1991; Hewelt *et al.*, 1994), and (v) the genetic background (Pandolfini *et al.*, 2002).

Introduction of homologous or heterologous sequences into the plant genome is the usual way to get overexpression of the corresponding gene in the transgenic plants, although transgene copy number can be both positively and negatively correlated with the level of expression (Hobbs *et al.*, 1992; Stöger *et al.*, 1998). The insertion of the transgene, however is not always followed by its expression as expected. Gene silencing can occur e.g. after an initial high expression level attributed to the application of strong promoters such as CaMV 35S (Elmayan and Vaucheret, 1996). Transcriptional gene silencing (TGS) is resulted primarly by the methylation of the promoter

sequence, while post-transcriptional gene silencing (PTGS) acts, among others, via cosuppression of homologous endogenous genes by the transcribed sense transgene (for reviews, see Fagard and Vaucheret, 2000; Iyer *et al.*, 2000). Insertion of multiple copies of the transgene, as the result of direct gene transfer methods, is considered to be one of the main sources of silencing, co-suppression, however, single-copy inserts can also exert the same effect (for review, see Iyer *et al.*, 2000).

Antisense RNA, as the transcript of an introduced antisense transgene sequence, can inhibit gene expression by binding to specific complementary reginons of the target RNA. Antisense strategies are usually used among others, (i) to produce mutants, (ii) to observe steps in metabolic pathways, (iii) to identify gene functions (iv) to determine sequence/promoter specifity and (v) transcript/protein relationships, (vi) to regulate plant development as well as (vii) to improve crops. As mechanisms of action both transcritional/posttranscritional and translational control can be supposed (for reviews, see Bourque, 1995). Among cereals, relatively few transgenic plants expressing antisense genes/cDNAs have been described. In barley, the antisense-method is mainly used in transient expression systems (Huntley and Hall, 1993; Schweizer *et al.*, 2000). Transgenic barley plants expressing antisense transgenes have been reported in two cases only. Transgenic barley plants produced by heterologous transformation with antisense *cnx-1* gene from *Arabidopsis* exhibited reduced nitrate-reductase activity (Koprek, 1996), and homologous transformation with an antisense SnRk1 protein kinase sequence resulted in abnormal pollen development and male sterility (Zhang *et al.*, 2001).

2.2.3. Jasmonates

Since the first identification of jasmonic acid (JA) and its methyl ester (JM) in a fungal culture filtrate and in *Jasminum grandiflorum* L., respectively (Aldridge *et al.*, 1971; Demole *et al.*, 1962), several physiological roles of jasmonates in plants have been elucidated. First, promotion of senescence and inhibition of seedling growth were described (Ueda and Kato, 1980, 1982). Later on, inhibitory effects on root growth, pollen germination and photosynthetic activities have also been observed and exogenously applied jasmonates were found to promote fruit ripening, tuberization and accumulation of secondary metabolites (for reviews, see Sembdner and Parthier, 1993; Creelman and Mullet, 1997a,b; León and Sánchez-Serrano, 1999; Wasternack and Hause 2002).

Jasmonates are ubiquitously occurring plant hormones in angiosperms, gymnosperms, ferns, algae and fungi (Ueda and Kato, 1980; Dathe *et al.*, 1981; Meyer *et al.*, 1984; Miersch *et al.*, 1987; Ueda *et al.*, 1991; Yamane *et al.*, 1981). In higher plants the highest endogenous levels of jasmonates have been detected in young dividing tissues such as the stem apex, root tips, young leaves, flowers and immature fruits (Sembdner and Parthier, 1993; Creelman and Mullet, 1995; Hause *et al.*, 1996). A rise in their endogenous levels could be observed in response to wounding (Peña-Cortés *et al.*, 1995; Bergey *et al.*, 1999; León *et al.*, 2001), tendril coiling (Weiler *et al.*, 1993), water deficit (Lehmann *et al.*, 1995), elicitors of pathogen defense (Gundlach *et al.*, 1992; Nojiri *et al.*, 1996) and the pathogen itself (Penninckx *et al.*, 1996). Usually, elevated JA levels during developmental processes or in response to biotic and abiotic stresses are accompanied with altered gene expression (see 2.2.4.).

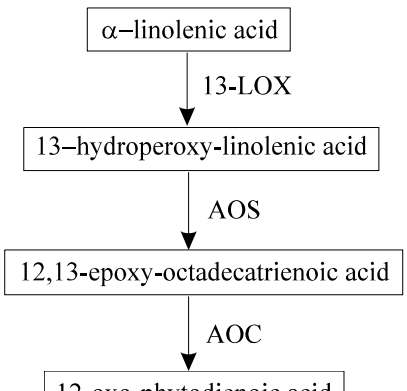


Figure 1. The biosynthetic pathway of jasmonic acid, originally proposed by Vick and Zimmermann (1984).

Jasmonates are linolenic acid (α-LeA)-derived, cyclopentanone compounds. The proposed pathway of their biosynthesis is shown in Figure 1. α-LeA is converted into 13-hydroperoxy-linolenic acid by a 13-lipoxygenase (LOX). Subsequent reactions catalyzed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) result in the cyclic 12-oxo-phytodienoic acid (OPDA). Following a reduction by OPDA-reductase and three steps of β-oxidation, (+)-7-*iso*-JA is formed, which is easily transformed to (−)-JA by spontaneous isomerization. This results in a molar ratio of about 9:1 (JA:7-*iso*-JA) within the plants. The pool of α-LeA for JA synthesis is provided by the action of ω-3 fatty acid desaturases on linoleic acid present in membrane lipids. The following multi-step enzymatic processes most probably take place in the chloroplast, while OPDA-reduction as well as β-oxidations are supposed to occur in the peroxisomes (for reviews, see Schaller, 2001; Feussner and Wasternack, 2002). Most of the enzymes involved in JA biosynthesis (LOX, AOS, AOC, OPDA-reductase) are transcriptionally up-regulated upon treatment with jasmonates or octadecanoids as well as upon exposure to biotic and abiotic factors leading to a rise in endogenous levels of octadecanoids and jasmonates (for reviews, see Wasternack and Hause, 2002).

Characterization of enzymes involved in the biosynthesis of jasmonates was performed for a number of plant species (for reviews, see León *et al.*, 2001; Schaller, 2001; for AOC, see Ziegler *et al.*, 2000). AOS represents the first enzyme within the AOS (jasmonate) branch of the LOX-pathway (Feussner and Wasternack, 2002). Its cDNAs have been cloned from flax (Song *et al.*, 1993), guayule (Pan *et al.*, 1995), *Arabidopsis* (Laudert *et al.*, 1996), tomato (Sivasankar *et al.*, 2000) and barley (Maucher *et al.*, 2000). In barley, two cDNAs coding for AOS (AOS1 and AOS2) have been cloned. The AOS protein of barley, as most AOSs analyzed to date, was detected in chloroplasts. The accumulation of AOS mRNA was observed upon treating barley leaf segments with sorbitol and glucose. These treatments also led to increased levels of jasmonates thus indicating the role of AOS in JA biosynthesis. In barley seedlings AOSs are expressed in parenchymatic cells around the vascular bundles of the scutellar node and leaf base (Maucher *et al.*, 2000), tissues exhibiting elevated JA levels (Hause *et al.*, 1996).

To date, endogenous jasmonate levels have been modified by the expression of the fatty acid ω3 desaturase, the LOX and the AOS cDNAs in transgenic dicotyledonous plants. The antisense-mediated depletion of a desaturase in transgenic potato plants led to the reduction in jasmonate content upon wounding (Martín *et al.*, 1999). Similarly, wound-induced JA accumulation decreased in transgenic *Arabidopsis* plants upon co-suppression-mediated down-regulation of LOX2 (Bell *et al.*, 1995). In both cases, however, the basal JA-level was unchanged. This suggests that either the levels of desaturase and lipoxygenase activities were not reduced sufficiently or additional enzymes are involved in JA-biosynthesis (Hedden and Phillips, 2000). To obtain elevated levels of

jasmonates, AOS was constitutively overexpressed in different plants. Surprisingly, this did not alter the basic JA-levels of untreated tobacco and *Arabidopsis* plants (Wang *et al.*, 1999a; Laudert *et al.*, 2000), whereas in potato untreated leaves showed increased JA levels (Harms *et al.*, 1995). In the latter case, however, JA-responsive gene expression appeared only upon wounding. This suggests that the elevated JA levels were sequestrated in the unwounded transgenic potato leaves (Harms *et al*., 1995).

2.2.4. Jasmonate-induced gene expression in barley

The level of jasmonates can rise endogenously upon various biotic and abiotic stresses. Such an endogenous change as well as the exogenous application of jasmonates are accompanied by alterations in the expression of various groups of genes (for reviews, see Creelman and Mullet, 1997a,b; Wasternack and Parthier, 1997; Wasternack and Hause, 2002). Jasmonates induce the synthesis of proteins involved in plant defense and signal transduction of stress responses, such as proteinase inhibitors, thionins, defensins, ribosome-inactivating proteins, chalcone synthase, lipoxygenase and calmodulin. Furthermore, genes coding for enzymes of jasmonate biosynthesis and secondary metabolism as well as seed and vegetative storage proteins are also JA-inducible. In contrast, the formation of some proteins mainly involved in photosynthesis is repressed in response to jasmonates (Reinbothe *et al.*, 1994).

The role of JA as a "master switch" has been illustrated in barley (Wasternack and Parthier, 1997). In leaf segments a thionin of 6 kDa (JIP6) (Andresen *et al.*, 1992), a 23 kDa (JIP23) and a 37 kDa protein (JIP37) (Weidhase *et al.*, 1987; Lehmann *et al.*, 1995), a 60 kDa protein (JIP60) with ribosome-inactivating properties (Chaudry *et al.*, 1994, Görschen *et al.*, 1997a) and several LOX forms (Feussner *et al.*, 1995; Vörös *et al.*, 1998) are synthesized upon treatment with jasmonates. JIPs are inducible not only by exogenous application but by endogenous rise of jasmonates as well. In contrast, *LOX2:Hv:1* (Vörös *et al.*, 1998) and a group of jasmonate-regulated genes (*jrg5*, *jrg10*, *jrg12*; Lee *et al.*, 1996) are exclusively inducible by exogenous JA.

Whereas putative functions for thionin, JIP60 and LOX forms could be proposed, the possible functions of JIP23 and JIP37 are poorly understood (Andresen *et al.*, 1992; Hause *et al.*, 1996, 1999; Leopold *et al.*, 1996). JIP37 shows partial homology to a phytase from maize (Maugenest *et al.*, 1997), but up to date no similarities to published sequences have been found for JIP23 (Andresen *et al.*, 1992). Genes coding for JIP23 were found in all cereals tested (Hause *et al.*, 1999), but only two examples are known on homologous sequences in dicots: in *Mesembryanthemum cristallinum* (H. J. Bohnert and M. Ibdah, pers. comm.) and in *Atriplex canescence* (Cairney *et al.*, 1995). In *M. cristallinum* the expression of *jip23* is related to abiotic stress (UV and salt).

Expression of genes coding for JIP23 can be induced by small amounts of exogenously applied jasmonates as well as by a low threshold of endogenous jasmonates exerted by various stress factors (Lehmann *et al.*, 1995; Kramell *et al.*, 2000). Usually up to 6 isoforms of JIP23 are synthesized upon jasmonate treatment, and they can be detected in barley seedlings as well. JIP23 and its mRNA occur specifically in cells and tissues exhibiting high osmolarity, e.g. the scutellum, scutellar node and the companion cells of phloem. This suggests that genes coding for JIP23 might be expressed in response to osmotic stresses as it appears during solute transport in developing seedlings (Hause *et al.*, 1996). While all 80 barley cultivars exhibited *jip23* expression during germination, some of them - lacking at least one JIP23 gene - failed to express *jip23* upon treatment of primary leaves with jasmonates as well as upon treatment with 1 M sorbitol. This suggests that different genes might be responsible for JA-induced expression in differentiated leaves and for developmentally regulated expression (Hause *et al.*, 1999).

Attempts have been made to obtain more information about the possible functions of JIP23 via heterologous expression of barley JIP23 cDNA in tobacco. One cDNA was sufficient to generate all the six JIP23 isoforms suggesting that JIP23 was modified posttranslationally. In transgenic tobacco plants overexpressing JIP23, several proteins such as the subunits of RuBPCase were down-regulated at the level of translation. The data suggest that discrimination among certain tobacco transcripts during translation initiation is caused by barley JIP23 (Görschen *et al.*, 1997b).

3. MATERIALS AND METHODS

3.1. INDUCTION OF HAPLOID EMBRYOGENESIS IN MICROSPORE CULTURE

3.1.1. Materials

3.1.1.1. Plant material

Barley. The experiments on the evaluation of different culture media in barley microspore culture were carried out using two genotypes: 'Igri' is a two-rowed winter-type, while 'Kymppi' is a two-rowed spring-type barley. Seeds were sown into a peat-soil mix and incubated in a greenhouse at room temperature. Two-three weeks after germination 'Igri' seedlings of 2-3 leaves were vernalized at 4 °C under continuous fluorescent light (40 $\mu molm^{-2}s^{-1}$) for 8 weeks. Donor plants were grown in a controlled greenhouse in the years 1997-1998. From the tillering stage on, fertilizer (Volldünger) was applied weekly.
Donor spikes were collected when the anthers in the most mature florets contained microspores of the mid- to late-uninucleate stage. During cold pretreatment a slow development of microspores was observed. Thus, late uninucleate to early binucleate stage was considered to be appropriate for isolation. Tillers being in the early booting stage with awns emerged about 0.5-0.8 cm from the flag leaf were cut between the 2nd and 3rd node and put into Erlenmeyer flasks containing fresh tap water. All leaves but the flag leaf were removed and the tillers were covered with a PVC bag to maintain high humidity (ca. 80% RH). Cold pretreatment of the donor spikes was performed under a dim fluorescent light at 4 °C for 14-21 days.

Triticale. Five complete hexaploid (2n=6x=42, AABBRR) winter triticale genotypes were involved in the experiments: one cultivar ('Presto') and four F_1 combinations ('Tewo x Moniko', 'Presto x Moniko', 'Presto x Novisadi', 'Novisadi x Moniko'). The donor cultivars used in the crosses are of Polish ('Presto', 'Moniko', 'Tewo') and Yugoslavian ('Novisadi') origin and all are registered cultivars in Hungary. Donor plants were grown in the field nursery during the growing season of 1995-1996. Standard herbicides have been applied according to the weed control protocol of the institute (Cereal Research Non-profit Company, Szeged). Preliminary experiments on the methods of pretreatment and microspore isolation were performed with four genotypes ('Tewo'; 'Moniko x Tewo', 'Tewo x Moniko', 'Novisadi x Tewo' F_1).

The collection and cold pretreatment of the donor spikes happened similarly to the method described above for barley. Spikes containing anthers with mid- to late-uninucleate microspores were collected when the tillers were in the late booting stage (sheath of the flag leaf open, upper spikelets emerged).

3.1.1.2. Culture media

Barley. In the evaluation of different induction media the following basic media were used (Appendix 1):
- N24A2.7G3 (further referred to as N24-BA), an LA_3 based medium with optimized N-composition (Mordhorst and Lörz, 1993) and
- 190-2 medium (further referred to as 190-BA) originally invented in wheat anther culture (Zhuang and Jia, 1983).
Both media were supplemented with 3 mM L-glutamine and 1 mgL^{-1} (4.4 μM) BAP (Mordhorst and Lörz, 1993). To study the necessity of hormones in the induction of androgenesis both media were prepared without BAP as well (N24-0 and 190-0, respectively).

Each medium contained 175 mM maltose (Scott and Lyne, 1994) and pH was adjusted to 5.8 with 1 M KOH. Osmotic pressure was checked in each preparation using an osmometer. The media were filter-sterilized and stored at room temperature.
Induced ELSs were incubated on induction media of reduced maltose content (80 mM) solidified with 0.2% Gelrite.
Plants were regenerated on hormone-free LA_3 medium supplemented with 80 mM maltose (Mordhorst and Lörz, 1992, 1993).

Triticale. For the evaluation of the effects of different hormone compositions on the induction of androgenesis, 190-2 medium supplemented with 3 mM L-glutamine and the following growth regulator combinations were applied:
- 1.5 mgL^{-1} (6.8 μM) 2.4-D and 0.5 mgL^{-1} (2.3 μM) kinetin (190-D/K),
- 10 mgL^{-1} (73 μM) PAA (190-PAA),
- no hormones (190-0).

Further preparation details were the same as detailed at barley.
Induced ELSs were cultured on solid induction medium as detailed at barley.
Plants were regenerated on hormone-free 190-2 medium without glutamine supplement (Zhuang and Jia, 1983; Pauk *et al*., 1991).

3.1.2. Methods

3.1.2.1. Determination of the developmental stage and the number of developing structures

The developmental stage of microspores was determined prior to collecting spikes and/or prior to the isolation of microspores. Anthers from a floret in the central part of the spike were squashed in a drop of water and examined under an inverted microscope.
The number of microspores was determined microscopically with a haemocytometer (Bürker). The different structures (dividing microspores, ELS etc.) developed in the cultures were counted in representative fields using an inverted microscope.

3.1.2.2. Isolation of microspores

In both triticale and barley, microspores were isolated via microblending segmented spikes based on the method described by Mordhorst and Lörz (1993). Pretreated spikes (1-10 pcs.) containing microspores of the late uninucleate to early binucleate stage (a slow progress in the development of microspores was observed during pretreatment) were removed from the leaf sheath and awns were cut down. Spikes were surface-sterilized in 2% sodium-hypochlorite for 20 min and rinsed three times with sterile water. Following sterilization they were cut into 1 cm segments and put into a 100 ml Waring Micro Blendor container (Eberbach Corp., Ann Arbour, Michigan, USA). Sixty ml of 0.3 M mannitol solution was added and microspores were isolated by blending twice for 5 sec at low speed. The quality of the maceration was visually monitored through the plastic cap of the vessel. The crude microspore suspension was filtered through 160 and 80 μm sterile nylon sieves to remove raw spike debris. The filtrate was divided between four centrifuge tubes (10 ml volume each) and centrifuged at 800 rpm for 5 min. The pellet was resuspended in 2 ml 0.3 M mannitol and the microspore suspension was carefully layered over a 0.58 M maltose solution. Following centrifugation at 600 rpm for 10 min, viable microspores were located in a band at the maltose/mannitol gradient interphase, while dead microspores and debris pelletted in the bottom of the tube. Viable cells were collected with a Pasteur pipette. They were resuspended (washed) in 0.3 M mannitol (8 ml/tube) and spun down at 600 rpm for 5 min.

3.1.2.3. Culture of microspores

Following the last centrifugation, pelletted microspores were resuspended in 1 ml of the culture medium. The quantity of microspores was determined as detailed above. Viable microspores were identified in triticale by staining with fluorescein diacetate (Widholm, 1972).
Culture density was adjusted to 0.7-1 x 10^5 microspore/ml by adding induction medium. Two ml aliquots of the microspore suspension were cultured in Petri dishes (∅ 60 mm, Greiner). Four to seven replicates per medium type (barley) or per genotype (triticale) were prepared in the different experiments.
Cultures were kept at 28 °C and 80% relative humidity in the dark. After 3-5 weeks of culture, embryo-like-structures (ELS) were plated on induction medium solidified with 0.25% Gelrite. Transfer happened using forceps in the case of slowly developing cultures yielding few ELSs. If high numbers of ELSs were formed, embryoids were transferred by pipette. ELSs were cultured at 28 °C under a 16 h photoperiod (20 $\mu molm^{-2}s^{-1}$).

3.1.2.4. Regeneration of plants

Barley. To prove that the microspore cultures are capable of generating complete plants with shoots and roots, 5-10 plantlets (ca. 2 cm height) per type of medium were transferred from solid induction medium to LA_3 regeneration medium and kept under unaltered conditions. Following tillering and rooting, plantlets were discarded without transplanting into soil, because growing up adult plants was not our aim in the barley microspore cultures.

Triticale. ELSs reaching the bipolar stage were transferred to 190-2 regeneration medium in glass culture tubes (Pauk *et al.*, 1991). The cultures were kept at 25 °C and a 16 h photoperiod. Four to five weeks after subculture the well-tillered and rooted plantlets were transplanted into non-sterilized peat/sand soil (1:1). During the following two weeks the plantlets were acclimatized in a growth cabinet (Conviron) at 25 °C, 80% relative humidity and a 16 h photoperiod (200 $\mu molm^{-2}s^{-1}$). Acclimatized plants were transferred to a conditioned greenhouse.
Vernalization was carried out in a cold chamber at 2-4 °C under continuous fluorescent light (40 $\mu molm^{-2}s^{-1}$) for six weeks.
Prior to vernalization the ploidy level of the individual plants was determined and the haploid plants were treated with colchicine to double chromosome number (Henry and de Buyser, 1980).

3.1.2.5. Determination of ploidy level

Ploidy level was determined in all plants regenerated from triticale microspore cultures. The method based on the length of stomatal guard cells (Borrino and Powell, 1988) was applied to each plant (126 pcs.), while exact chromosome number was determined in randomly chosen samples (58 pcs.) only.

The length of the stomatal guard cells was determined from a 10 mm distal leaf segment taken from microspore derived plants. Chlorophyll was extracted by incubating in 70 % ethanol overnight and the leaf segments were mounted in a drop of water on a glass slide with cover glass. The length of 10 stomatal guard cells per sample was measured (lengthwise of the guard cells) using an ocular micrometer. Plants exhibiting a stomatal guard cell length being 40-50% shorter than in control hexaploid plants, were considered to be haploids.

Chromosome numbers were determined from root tip preparations. Root tips of the donor plants were incubated in a cold chamber at 4 ºC for 24 hours. The pretreated tips were collected and treated in saturated oxiquinolin suspension for 5 h and fixed in 3:1 (v:v) ethanol:glacial acetic acid. Prior to staining in acetocarmine, root tips were hydrolysed in 1 M HCl at 60 ºC for 10 min. Squash

preparations were made in 45% acetic acid and chromosomes were counted in three well-spread cells per root tip.

3.1.2.6. Analysis of data

The evaluation of data started with the descriptive statistical analysis (mean, standard deviation, coefficient of variation) of the three androgenetic parameters (ELS/culture, albino plant % and green plant %) sorted by treatments (media). Standard deviation of data proved to be very high, therefore logarithmic transformation for data of ELS (given as non-proportional values) was performed prior to further analysis. Data of albino and green plants given as percentage were transformed using *arcsin* transformation. After transformation, data showed approximately normal distribution by using the paired two-sample t-test. Thus, further analysis was possible. Analysis of variance (ANOVA) was performed in order to analyze the effects of the three media on the three androgenic traits. In the case of the regeneration of albino and green plants, one-way multiple comparison of the means of different media was based on the LSD. The mean values in ELS production were compared in case of similar deviations by two sample t-test, and in case of different deviations by Welch-probe. ANOVA and other statistical tests (Fowler and Cohen, 1990) were computed using appropriate programmes from the MiniTab statistical package.

3.2. PREPARATION OF NOVEL VECTOR CONSTRUCTS

3.2.1. Materials

3.2.1.1. Plant material

For transformation studies the two-row spring-type barley (*Hordeum vulgare* L.) cultivar 'Salome' was used. Donor plants were grown in growth chambers (Heraeus Vötsch) under a 16 h photoperiod at 400 $\mu molm^{-2}s^{-1}$ light level, 12 °C and 70% relative humidity (Wan and Lemaux, 1994).

Barley seedlings used for protoplast isolation were grown on MS medium without hormones and sucrose at 25 °C under a 16 h photoperiod. Prior to laying on the medium mature caryopses were dehusked in 50% H_2SO_4 for 2 h on a rotary-shaker (120 rpm) and rinsed 10x in tap water. Sterilization in 3% Na-hypochlorite + 0.1% TWEEN 20 for 20 min in vacuum-exsiccator was followed by rinsing 5x in sterile distilled H_2O.

3.2.1.2. *Escherichia coli* strain

In all experiments the *E. coli* strain XL1-Blue MRF' (Bullock *et al.*, 1987; Stratagene) was used. The original strain was stored as stock (700 µl bacterium culture/300 µl 50% glycerol) at -80 °C.

3.2.1.3. Plasmids, cDNAs and oligonucleotides

In the preparation of plasmid vectors for plant transformation the **pUC18** (Norrander *et. al.*, 1983; MBI Fermentas) cloning vector containing an extensive multiple cloning site (MCS) was applied. It was used for various purposes: (1) blue/white screening, (2) integrating new restriction sites to DNA fragments and (3) subcloning DNA fragments for sequencing.

In the transient transformation experiments the following plasmids were used:

pAHC18 (Christensen *et al.*, 1992) contains the luciferase gene (*luc*) from the firefly, *Photinus pyralis* (Ow *et al.*, 1986) under the control of the maize ubiquitin (*Ubi-1*) promoter and first intron and has the *nos* terminator from *Agrobacterium tumefaciens*.

pAHC25 (Christensen *et al.*, 1992) contains the phosphinothricin-acetyl-transferase gene (*bar*) from *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) as selection marker and the β-glucuronidase gene (*uid*A) (Jefferson *et al.*, 1987). Both are under the control of the *Ubi-1* promoter and first intron and have the *nos* terminator.

Plasmids and cDNAs used for the preparation of vectors for stable transformation experiments:
pAHC20 (Christensen *et al.*, 1992) contains the *bar* gene as selection marker under the control of the *Ubi-1* promoter and first intron and has the *nos* terminator.

pWD26.41 (Dröge *et al.*, 1992) contains the *pat* resistance gene from *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988) as selection marker under the control of the 35S promoter from the cauliflower mosaic virus (Gardner *et al.*, 1981) and has a *nos* terminator.

Plasmids pAHC18, pAHC20, pAHC25 and pWD26.41 were a kind gift from Robert Hänsch (Botanical Institute, TU Braunschweig, Germany).

AOS1 cDNA of 1819 bp (Accession: AJ250864) was prepared from barley leaves treated with JM (Maucher *et al.*, 2000).

JIP23-3 cDNA of 1111 bp was prepared from barley seedlings. Among the three JIP23 cDNAs prepared simultaneously, JIP23-3 exhibited the highest homology to the others (Hause, unpublished).
Both cDNAs were cloned in the pBK-CMV vector (Stratagene).

Nucleotide sequence and application of the oligonucleotides used are summarized in Table 4.

Table 4. Nucleotide sequence and application of oligonucleotides

Name	Direction	Nucleotide sequence	Application
mcs-1	forward	5'-TGC CCG GGC ACT AGT ATC GAT CTA GAG CGG CCG CAT GCA-3'	Build multiple cloning site (MCS) in the new vectors with *Ubi-1* promoter.
mcs-2	reverse	5'-TGC GGC CGC TCT AGA TCG ATA CTA GTG CCC GGG CAT GCA-3'	
sb-85[a]	forward	5'-AAT TTG CA-3'	*Eco*RI-*Pst*I ligation.
pat-12	forward	5'-CAA TCA CTA CAT CGA GAC GAG C-3'	Amplification of the *pat* gene fragment in PCR mapping.
pat-22	reverse	5'-AAG TCG CGC TGC CAG AAC-3'	

Nucleotide sequences are shown from 5' to 3', regardless of the actual orientation. The applications are summarized briefly, detailed description can be found in the text. [a] sb-85 is used by the kind permission of Stephan Bau (unpublished).

3.2.2. Molecular biological methods

For the centrifugation of volumes up to 1.5 ml a Sigma 2K15 centrifuge, for the centrifugation of bigger volumes Eppendorf 5810R and Sorvall RC-28S (>250 ml) centrifuges were applied. Rpm values given at the different methods refer to these devices.

3.2.2.1. Transformation of *E. coli* cells

Competent *E. coli* cells were prepared from slowly saturated *E. coli* cultures (Inoue *et al.*, 1990). The cells can be stored for several months before usage. Twelve colonies of the Tetracyclin-

resistant *E. coli* strain (XL1-Blue MRF') were cultured in 250 ml SOB medium without antibiotics at 18 °C, 200 rpm. At OD_{600} = 0.5 the culture was incubated on ice for 10 min followed by centrifugation for 10 min at 4 °C, 3,000 rpm. The pelletted cells were resuspended in 60 ml TFB-buffer followed by incubation on ice for 10 min and centrifugation for 10 min at 4 °C, 3,000 rpm. The pellet was resuspended in 20 ml TFB-buffer/1.5 ml DMSO and incubated on ice for 10 min. Aliquots of 600 µl were frozen in liquid N_2 and stored at –80 °C.

For transformation cells were thawn on ice. DNA (5-10 µl of the ligation-mix) was thoroughly mixed with 150-200 µl of the cell suspension. After incubation on ice for 30 min, cells were subjected to heat-shock at 42 °C for 30 sec and further incubated on ice for 5 min. After adding 800 µl SOC medium (SOB + 20 mM glucose) without antibiotics, the cell suspension was cultured for 30-60 min at 37 °C, 200 rpm. Transformed cells (100-300 µl of suspension) were selected on LB-plates containing antibiotics.

SOB medium:
20 g/l tryptone
5 g/l yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
pH 6.8-7.0

TFB-buffer:
10 mM PIPES
250 mM KCl
55 mM $MnCl_2x4H_20$
15 mM $CaCl_2x2H_2O$
pH 6.7

3.2.2.2. Isolation and purification of plasmid DNA from *E. coli*

Isolation of plasmid DNA. The mini- and maxiprep methods applied for the isolation of plasmid DNA are based on the rapid alkaline extraction procedure (Birnboim, 1983). For miniprep 1-1.5 ml of an overnight culture of a single *E. coli* colony in LB medium was centrifuged for 2 min at 4 °C, 15000 rpm. The pelletted cells were resuspended in 150 µl Resuspension Buffer. Equal volumes of Lysis Buffer and Neutralization Buffer were added subsequently by careful mixing. The incubation on ice for 20 min was followed by centrifugation for 10 min at 4 °C, 15000 rpm. The supernatant containing the isolated plasmid DNA was added to 1000 µl 96% ethanol (2.5 x buffer volume), incubated on ice for 10 min and centrifuged for 10 min at 4 °C, 15000 rpm. The pellet was washed with 500 µl 70% ethanol. After drying plasmid DNA was dissolved in 30-50 µl TE or H_2O.

For maxiprep 2 ml of an overnight culture was added to 30 ml LB medium with antibiotics and cultured at 37 °C, 200 rpm. At OD_{600} = 0.6 the cultures were diluted to 500 ml with LB medium and incubated under the same conditions. At OD_{600} = 0.4 chloramphenicol was added to get a final concentration of 1.7 % and the culturing was continued for 14-16 hours. Isolation steps and buffers used were the same as for miniprep with the following differences: (i) 5 ml of each buffers were used, (ii) DNA was precipitated by using 10.5 ml iso-propanol (0.7 x buffer volumes) instead of 96% ethanol and (iii) plasmid DNA was dissolved in 100-500 µl TE.

Resuspension Buffer:
50 mM Tris-HCl pH 8.0
10 mM EDTA pH 8.0
100 µg/ml RNase A

Lysis Buffer:
200 mM NaOH
1% SDS

Neutralization Buffer:
3.0 M K-acetate pH 5.5

Purification of plasmid DNA with the Bio-Rad Quantum Prep® Plasmid Miniprep Kit and with the QIAGEN Plasmid Midi Kit. Plasmid DNA for nucleotide sequencing was prepared from overnight cultures according to the manufacturer's Instruction Manual of the Bio-Rad Quantum Prep® Plasmid Miniprep Kit. The purification of plasmid DNA isolated by maxiprep was performed according to the QIAGEN Plasmid Handbook (1997).

Purification of DNA using the phenol extraction method. In order to remove protein contaminants from prepared DNA or to stop enzymatic reactions, DNA solutions or reaction mixes were mixed with an equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1, v:v:v) and centrifuged for 2 min at 15000 rpm. In the case of low DNA concentration the extraction was repeated with the lower (phenol-containing) phase, in the case of high protein content with the upper (DNA-containing) phase. Phenol residues were removed by mixing and centrifuging the upper phase with equal volume of chloroform/isoamyl-alcohol (24:1, v:v). Purified DNA was precipitated with 0.1 x vol. 3 M Na-acetate (pH 5.2) and 2.5 x vol. 96% ethanol at -20 °C for 30 min. After centrifugation for 30 min at 4 °C and 15 000 rpm, DNA pellet was washed with 70% ethanol for 5 min at 4 °C, 15 000 rpm. Dry pellet was dissolved in TE.

3.2.2.3. Restriction analysis

The optimal conditions (salt concentration and composition of the buffer, incubation temperature) for digestion with one or two restriction enzymes were set according to the recommendations of Boehringer (Mannheim) and/or Fermentas (Vilnius, Lithuania). In general, ca. 1 U enzyme per 1 µg plasmid-DNA was used in a total volume of 15 µl of the appropriate digestion buffer (SuRE/Cut Buffers from Boehringer or Five Buffer Plus System from Fermentas). The time of the digestion varied between 1 hour and overnight.

To determine the optimal conditions needed for partial digestion two methods were used: (i) the time of reaction was varied while the other conditions remained constant or (ii) a set of serial dilutions of the enzyme were prepared (Ausubel *et al.*, 1999). The enzymatic reaction was stopped by heat inactivation at 65 °C or by chelating Mg^{2+} with 12.5 mM EDTA.

3.2.2.4. Gel electrophoresis and extraction of DNA from agarose gel

DNA fragments were separated by electrophoresis in 0.7-1.0% agarose gels in 1x TAE buffer supplemented with 0.05% (w/v) ethidium bromide (Sambrook *et al.*, 1989). Smart Ladder (Eurogentec) or λDNA *Eco*RI/*Hin*dIII (Fermentas) were used as DNA molecular length markers according to the manufacturers' instructions. DNA samples were loaded onto the gel in 1x Loading Dye Solution (Fermentas). Voltage and running time were set according to the size of the gel (Sambrook *et al.*, 1989). The DNA fragments were visualized and photographed in a UV transilluminator.

DNA fragments were cut out of the agarose gel and extracted by using the QIAquick Gel Extraction Kit protocol (QIAquick Spin Handbook, 1997).

3.2.2.5. Dephosphorylation and ligation

Prior to ligation vector plasmid fragments were treated with Calf Intestine Alkaline Phosphatase (CIAP) in order to prevent self-ligation of termini. Up to 1 µg fragment DNA, 10 U CIAP (Fermentas) in 1x CIAP Buffer was used independently of the size of the fragments. The treatment was performed at 37 °C for 30 min and stopped by phenol extraction followed by precipitation with ethanol (see 3.2.2.2.).

Optimal transformation efficiency was achieved when the insert:vector molar ratio was 3:1 in the ligation mix. 1U T4 DNA ligase (Fermentas) per 1 µg DNA was used in a final volume of 20 µl of the 1x ligase buffer. The ligation was performed at 14 °C for 16-20 h.

3.2.2.6. Colony hybridization

The method is based on that described by Hanahan and Meselson (1980, 1983) for plasmid screening at high colony density adapted by Sambrook *et al.* (1989).

Colony-lifting. Freshly transformed *E. coli* cells were plated on a moderately-charged nylon membrane (Schleicher & Schuell Nitrane® N) placed on selective LB medium. After 2-3 hours of incubation at 37 °C (diameter of colonies ≤0.5 mm) the membrane was peeled off the medium and two copies were prepared successively by evenly pressing the replicas to the master-membrane. The replicas were laid on selective LB medium and incubated for 1.5-3 hours. After that they were removed from the medium and prepared for hybridization as follows (3 ml solution/membrane):
- lysis of cells in 10% SDS for 3 min,
- denaturation of DNA in 1.5 M NaCl/0.5 M NaOH for 3 min,
- neutralization in 1.5 M NaCl/0.5 M Tris-HCl pH 8.0 for 5 min,
- rinsing in 0.2 M Tris-HCl pH 7.5/2 x SSC-buffer for 30 sec,
- air drying on filter paper,
- immobilizing DNAs on the membrane by baking at 80 °C for 2 h.

Preparation of radioactively labeled DNA probes. Dephosphorylated single-stranded oligonucleotide (mcs-2) (Table 4) of 39 bases was used as probe. Via forward reaction catalyzed by T4 polynucleotide kinase the terminal phosphate of [γ-^{32}P]ATP was transferred to the 5'-hydroxyl termini of the probe. The 25 µl reaction mix contained 1x reaction buffer, 20 pmol oligonucleotide, 16 pmol (50 µCi) [γ-^{32}P]ATP (3000 Ci/mmol) and 1.3 U T4 PNK. It was incubated at 37 °C for 60 min and stopped by heating to 75 °C for 10 min. Before adding to the hybridizing solution unincorporated [γ-^{32}P]ATPs were removed and the labeled oligonucleotides were desalted in a NAP™-5 Column (Pharmacia Biotech) containing Sephadex® G-25 Medium according to the manufacturer's instructions.

Prehybridization, hybridization and autoradiography. The membranes were prehybridized in Prehybridization Solution at 60 °C for 1 h. After adding 25 µl of the ^{32}P-labelled DNA probe to the prehybridizing solution the hybridization was carried out at 60 °C overnight. The membranes were washed in 6x SSC/0.1% SDS for 2x15 min at room temperature and for 2 x 20 min at 60 °C. Membranes were exposed to X-ray film at -80 °C overnight. In the case of repeated hybridization the filters were washed in boiling 0.05x SSC/0.1% SDS for 2 x 3 min after autoradiography to remove the previously hybridized probes. Following autoradiography, colonies on the master membrane respecting to signals on both of the replicas were cultured and tested as described in 3.2.2.2.-3.2.2.4.

Prehybridization Solution:
6x SSC
100x Denhardt's Reagent
100 µg/ml boiled herring sperm DNA
0.5% SDS

Denhardt's Reagent (100x):
2% Ficoll 400
2% PVP
2% BSA fraction V

3.2.2.7. Preparation of total plant DNA

Total DNA of putative transgenic barley plants was isolated using the quick miniprep method (Dellaporta *et al.*, 1983). Young leaves (100 mg) of *in vitro* regenerated plantlets were collected and frozen in liquid N_2. Following thorough grinding with a glass rod the samples were incubated in 1 ml Extraction Buffer at 65 °C for 10 min. After adding 300 µl 5.0 M K-acetate (pH 4.8) the extracts were incubated on ice for 10 min and centrifuged for 10 min at 4 °C, 15 000 rpm. Proteins were

removed from the supernatant by mixing with 800 µl phenol-chloroform-isoamylalcohol followed by centrifuging for 5 min at 12000 rpm. DNA was precipitated by adding 1 ml iso-propanol to the upper phase followed by centrifugation for 5 min at 15000 rpm. After washing with 70% ethanol and air-drying the DNA pellet was dissolved in 50-100 µl TE.

Extraction Buffer for plant DNA:
100 mM Tris-HCl pH 8.0
50 mM EDTA pH 8.0
500 mM NaCl
1.5% SDS
10 mM mercapto-ethanol

3.2.2.8. Polymerase chain reaction (PCR)

PCR reactions were set up according to Sambrook *et al.* (1989). Fifty µl reaction mix contained 0.1 µg template DNA, downstream and upstream primers 300 nM each, 0.2 mM dNTP, 1.25 U *Taq* DNA Polymerase (Life Technologies) and 1.5 mM $MgCl_2$ in 1x PCR buffer. Following an initial denaturing step at 94 °C for 3 min, the amplification cycle (94 °C for 15 sec, 52 °C for 30 sec and 72 °C for 2 min) was repeated 35 times. The reaction was finished with a final elongation step at 72 °C for 10 min. PCR reactions were performed by using an Eppendorf Mastercycler.

3.2.2.9. Protein isolation and Western-blot analysis

Extraction of proteins. Proteins were extracted from the protoplasts via the method of Meyer *et al.* (1988). Two ml 10 mM Tris-HCl (pH 8.0) and 3 ml phenol (saturated with 0.1 M Tris-HCl pH 8.0) were added to 5 ml protoplast culture and stirred for 30 min at room temperature. Following centrifugation at 5000 rpm for 10 min, proteins were precipitated by the addition of 4x volumes of 0.1 M NH_4-acetate in methanol to the phenol phase and by incubation at -20 °C for 4 h. After sedimentation of proteins by centrifugation at 5000 rpm for 10 min the protein pellet was washed 3 times with 0.1 M NH_4-acetate in methanol. The air-dried pellet was dissolved in SDS Sample Buffer facilitated by heating to 90-100 °C for 3 min.

Protein concentration was determined by the method of Esen (1978) using SDS Sample Buffer (68 mM Tris-HCl pH 8.0, 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol) and standard BSA solution (2 mg/ml) for calibration. 5 µl protein samples were pipetted to filter paper discs and incubated in the Staining Solution (25% iso-propanol, 10% acetic acid, 0.5 g/l Coomassie Brilliant Blue R-250) for 20 min. After washing 3 times with dd. H_2O (1 x cold, 2 x boiling) and drying, stained proteins were eluted from the filter discs with 3 ml 0.5% SDS solution at 55 °C for 20 min. Protein concentration was determined spectrophotometrically at 578 nm.

SDS Sample Buffer:
68 mM Tris-HCl pH 8.0
10% glycerol
2.3% SDS
5% β-mercaptoethanol

Staining Solution:
25% iso-propanol
10% acetic acid
0.5 g/l Coomassie Brilliant Blue R-250

SDS-polyacrylamide-gelelectrophoresis (PAGE) and Western-blot. Total protein (1 µg) was separated in a 12% polyacrylamide gel (Laemmli, 1970). As molecular weight marker 5 µl Prestained Protein Molecular Weight Marker (Fermentas) was used. Electrophoretic transfer of the proteins onto BA 85 nitrocellulose membrane (Schleicher and Schuell, Darmstadt) was performed by semi-dry electroblotting (Kyhse-Andersen, 1984) using Blot Buffer.

For immunostaining blots were equilibrated in TBST and blocked with 1% BSA in TBST for 1 h. Blots were incubated for 2 h with the rabbit monospecific polyclonal antibody raised against JIP23 (diluted 1:10000 in 1% BSA/TBST). After washing (4 x 10 min) incubation with the anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer, Mannheim; diluted 1:2000 in 1% BSA/TBST) was performed for 1 h. Blots were equilibrated in Reaction Buffer for 5 min and stained with p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) substrate solution (45 µl NBT stock/35 µl BCIP stock in 10 ml Reaction Buffer; stock solutions: 50 mg/ml of NBT or BCIP, dissolved in 70% and 100% DMF, respectively). The staining reaction was stopped by washing the filters with 10 mM Tris-HCl pH 8.0/1 mM EDTA.

Blot Buffer:	**TBST:**	**Reaction Buffer:**
25 mM Tris	20 mM Tris-HCl pH 7.5	100 mM Tris-HCl pH 9.5
0.1 M glycine	150 mM NaCl	100 mM NaCl
20% methanol	0.05% TWEEN 20	50 mM $MgCl_2$
0.075% SDS		

3.2.2.10. PAT-assay

Protein extracts of transformed mesophyll protoplasts (3.2.3.1.) and those of putative transgenic plants (3.2.3.4.) were incubated with [^{14}C] acetyl-CoA and PPT followed by thin-layer chromatography (de Block *et al.*, 1987, modified by Schulze, pers. comm.). The PAT-assays were performed at the Botanical Institute of the TU Braunschweig by Jutta Schulze.

3.2.3. Methods of plant cell and tissue culture

The composition of media and buffers used is given in Appendix 4 and 5.

3.2.3.1. Isolation and transformation of barley mesophyll protoplasts

The method of protoplast isolation based on that described by Schulze (unpublished). The recommended enzyme composition (4% cellulase and 0.02% pectinase), however, did not result in suitable amounts of protoplasts for our transformation experiments. In a set of trials combinations of different concentrations of cellulase (2%, 4%) and pectinase (0.01%, 0.02%, 0.06%) were tested. We found that the only combination resulting in protoplasts of suitable amount and quality was that of 2% cellulase (1 U/mg; Serva) and 0.02% pectinase (1 U/mg; Merck), therefore this was used in the transformation experiments.

Fully developed leaves of 7-10 days old seedlings were cut into pieces of ca. 2 mm. Approximately 400 mg of the leaf material was incubated in 5 ml enzyme solution (EW) on a rotary shaker (50-60 rpm) at 25 °C in the dark for 15-16 h. Protoplasts were separated from the leaf debris by filtering through a nylon mesh (56 µm) and rinsed with 2x5 ml washing solution (WLW). Following centrifugation at 600 rpm for 5 min the pelletted protoplasts were gently resuspended in 1 ml WLW. The protoplast suspension was layered on 10 ml 0.6 M sucrose and centrifuged for 10 min at 600 rpm. Viable protoplasts could be collected from the green band located in the upper (WLW) phase, whereas dead protoplasts and debris were pelletted. The protoplast suspension was diluted with 10x volume of WLW and centrifuged for 5 min at 600 rpm. To the pelletted protoplasts 1 ml WLW was added and the protoplast concentration was determined in a haemocytometer (Thoma). Following centrifugation at 600 rpm for 5 min the supernatant was thoroughly removed and the protoplast density was set to $1x10^6$/ml with MaMg/G solution. 500 µl aliquots of the protoplast suspension were used for transformation as follows (Negrutiu *et al.*, 1987):
- heat shock at 45 °C for 5 min in water bath,
- 30 sec on ice,
- adding 10 µg DNA/5x 10^5 protoplasts,

- adding 500 µl Ca/PEG solution,
- incubation at room temperature for 20 min (with occasional shaking).

Increasing amounts of WLW (1, 2, 3 and 4 ml) were added to the suspension at intervals of 2 min. Protoplasts were pelletted by centrifuging at 600 rpm for 5 min and resuspended in the culture medium (T/G) at a density of $2x10^5$/ml. Proteins for PAT-assay (3.2.2.10.) and Western-blotting (3.2.2.9.) were extracted from the protoplasts after 2, 3 and 6 days of cultivation at 25 °C in the dark.

3.2.3.2. Maize (*Zea mays* L.) suspension cultures

Suspension cultures of the maize genotype 'Black Mexican Sweet' (BMS) were cultured in 50 ml of L32 liquid medium under continuous shaking (120 rpm) at 25 °C in the dark. The medium was exchanged weekly retaining 1/3 of the original culture. For transient transformation experiments ca. 3.5 ml of the suspension was evenly spread over a sterile filter paper disc (∅ 5 cm) and placed on solid L32 medium. The bombardment was performed one day after plating.

3.2.3.3. Barley callus cultures and plant regeneration via somatic embryogenesis

Donor spikes were collected ca. 14 days after anthesis, wrapped in moist paper towels and stored at 4 °C for up to 5 days. After dehusking, the caryopses were surface-sterilized with 70% ethanol for 1 min, followed by stirring in 3% sodium hypochlorite + 0.1% Tween 20 for 15 min and rinsing 5x with sterile distilled water.

Immature embryos of 1.8-2.5 mm were aseptically excised from the caryopses under a stereomicroscope. After removing the embryo axis the isolated scutella were placed on 91/4 (2 mgL^{-1} 2,4-D) or 91/4m3 medium (3 mgL^{-1} 2,4-D) (Schulze, pers. comm.), with the scutellum exposed and the adaxial side in contact with the medium. Prior to bombardment 25 scutella close together in the middle of a 6 cm diameter Petri dish were cultured at 25 °C in the dark for 1 day. When cultured for 4-6 days under the same conditions, 25 or 45 scutella were placed apart from each other in Petri dishes of 6 or 9 cm diameter, respectively.

Calli formed on the surface of the scutella were transferred to fresh medium after about 2 weeks. Embryogenic structures formed were transferred to 91/4m1 medium of lower hormone concentration (1 mgL^{-1} 2,4-D). Appearing shoot primordia were transferred to MS0.5 medium containing 0.5 mgL^{-1} 2,4-D and lacking casein-hydrolysate, and cultured in diffuse light. Plant regeneration was performed on hormone-free MS0 medium containing 30 g/l sucrose instead of maltose under 6 h photoperiod at 160 $\mu molm^{-2}s^{-1}$ light level and 23 °C.

Osmotic treatment of the scutella. To decrease tissue damage upon bombardment, the osmotic treatment of scutella (Altpeter *et al.*, 1996a; Vasil and Vasil, 1999a) was applied in one set of experiments. Scutella were incubated on 91/4m medium for 5-7 days. Four to six hours prior to bombardment, scutella exhibiting initial steps of callus development were transferred to an identical medium supplemented with osmotica (0.2 M sorbitol + 0.2 M mannitol). After bombardment the scutella stayed still 16-20 hours on this medium then they were exposed to selection as described in 3.2.3.4.

3.2.3.4. Selection of the bombarded scutella

The *pat* and *bar* resistance genes incorporated into the vectors used for stable transformation (pUAOSsense, pUAOSantisense, pUJIPsense, pUJIPantisense, pAHC25) confer resistance against phosphinothricin (PPT). The herbicide 'Bialaphos' (a PPT-derivative containing two L-alanine

residues, produced by fermentation of *Streptomyces hygroscopicus*) was used as selective agent in a concentration of 3 mg/ml. Bombarded scutella were transferred to 91/4m medium supplemented with Bialaphos 2 days after bombardment. The transfer to fresh medium was repeated 1-2 times every 14 days. The third selection step was performed, however with scutella showing embryogenic callus formation. Further transfer of calli to media of reduced hormone content happened like described in 3.2.3.3. The only difference was the addition of 3 mg/l Bialaphos to the media (91/4m1bi and MS0.5bi). Plant regeneration was performed as described in 3.2.3.3., at a Bialaphos concentration of 1 mg/l (MS0bi).

3.2.4. Particle bombardment using the particle inflow gun

3.2.4.1. Coating of the gold particles

The coating of the gold particles with plasmid DNA was based on the method of Klein *et al.* (1988a,b) which was modified and established for the PIG by Koprek (1996). For 12 bombardments 6 mg gold particles (0.3-3 µm, Chempur or 1.0 µm, Bio-Rad) were suspended in 200 µl 70% ethanol by vortexing and sterilized for 10 min. After centrifugation at 4000 rpm for 30 sec, the pelleted gold particles were rinsed by vortexing in 100 µl sterile distilled water followed by centrifugation at 800 rpm for 15 sec. Gold particles were resuspended in 100 µl 50% sterile glycerol by sonication and put on ice for ca. 15 min. Ten µg DNA (ca. 1 µg/µl) was added to the gold suspension by careful mixing followed by 15 min incubation on ice. One hundred µl 2.5 M $CaCl_2$ and 40 µl 0.1 M spermidine were added in subsequent steps and carefully mixed by finger vortexing. After ca. 10 min sedimentation 200 µl of the supernatant was carefully removed. Prior to bombardment 4 µl gold suspension was pipetted into the center of an aluminum foil disc fitting in the syringe filter unit of the particle inflow gun (Koprek, 1996).

During the optimization of the bombardment parameters (see 4.2.4.1. and 4.2.4.2.), two further methods were applied. The coating protocol was supplemented with additional steps based on a method established for the PDS-1000 (Koprek, 1996). The coated gold particles in glycerol were centrifuged for 15 sec at 800 rpm and washed subsequently with 200 µl 70% ethanol and 100 µl 100% ethanol. Gold particles were resuspended in 45 µl or 150 µl 100% ethanol by 3 sec sonication. According to the bombardment protocol, (i) 3 µl suspension was placed in the center of an aluminum foil disc and let dry (Pauk, pers. comm.) or (ii) 10 µl suspension was pipetted onto a metal filter disc fitting in the filter unit (Stenzel, 1998).

Reduction of the amount of gold to decrease tissue damage. To decrease tissue damage caused by bombardment, the amount of gold was reduced from 6 mg (500 µg/shot) to 3 mg (250 µg/shot). This resulted in a reduced number of blue spots/scutellum in the histochemical GUS-assay (data not shown). Altogether 14725 scutella were bombarded with 250 µg gold/shot in the stable transformation experiments.

3.2.4.2. The setup of the particle inflow gun

The particle inflow gun (PIG) used in the experiments is based on the model described by Finer *et al.* (1992). It was built in the workshop of the Leiden University, The Netherlands. The vacuum chamber of the PIG is equipped with separate vents for the vacuum outlet and the air inlet and a vacuum gauge, too. The helium pressure can be set directly on the flask. The helium is driven to the compression unit through a solenoid controlled by an electric switch. After the activation of the solenoid, helium streams into the filter unit connected to the compression unit. Here it accelerates the gold particles placed either on the lower surface of an aluminum foil disc or on the upper surface of a wire screen fitted into the filter unit (see 3.2.4.1.). The accelerated particles are

dispersed by a wire mesh (∅ 5 cm) placed in the center of the upper shelf of the vacuum chamber. Besides dispersion, this mesh protects the target tissues placed on the lower shelf against direct helium pressure. Shelves can be adjusted at distances of 0, 4, 7, 10 and 13 cm from the tip of the filter unit (Fig. 14).

3.2.5. Assay for transient *luc* expression

Luciferase catalyzes the ATP-dependent oxidative decarboxylation of luciferin. The *luc* activity is measured by the amount of emitted light during the reaction (Luehrsen *et al.*, 1992).
Two days after bombardment ca. 300 mg BMS cells or 1-5 scutella were collected from the entire surface of the plate. Six hundred µl 0.1 M NaH_2PO_4 (pH 7.8) was added to the BMS cells followed by vortexing for 5 sec and centrifugation for 2 min at 10000 rpm, 4 °C. The pellet was resuspended in 200 µl extraction buffer and the cells were homogenized by sonication for 2 x 10 sec (1 min break). After centrifugation for 15 min at 10,000 rpm, 4 °C, 450 µl of the supernatant (protein extract) was put on ice. Barley scutella and leaves of tobacco plants expressing *luc* constitutively were homogenized in 200 µl extraction buffer with a pre-cooled thorn followed by sonication for 10 sec. To measure luciferase activity, 100 µl extract was given to 350 µl reaction buffer in a 10 ml tube and placed into the luminometer (Lumat LB 9501, Berthold). One hundred µl luciferin solution was automatically injected into the probe and the light emission was measured for 10 sec.

The protein concentration of the samples was determined according to Bradford (1976). Five µl extract was mixed with 1000 µl Bio-Rad protein reagent (diluted 4:1, v/v, with H_2O). After 10 min incubation at room temperature the extinction was measured at 595 nm against a clear sample. The extinction value of 0.1 corresponds to 2 µg protein in the assay. Results were expressed in terms of relative light units (RLU) per milligram protein.

Extraction buffer:
100 mM NaH_2PO_4
5 mM DTT

Luciferin stock-solution:
10 mg D-Luciferin
3.57 ml 25 mM Glycylglycine pH 7.8
aliquoted by 40 µl, stored at -20 °C

Luciferin solution:
40 µl Luciferin stock-.solution
2 ml 25 mM Glycylglycine pH 7.8

Reaction buffer:
15 mM $MgSO_4$
25 mM Glycylglycine pH 7.8
5 mM ATP

3.2.6. Histochemical assay for transient β-glucuronidase expression

The histochemical staining is based on the release of a water-insoluble indigo blue dye following the addition of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA), a substrate cleaved by β-glucuronidase at the β1 glucuronic bond between its glucuronic acid and 5-bromo-4-chloro-3-indolyl parts. The number and the distribution of the blue spots can be used as parameter for transformation efficiency.

The staining of the bombarded BMS suspension cells was carried out according to Jefferson *et al.* (1987). Two days after the bombardment 500 µl staining solution containing X-GlcA was pipetted onto the plated suspensions followed by overnight incubation at 37 °C in the dark. For the assay of scutella the improved protocol for eliminating endogenous β-glucuronidase background in barley was used (Hänsch *et al.*, 1995). Two days after the bombardment the scutella and the staining solution were pretreated for 60 min at 55 °C before pipetting 400-500 µl of the solution onto the scutella on the plate. The evaluation by counting the blue spots on the scutella's surface was carried out after overnight incubation at 55 °C in the dark.

Staining solutions for histochemical GUS-assays

from BMS suspensions (Jefferson *et al*., 1987)**:**

50 mM Na-phosphate buffer pH 7.4
10 mM Na_2-EDTA
0.1% Triton X-100
0.4% sarcosyl
2 mM X-GlcA
2% DMSO
10 mM β-mercaptoethanol

from scutella (Hänsch *et al*., 1995)**:**

100 mM Na-phosphate buffer pH 7.4
10 mM Na_2-EDTA
0.5 mM $K_3Fe(CN)_6$
0.5 mM $K_4Fe(CN)_6$
0.4 M sorbitol
2 mM X-GlcA
2% DMSO
10 mM β-mercaptoethanol

4. RESULTS

4.1. INDUCTION OF HAPLOID EMBRYOGENESIS IN MICROSPORE CULTURE

4.1.1. The microspore culture of triticale

In this part of the work the main goal was to establish a protocol for the culture of isolated microspores of triticale. The elements of the novel protocol (e.g. pretreatment, isolation of microspores, composition of induction and regeneration media) were based on those established for barley and wheat microspore culture in our laboratory (data not shown).

In the preliminary experiments the method based on pretreatment of isolated anthers was intended to be adapted. Triticale microspores isolated from anthers pretreated in 0.3 M or 0.45 M mannitol, however, did not give a satisfactory response and the plant regeneration capacity was very poor (Table 5). Microblending of spikes adapted by us for the isolation of barley and wheat microspores, however, was found to be a suitable method for initiating triticale cultures. As induction medium, first the most widely used medium of barley microspore cultures, FHG (see 2.1.1.1.) was tried without any response. Successfully were adapted and used in later experiments, however, the 190-2-based media, which were earlier used in wheat microspore culture (Puolimatka *et al.*, 1997). Preliminary experiments showed that hormone compositions used in wheat microspore culture (2,4-D + kinetin; PAA) should be used also for triticale. Induction media supplied with BAP, as applied for barley, did not give satisfactory results here (data not shown). To study hormone-free induction of androgenesis, an induction medium lacking hormone-supplement was also introduced (190-0).

Table 5. Summary of the preliminary experiments performed by microblending of pretreated anthers

Genotype	No. of replicates (10^5 ms)	Total ELS	Total plants	
			Albino	Green
'Tewo'	2	-	-	-
'Tewo x Moniko' F_1	4	-	-	-
'Moniko x Tewo' F_1	9	750	54	3
'Novisadi x Tewo' F_1	7	104	8	3

Anthers were pretreated in 0.3 or 0.45 M mannitol, microspores were cultured in 190-D/K medium.

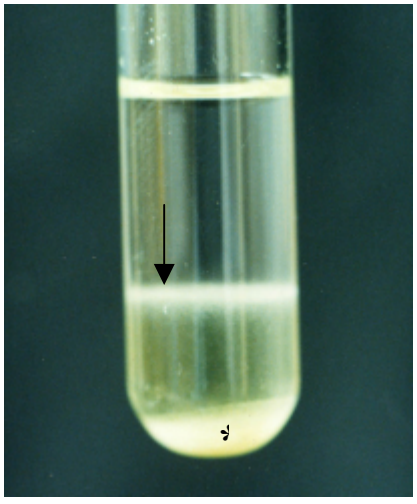


Figure 2. Separation of viable (arrow) and dead (star) microspores via density gradient centrifugation in 0.3 M mannitol (upper phase) and 21% maltose (lower phase).

4.1.1.1. Characteristic stages of triticale androgenesis in microspore culture

Triticale microspores were isolated and cultured according to a novel protocol (Fig. 4). Viable mid- to late-uninucleate microspores (Fig. 3a) could be recovered from microblended spikes after the crude macerate had been sieved twice and centrifuged with a maltose/mannitol gradient (Fig. 2). Depending on the genotype and on the homogeneity of the microspore population of the spikes, the average number of isolated microspores per spike varied on a broader or narrower range (Table 6).

Table 6. Number of isolated microspores per spikes

Genotype	No. of spikes	Isolated microspores per spike (x 10^3)
'Presto'	8	220 - 280
'Tewo x Moniko' F_1	4	260 - 280
'Presto x Novisadi' F_1	4	290 - 295
'Presto x Moniko' F_1	5	285 - 290
'Novisadi x Moniko' F_1	9	225 - 384

Spikes were homogenized by microblending.

Characteristic stages of triticale androgenesis were studied in cultures of 'Tewo x Moniko' F_1 (Table 7). Fluorescein diacetate (FDA) staining showed that 64% of the freshly isolated microspores were viable. After two days of culture, cell division could be observed at 25% of the viable micropores. The non-responding microspores exhibited plasmolysis. Nine percent of the dividing cells gave rise to multicellular colonies. These structures became visible under an inverted microscope within one week after isolation (Fig. 3b). Thirty-five percent of the multicellular colonies were able to burst through the exine and develop into globular structures (Fig. 3c). Within the next 2-3 weeks, they quickly developed into embryo-like structures (ELS) which were easily detectable on the surface of the culture medium (Fig. 3d). Merely 0.09% of the initial microspore population gave rise to ELS (Table 7).

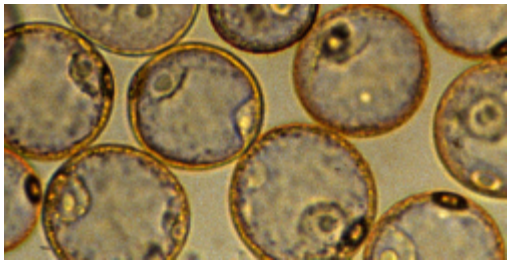
Table 7. Developmental parameters of triticale microspores

Developmental stage	No. of structures/culture	Percentage (%)	
		A*	B**
Cultured microspores	9.5 x 10^4	100	100
FDA stained microspores, after isolation	6.1 x 10^4	64	64
Viable microspores, 1 day after isolation	1.5 x 10^4	25	16
Multicellular colonies, 1 week after isolation	1.3 x 10^3	9	1.4
Globular structures, 4th week of culture	4.5 x 10^2	35	0.47
Plating efficiency, 6th week of culture	87	19	0.09

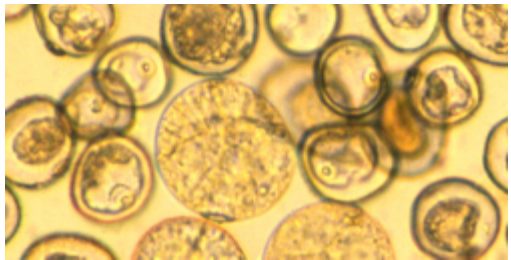
'Tewo x Moniko' F_1, means of six cultures. Plating efficiency = ELS/culture.

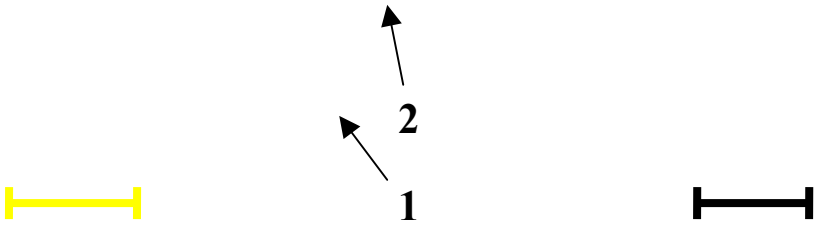
$$* A = \frac{\text{No. of structures}}{\text{No. of previous developmental structures}} \times 100 \quad ** B = \frac{\text{No. of structures}}{\text{No. of cultured microspores}} \times 100$$

a)

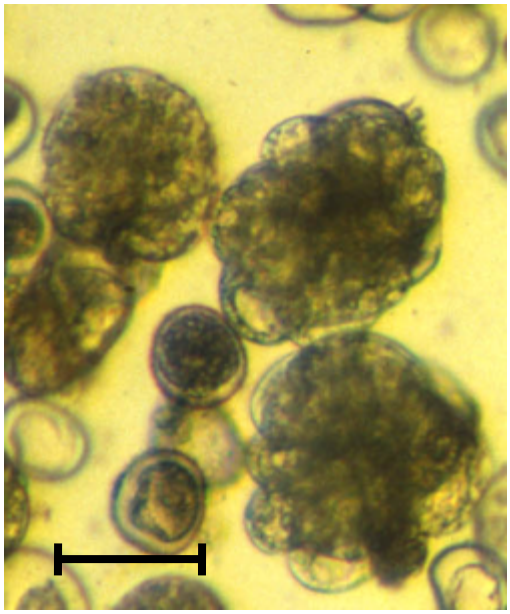


b)





c)



d)

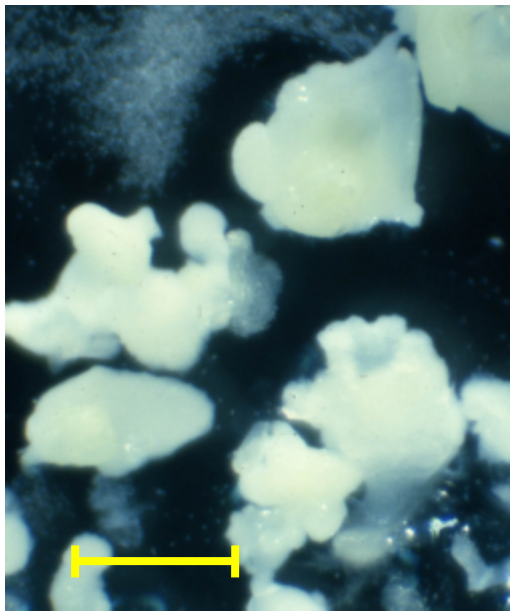


Figure 3. Developmental stages of isolated microspores of triticale in culture ('Tewo x Moniko' F_1, 190-0 medium): **a)** freshly isolated microspores of the **(1)** late-uninucleate and **(2)** early-binucleate stages, bar = 50 µm; **b)** dividing and plasmolyzed, dead microspores, one week after isolation, bar = 50 µm; **c)** microspore-derived multicellular structures (aggregates), 14 days after isolation, bar = 50 µm; **d)** ELSs (embryoids) in the induction medium, prior to transfer to solid medium, 35 days after isolation, bar = 2 mm.

During the fifth week of culture, ELSs were plated on solidified induction medium. This step was repeated with the slower growing structures in two weeks. After one-two weeks on solidified induction medium, ELSs were transferred to regeneration medium where they continued to develop. Well-developed embryoids were separated and transferred into individual regeneration vials. Here they germinated and 19% of them developed into green or albino plantlets. After the 8-9th week of culture, well-tillered and well-rooted regenerants were transplanted into soil and grown further in the greenhouse. Following the determination of ploidy-level, haploid regenerants were subjected to colchicine treatment. After vernalization (4 ºC, 6 weeks) and further cultivation in greenhouse, the regenerants produced sterile, semi-fertile (Fig. 6) and fertile spikes.

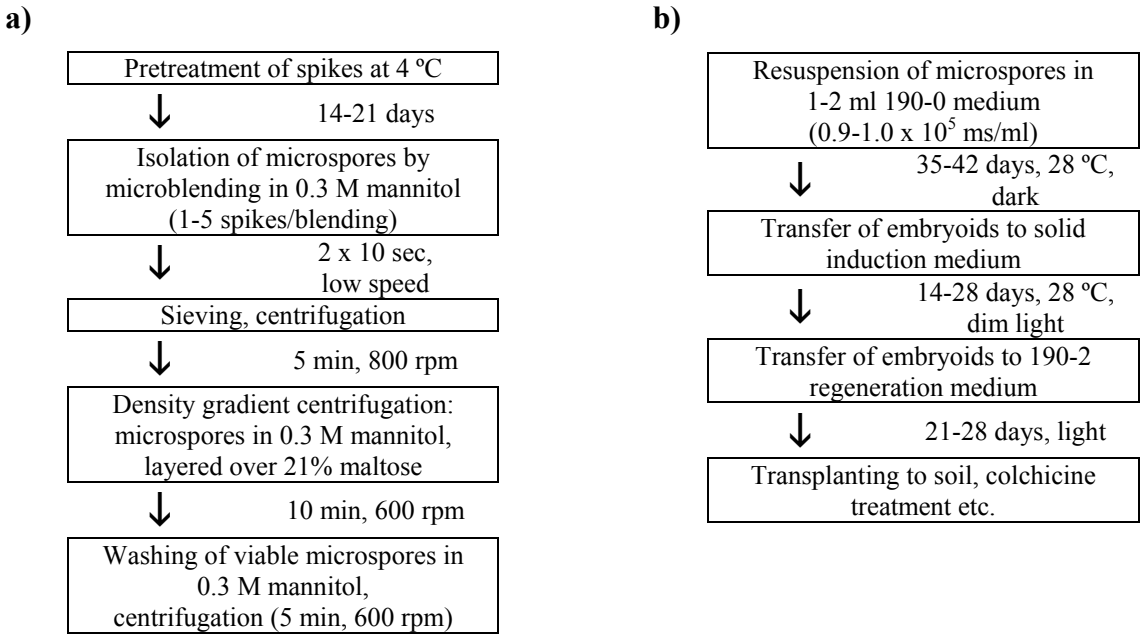


Figure 4. Isolation **(a)** and culture **(b)** of triticale microspores.

4.1.1.2. Influence of the hormone content of the induction medium on triticale androgenesis

The influence of genotype and medium on ELS production in microspore culture was studied in experiments with one triticale variety and three F_1 combinations (Table 8). Culture media both with (190-PAA, 190-D/K) and without (190-0) hormone supply supported the induction of ELSs in each genotypes tested (Table 8). The highest number of ELS per culture (118) was recorded for 'Presto x Moniko' in the 190-0 hormone-free induction medium. The ELS-induction capacity of this medium, however, varied on a broad range of 3 to 118 ELS/culture in the different genotypes. In the two media supplemented with hormones (190-PAA, 190-D/K) 5.8 to 20.7 ELS/culture has been detected (Table 8).

Table 8. Effect of genotype and culture medium on induction of ELS in triticale microspores

Genotype	Medium	No. of replicates	Plating efficiency
'Presto'	190-0	3	20.0
	190-PAA	4	20.7
'Tewo x Moniko' F_1	190-0	6	11.3
	190-D/K	5	5.8
'Presto x Novisadi' F_1	190-0	1	3.0
	190-D/K	6	15.8
'Presto x Moniko' F_1	190-0	8	118.0
	190-PAA	1	7.0

Plating efficiency = ELS/culture. Culture (replicate) = 10^5 microspores.

The effect of different culture media on the production of ELSs, green and albino plants has been evaluated in cultures of 'Novisadi x Moniko' F_1 (Table 9). 190-0 medium had a significant impact on the ELS production. In contrast, the effect of this medium on the regeneration of albino plantlets was not significant and there was only a weak effect on the production of green plantlets. The addition of PAA or 2,4-D and kinetin had no significant effect on any of the three parameters scored (Table 9).

Table 9. Values of mean (x), standard deviation (s), and coefficient of variation (CV%) for culture characteristics in three induction media

Characteristics	Medium	x*	s	CV%
ELS (number/culture)	190-0	4.64 a	0.95	20.5
	190-D/K	3.44 b	0.33	9.6
	190-PAA	2.78 bc**	0.37	l3.3
Albino plantlets (%)	190-0	28.00 a	9.12	32.6
	190-D/K	17.56 a	10.61	60.4
	190-PAA	34.83 a	18.51	53.1
Green plantlets (%)	190-0	14.84 a	10.84	73.0
	190-D/K	14.68 ab	8.85	60.3
	190-PAA	6.32 b	5.99	94.8

'Novisadi x Moniko' F_1, seven replicates. Based on transformed data (logarithmic: ELS; *arcsin*: albino and green plantlets); * Population means within a box having the same letters are not significant at p=5% level according to F protected LSD test. ** Significant at p=5% level according to two sample t-test.

ANOVA showed that the effect of media on the three traits (ELS, albino and green plant %) was different. In the case of ELS, the variance caused by differences of media was 16 times higher than variance of error (Table 10). This value was significantly lower in the case of albino and green plantlet regeneration. The effect of medium was the most significant for ELS production (p=0.01). For green plantlet regeneration it was p=0.05, while the level of significance for albino plantlet regeneration was only p=0.1. It means that induction media had a strong effect on the number of ELSs. Considering albino and green plant regeneration, however, the effect of induction medium was less significant.

Table 10. Mean squares (MS) from the analysis of variance for standard deviation (s), and coefficient of variation (CV%) of culture characteristics

Source of variation	df	ELS		Albino plant %		Green plant %	
		MS	F-test	MS	F-test	MS	F-test
Medium	2	6.179	16.17***	530	2.95*	284.7	3.69**
Error	18	0.382		179		77.2	

'Novisadi x Moniko' F_1, seven replicates. Significant at the *p=10%, **p=5%, ***p=1% level.

The effect of different media on embryo production (ELS) was statistically confirmed. Values measured on 190-0 medium are higher than those on the other two media. 190-PAA medium gave the poorest result, although it cannot be proven on 95% confidence level by ANOVA. Because standard deviation of data measured on 190-0 medium is many times higher than those measured on the two other media, means of the different media were compared using Welch-test (Table 9). The results confirmed that 190-0 medium caused a higher level of embryogenesis and its effect is significantly stronger than those of both the 190-2,4D/K (p=5%) and the 190-PAA (p=1%) media. Two sample t-test showed the differences between 190-2,4D/K and 190-PAA media to be significant. As there was no significant difference between the standard deviations of regenerated albino and green plants on the three different media, one way ANOVA was sufficient for the evaluation of the effect of media (Table 10). Different media caused no significant difference in the number of albino plants, but there was a significant difference on the 95% confidence level between 190-0 and 190-PAA media with respect to green plant production (Table 9).

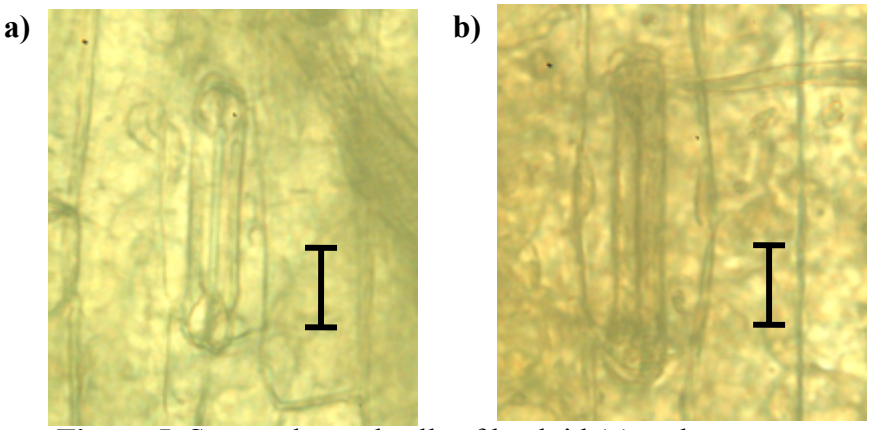


Figure 5. Stomatal guard cells of haploid **(a)** and spontaneously rediploidized **(b)** triticale plants. 'Novisadi x Moniko' F_1, bar = 10 μm.

4.1.1.3. Ploidy level of the green plantlets

A total amount of 126 green plants were regenerated and transferred to soil in the different experiments (Table 11). Regenerants were derived primarly from F_1 combinations and were therefore of potential breeding value. Before vernalization, the ploidy level of plants was determined by measuring the stomatal guard cell size and by counting chromosome number in root tip cells. Stomatal guard cells of the putative haploids were 40-50% shorter than those of the spontaneously diploidized or natural control hexaploids. Average stomatal guard cell sizes ranged 21-32 μm for haploid and 35-39 μm for spontaneous diploid plants (Figure 5; Appendix 1). Mixoploids had either guard cells of intermediate length or guard cells of both haploid and diploid size were identified in different parts of the same leaf. After this relatively quick test, 113 plants were found to be haploid, 7 were mixoploids and 6 were spontaneous diploids. For 45 haploids for all mixoploids and diploids, chromosome numbers were exactly determined from root tip preparates. A significant correlation was determined between the results of the two methods for assessing ploidy level.

Fifty-seven (45%) of the regenerants set seed after the colchicine-treatment of haploid plants (Table 11; Figure 6). From the 'Presto x Novisadi' F_1 no fertile plants were obtained. The average number of seeds per spike ranged between 13 and 20 (Table 11). Except for one 'Presto' plant, all of the spontaneously rediploidized plants were fertile. There was no significant difference in the seed set

of spontaneously rediploidized plants and of those rediploidized by colchicine treatment (Appendix 1).

Figure 6. Partially fertile spike of a triticale plant derived from microspore culture. 'Novisadi x Moniko' F_1.

Table 11. Ploidy level of regenerants from triticale microspore culture

Genotype	Transplanted plantlets	Fertile plants*	Seed/spike	Ploidy level **		
				n (3x)	mixoploid	2n (6x)
Presto	18	9 (50%)	20	15 (10)	1 (1)	2 (2)
'Tewo x Moniko' F_1	12	8 (67%)	18	11	1 (1)	-
'Presto x Novisadi' F_1	6	-	-	6	-	-
'Presto x Moniko' F_1	19	9 (47%)	13	19 (7)	-	-
'Novisadi x Moniko' F_1	71	31 (43%)	20	62 (28)	5 (5)	4 (4)
Total	126	57 (45%)		113 (45)	7 (7)	6 (6)

* In parenthesis are the fertile plant per total plant values. ** Numbers of root tips evaluated are in parentheses. Fertility of plants determined after colchicine treatment, on the basis of seed set.

The results of the experiments performed with microspore cultures of triticale can be summarized as follows:

i. An effective method for regeneration of haploid/dihaploid plants from isolated microspores has been developed for triticale.

ii. Hormone-free induction medium had a significant impact on ELS production. Its effect on green and plant regeneration, however, was not strong. Media supplemented with hormones had no significant effect on any of the three parameters.

4.1.2. Barley microspore culture

In this part of the work the primary goal was to study the induction of androgenesis and plant regeneration in barley microspore cultures without exogenous hormone-supplement. Furthermore, the hormone-free induction medium (190-0) efficiently used in triticale microspore culture (see 4.1.1.) was also tested in barley, comparing its effect on plant regeneration with those of an induction medium optimized for barley microspore culture (N24-BA). The studies were performed in two steps:

i. Isolated microspores of cultivar 'Igri', the model genotype of barley haploid tissue cultures, were induced in N24-BA medium as well as in modified 190-2 medium, both with and without hormone supplement (Table 12, Fig. 7). N24-BA is a medium of optimized nitrogen composition. It was established exclusively for microspore culture of barley and it contains 1 mgL^{-1} BAP (Mordhorst and Lörz, 1993). Hormone-free N24 is designated as N24-0. 190-BA and 190-0 media - with and without BAP-supplement, respectively - are based on 190-2 medium invented for wheat anther culture (Zhuang and Jia, 1983, Pauk *et al*., 1991). Furthermore, 190-0 and its variants supplemented with other hormones (2,4-D, kinetin or PAA) were successfully used in microspore culture of triticale (see 4.1.1.2.).

ii. Isolated microspores of the cultivars 'Igri' and 'Kymppi', a genotype successfully involved in microspore culture (Salmenkallio-Marttila, 1995; Ritala *et al.*, 2001), were induced in N24-BA medium as well as in 190-0 medium. N24-BA is an optimized induction medium for barley microspore culture (see above), while 190-0 was found to induce the highest number of embryoids in triticale microspore culture (Tables 8 and 9).

4.1.2.1. Isolation and culture of barley microspores

Microspores of the late-uninucleate to early-binucleate stage were isolated by microblending of cold-pretreated spikes. It was observed that blending of up to 30 spikes at the same time and repeated blending of the sample (as suggested by Mordhorst and Lörz, 1993) did not result in a higher yield of isolated microspores (data not shown). If the spikes contained microspores of the required stage of development, blending of a single spike could result in 0.7-0.9 x 10^5 microspores (cv. 'Kymppi'), a sufficient amount for one culture plate. To offer an amount of microspores, possibly sufficient for more plates, five spikes were blended two times for 5 sec at low speed. Following cleaning of microspore suspension by sieving and density-gradient centrifugation, microspores were cultured at a density of 0.7-1.0 x 10^5/ml.

Non-responding microspores exhibited plasmolysis in the first four days of culture. Cell aggregates originating from dividing microspores could be observed after 7-14 days of culture, depending on genotype. In spite of this early proliferation, however, at several genotypes studied in the preliminary experiments, the aggregates arrested in development and died showing a necrotic symptom. No plasmolysis could be observed in these cases (data not shown). At cultivars 'Igri' and 'Kymppi', the first embryo-like structures (ELS) could be observed after 21-28 days of culture, irrespective of genotype and medium. These embryoids were transferred to solid induction medium after 32-38 days of culture. Slower developing structures were transferred 7-14 days later. In the case of a lower number of embryoids (up to 150), the transfer happened by using a forceps. At a higher number of ELSs, a Pasteur-pipette made of plastic was used for transfer. The highest number of embryoids was detected in 190-0 medium (76 ELS) at the first transfer. It was followed by N24-BA (35 ELS) and N24-0 (22 ELS). The lowest number of embryoids was registered in 190-BA (11 ELS). This order has been determined in two experiments. Further transfers were performed with pipette, thus total embryo production could not be calculated.

Germination of embryoids on solid induction media could be observed from the seventh day following transfer, irrespective of cultivar and medium. These plantlets usually had no or very poor root system on the induction medium (Fig. 7). To check whether they can develop into complete plantlets, randomly chosen plantlets of up to 2 cm height were transferred to hormone-free LA_3 medium. The numbers of plantlets involved were 95 and 22 for cv. 'Kymppi' (from N24-BA and 190-0 medium, respectively) and 10, 20, 20 and 13 for cv. 'Igri' (from N24-BA, 190-BA, N24-0 and 190-0 media, respectively). Each case, the development of plantlets continued on LA_3, reaching a well-rooted state ready to be transplanted into soil.

4.1.2.2. Effects of different induction media on barley androgenesis

Table 12 and Figure 7 show green plant production from microspore cultures of 'Igri' induced in four different media. Due to the high values of CV% (>100%), statistical analysis of data was not possible (data not shown). Values of mean (x) are given for information only (Table 12).

Induction on N24-BA medium resulted in the highest number of green plants in each experiment except for No. 3. Except for the result in this experiment, 190-0 medium resulted in the lowest number of green plants. Although mean values suggest N24-0 being the second best medium, this result is based on one peak result (52 green plants/culture) achieved in Exp. 4. 190-BA medium gave better result than N24-0 in three experiments (Exp. 2, 3, 5), it was inferior in two cases (Exp. 4, 6) and the results were equal in the first experiment.

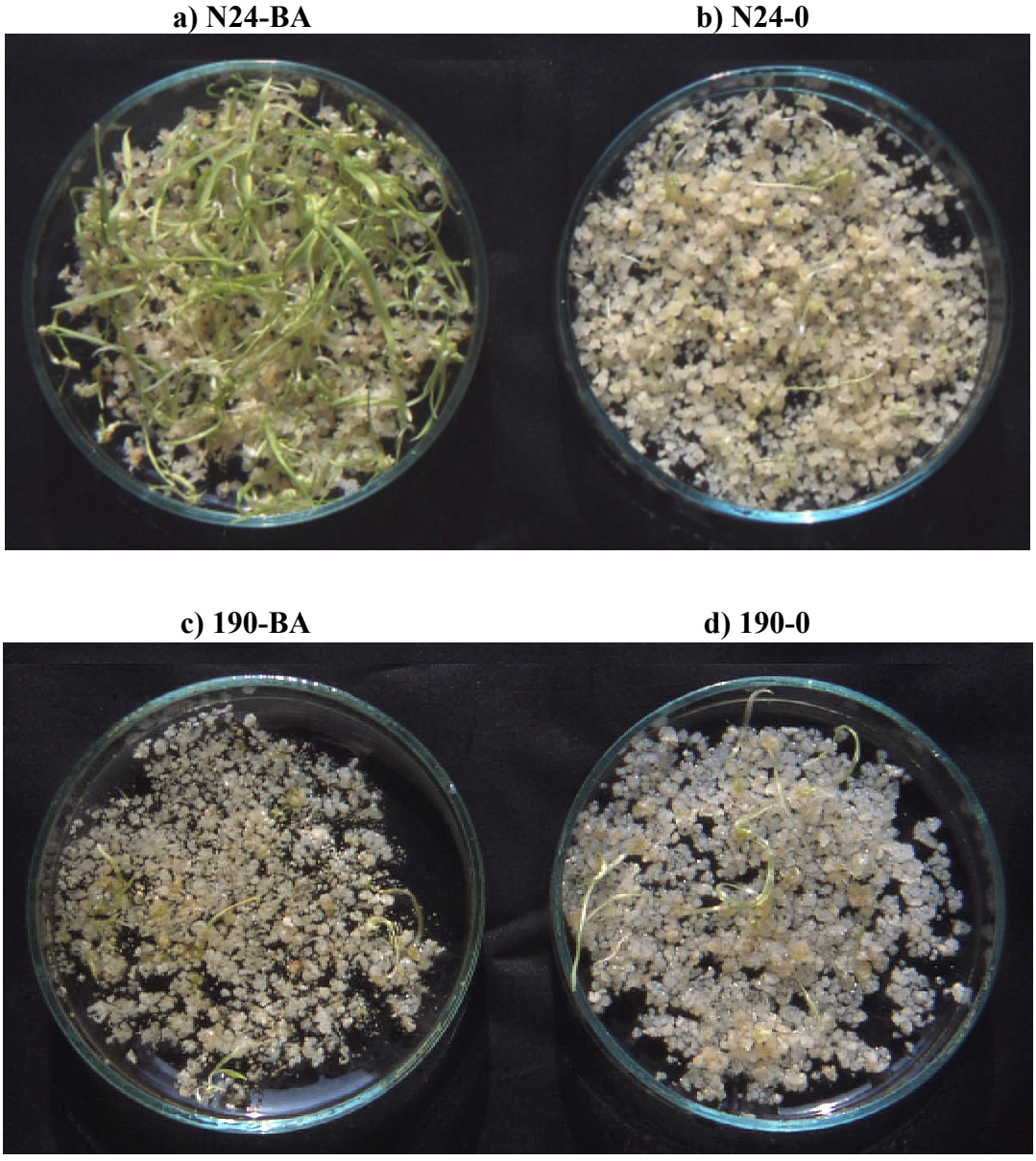


Figure 7. Regeneration of barley plantlets (cv. 'Igri') on **(a)** N24-BA, **(b)** N24-0, **(c)** 190-BA and **(d)** 190-0 medium.

Table 12. Effect of different induction media on the green plant regeneration in isolated microspore culture of cultivar 'Igri'

Exp.	Green plants/100,000 ms			
	N24-BA	190-BA	N24-0	190-0
1.	2	0	0	0
2.	38	13	0	1
3.	8	6	1	25
4.	93	3	52	0
5.	42	24	3	-
6.	259	3	4	-
x	73.7	8.2	10.0	6.5

Culture density: 100,000 ms/ml. Values of mean (x) are given for information only.

4.1.2.3. Hormone-free induction of androgenesis in microspore cultures of barley

The evaluation of the BAP-supplemented N24-BA and the hormone-free 190-0 media was performed in separate experiments. Thus, results are interpreted by giving numbers of green and albino plantlets originating from the same culture plate. Due to the low number of data, values of mean (x), standard deviation (s) and coefficient of variation (CV%) are given for information only.

Tables 13 and 14 show that cultivar 'Igri' regenerate 9-fold higher numbers of green plants per culture plate (294.3) than cultivar 'Kymppi' (33.8), if microspores are induced on N24-BA medium. In the case of 190-0 medium, however, there is no significant difference between the two cultivars. Thus, the regeneration capacity of N24-BA medium is 27-fold higher than that of 190-0 in the case of 'Igri'. In the case of 'Kymppi', however, only a 3-fold difference between the regeneration capacities of the two media could be observed.

Table 13. Regeneration of green and albino plants from microspore cultures of cultivar 'Igri' in **(a)** N24-BA and in **(b)** 190-0 induction media

a) N24-BA

No. of culture	Regenerated plants/100,000 ms	
	Green	Albino
1.	224	9
2.	353	12
3.	306	8
x	294.3	9.7
s	53.31	1.70
CV%	18.1	17.5

b) 190-0

No. of culture	Regenerated plants/100,000 ms	
	Green	Albino
1.	8	0
2.	14	1
x	11	0.5
s	3	0.5
CV%	27.3	100.0

Culture density: 100,000 ms/ml. Values of mean (x), standard deviation (s) and coefficient of variation (CV%) are given for information only.

The percentage of albino plants regenerated from N24-BA medium was 3% at 'Igri', while it was 52% at 'Kymppi'. On 190-0 medium, there was no significant difference between the two cultivars: percentage of albino plants was 4.3% for 'Igri' and 10% for 'Kymppi'.

Table 14. Regeneration of green and albino plants from microspore cultures of cultivar 'Kymppi' in **(a)** N24-BA and in **(b)** 190-0 induction media

a) N24-BA

No. of culture	Regenerated plants/100,000 ms	
	Green	Albino
1.	36	47
2.	52	47
3.	29	24
4.	18	29
x	33.8	36.8
s	12.34	10.40
CV%	36.50	28.26

b) 190-0

No. of culture	Regenerated plants/100,000 ms	
	Green	Albino
1.	0	0
2.	9	0
3.	26	4
x	11.7	1.3
s	10.78	1.89
CV%	92.1	145.4

Culture density: 100,000 ms/ml. Values of mean (x), standard deviation (s) and coefficient of variation (CV%) are given for information only.

4.1.2.4. Comparison of the nitrogen composition of N24-BA and 190-0 media

Due to the differences in the responses of barley cultivars to N24- and 190-2 based media (see 4.1.2.2. and 4.1.2.3.), the N-composition of 190-0 medium was calculated and it was compared with the optimized N-composition of the N24-BA medium (Table 15). This calculation was made to answer the question whether the N-composition of 190-2-based induction media successfully used in triticale microspore culture (see 4.1.1.) corresponds to the optimal nitrogen amount and proportion values determined for barley by Mordhorst and Lörz (1993).

Considering inorganic N sources, KNO_3 is the only common component in the two media. 190-0 medium contains an additional nitrate source [$Ca(NO_3)_2x4H_2O$] as well, and the ammonium source [$(NH_4)_2SO_4$] is also different from that of N24-BA (NH_4Cl). Total inorganic nitrogen content of 190-0 (13.8%) is 50% of that optimized in N24-BA medium (27%). This is due to the extremely low nitrate content of the former one. While ammonium contents are similar, NO_3^-/NH_4^+-ratio of 190-0 (78:22) is also below the optimal ratio (90:10) determined by Mordhorst and Lörz (1993).

In contrast to inorganic nitrogen, there is no difference in glutamine content of the two media (3 mM). Differences in inorganic N/organic N rates are not significant either. The rate calculated for 190-0 (84:16) is inside the optimal range (71:29 to 90:10) determined for N24-BA medium.

Table 15. Nitrogen composition of induction media

Component	N24A2,7G3	190-0
KNO_3	24.3	9.9
NH_4Cl	2.7	-
$(NH_4)_2SO_4$	-	3.0
$Ca(NO_3)_2x4H_2O$	-	0.9
total N_{inorg}	27.0	13.8
Glutamine	3.0	3.0*
total N	30.0	16.6
NO_3^-/NH_4^+	90:10	78:22
N_{inorg}/N_{org}	90:10	83:17

Values are given in mM nitrogen. For composition of the basic media, see Appendix 2. * Supplemented with 222 mgL^{-1} glutamine.

Due to the low concentration of inorganic nitrogen, the total N-content of 190-0 medium (16.6 mM) is significantly lower than that of N24-BA (30 mM). Furthermore, this value is below the minimum

nitrogen amount (20 mM) found to be necessary for plant regeneration at the high level (Mordhorst and Lörz, 1993). According to the nomination system of Mordhorst and Lörz (1993), 190-2-based media can be designated as N11A3G3.

The results of the experiments performed with microspore cultures of barley can be summarized as follows:

i. In microspore cultures of the barley cultivars ‘Igri’ and ‘Kymppi’, induction of androgenesis without exogeneous hormone-supplement is possible in both N24- and 190-2-based media. The regeneration capacity of embryoids derived on hormone-free induction media is significantly lower than the regeneration capacity of those from hormone-supplemented media.

ii. Both N24- and 190-2-based induction media are suitable for the induction of androgenesis in cultivars ‘Igri’ and ‘Kymppi’. The regeneration capacity of embryoids derived in N24 medium is higher than the regeneration capacity of those from 190-2, both with and without hormone-supplement.

iii. Regeneration rate of ‘Igri’ was significantly better than that of ‘Kymppi’ if microspores were cultured in N24-BA induction medium. Similar regeneration rates were received, however, in the case of induction in hormone-free 190-0 medium.

iv. Comparison of nitrogen composition shows that total N-content of the 190-0 medium (16.6 mM) is significantly lower than that of N24-BA (30 mM). This is due to its low nitrate, consequently, inorganic N-content. Ratio of inorganic N/organic N, however, is inside the optimal range.

4.2. PREPARATION OF NOVEL VECTOR CONSTRUCTS

The goal of this part of the work was to produce novel plasmid vector constructs which are suitable for the stable transformation of barley via particle bombardment. As this work meant the first step in a research project on the role of jasmonates in the development of barley, cDNAs coding for barley AOS or JIP23 were inserted into the novel vectors in sense or antisense orientation, respectively.

Additionally, a plant regeneration system based on somatic embryogenesis was adapted and bombardment parameters were optimized to enable the first use of the novel vectors in preliminary, stable transformation experiments.

4.2.1. Preparation of novel vector constructs

Plasmid vectors to be used for the stable transformation of barley scutella were designed to carry two individual parts: (i) the transgene of importance and (ii) the selection marker gene, both with the corresponding regulator sequences. The basic plasmid background containing the sequences coding for bacterial antibiotic resistance was derived from pAHC20. The cDNAs coding for AOS1 and JIP23, respectively, were cloned into this plasmid under the control of the *Ubi-1* promoter. Both cDNAs replaced the *bar* gene of the original plasmid. The *pat* selection marker gene driven by the 35S promoter was imported as a fragment of the pWD26.41 plasmid. The three main steps of cloning were as follows (Fig. 8):

1. The *bar* gene was removed from pAHC20 by partial digestion with the *Pst*I restriction enzyme. Multiple cloning site (MCS) of 35 base pairs, composed of oligonucleotides mcs-1 and mcs-2 (Table 4), was inserted between the *Pst*I sites. As the MCS (Fig. 11) completes the restriction sites with T instead of G, *Pst*I cannot recognize the original sites any more. Transformations were repeated three times with ligation mixes containing MCSs dephosphorylated or not-dephosphorylated prior to annealing, respectively. As miniprep assay was ineffective in finding colonies containing MCS, the clones were selected via radioactive colony-hybridization. Sixty-four positive clones were obtained, 35 of them originating from ligations with dephosphorilated MCSs. The restriction analysis of these clones confirmed the positive result at three clones of the latter type. The orientation of the MCS (sense or antisense) was determined by sequencing. For this purpose, MCS-containing fragments of the three selected clones were inserted into pUC18 cloning vectors by *Eco*RI ligation.

2. The modified pAHC20 clones (pAHC20m) containing the sense or antisense MCS were opened by *Hin*dIII and *Pst*I cleavage and the 1689 bp *Eco*RI-*Hin*dIII fragment of the pWD 26.41 plasmid was inserted. A 7 base oligonucleotide (sb-85) was necessary to make the *Pst*I-*Eco*RI ligation possible (Table 4).

3. Both the sense and the antisense clones were cut by *Not*I and *Spe*I (inside the MCS). Fragments of AOS1 (1886 bp) and JIP23 (989 bp) cDNAs digested with the same enzymes were inserted into the sense and antisense vectors, respectively.

In the further experiments the vectors containing the cDNAs in sense orientation are referred to as pUAOSsense and pUJIPsense and those containing them in antisense direction, as pUAOSantisense and pUJIPantisense (Fig. 9 and 10).

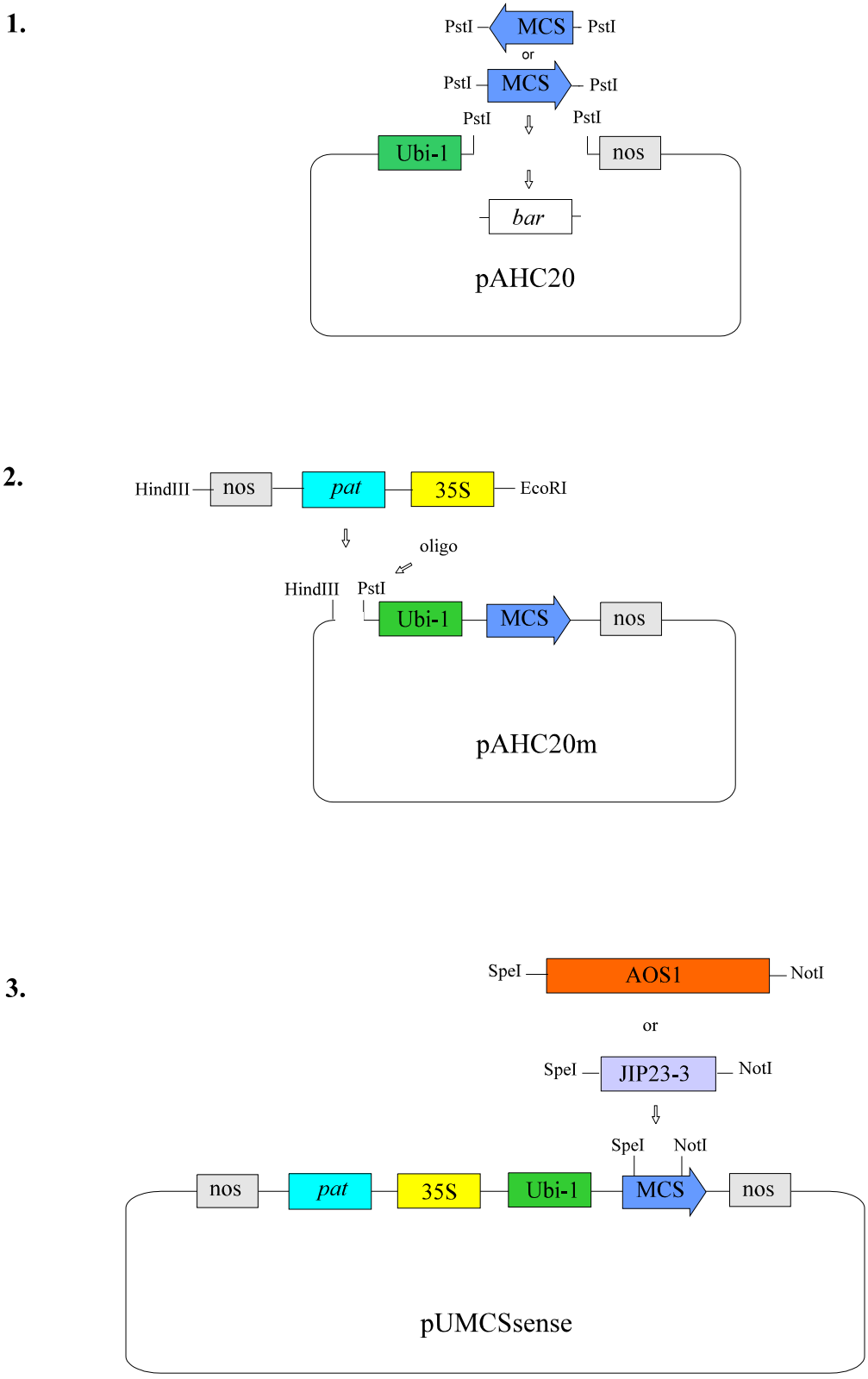


Figure 10. The scheme of preparation of the novel vector constructs.
See text for details. In steps 2 and 3 only the preparation of the sense constructs is shown.

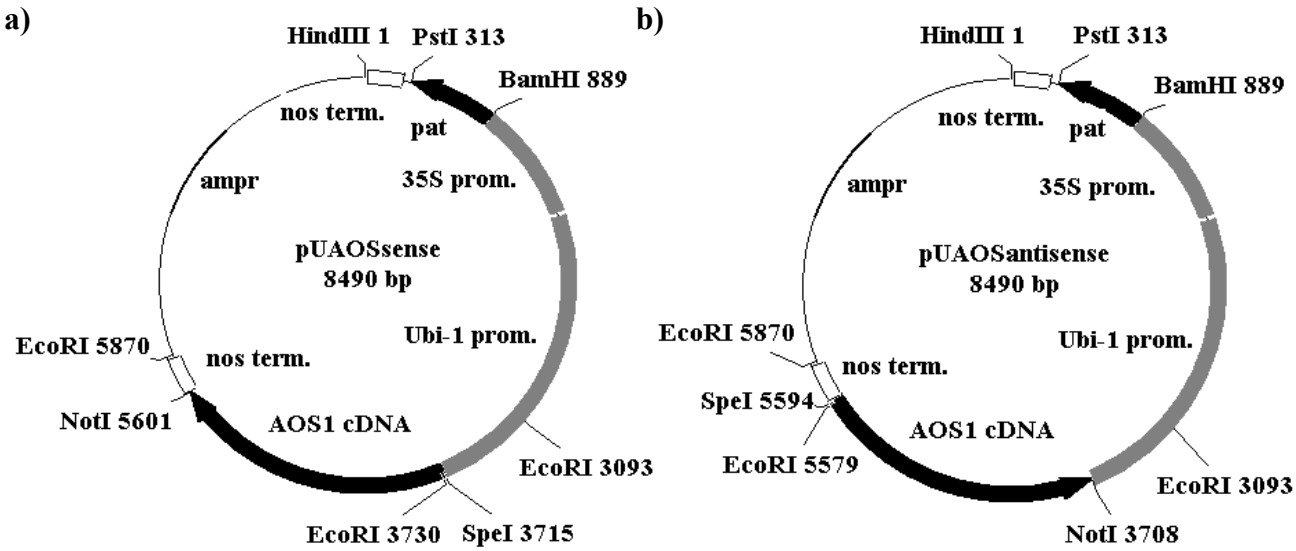


Figure 9. Plasmid vectors carrying the AOS1 cDNA in sense (a) and antisense (b) orientation.

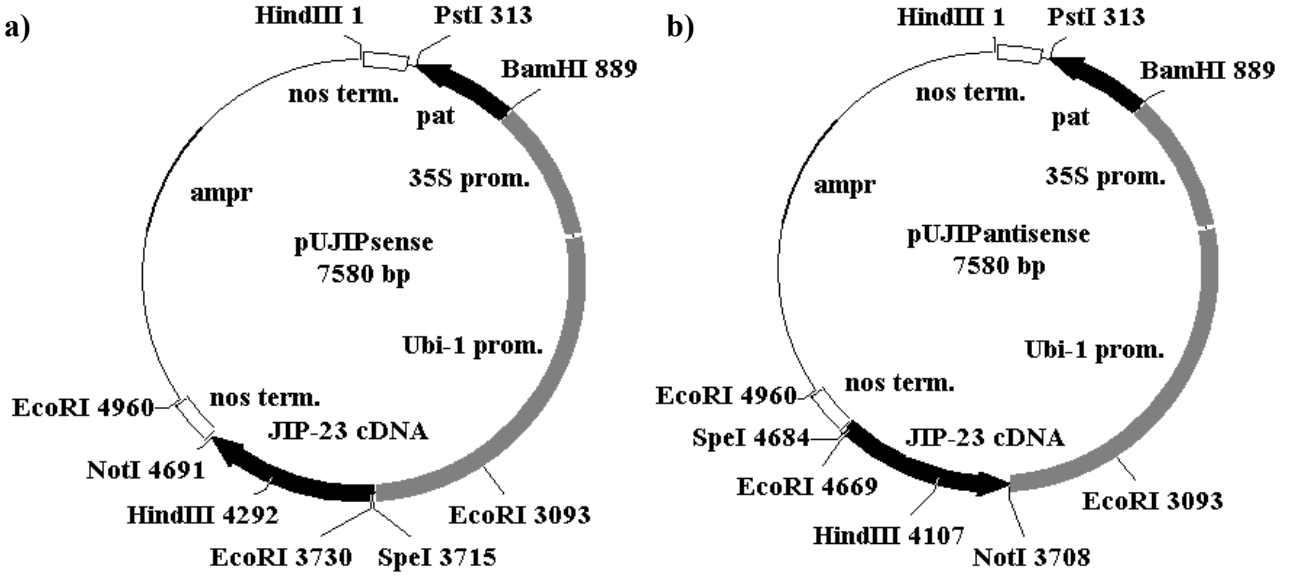


Figure 10. Plasmid vectors carrying the JIP23 cDNA in sense **(a)** and antisense **(b)** orientation.

The plasmid constructs derived after the second preparation step contain the MCSs in sense or antisense orientation (pUMCSsense and pUMCSantisense, respectively). The MCSs containing restriction sites for at least ten endonucleases (Fig. 11) make these vectors a useful tool for the cloning and expression of other genes/cDNAs than those described here. An example for such an application is the insertion of a barley LOX cDNA (Vörös *et al.*, 1998) into the sense construct. This latter vector was prepared for the request of the Botanical Institute, TU Braunschweig and was not used in our work (data not shown).

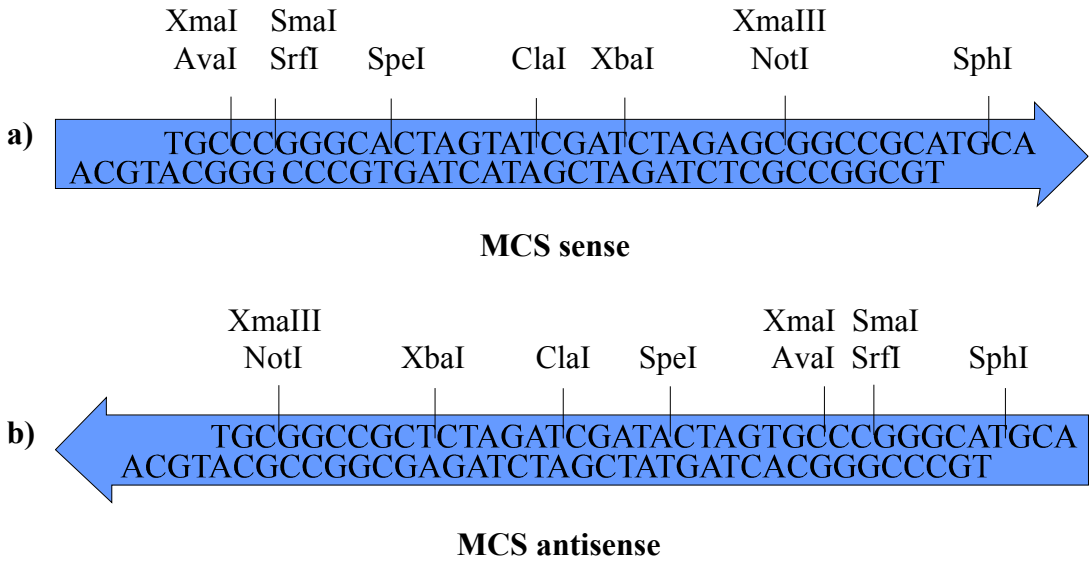


Figure 11. Restriction sites of the multiple cloning sites in sense **(a)** and antisense **(b)** orientation.

4.2.2. Assays for transient gene expression in barley mesophyll protoplasts

Before starting stable transformation experiments with the novel vector constructs, the expression of both the structural and the resistance genes in the genetic background of barley was checked. For transient expression mesophyll protoplasts were isolated from cultivar 'Salome', the model genotype of the studies on JAs and JIPs. This genotype was intended to give the target material for the first set of stable transformations as well. Vectors carrying the AOS1 and JIP23 cDNAs in sense orientation under the control of the *Ubi-1* promoter (pUAOSsense and pUJIPsense) were introduced into protoplasts by PEG-mediated transformation. The activity of PAT was detected by radioactive PAT-assay and the occurrence of JIP23 upon transformation was analyzed by Western-blotting.

4.2.2.1. Transient expression of the *pat* gene in barley mesophyll protoplasts

The expression of the *pat* gene controlled by the 35S promoter in mesophyll protoplasts of barley revealed the activity of the transgene in the genetic background of barley. Protein for the PAT-assay was extracted from the protoplasts after two days of cultivation.

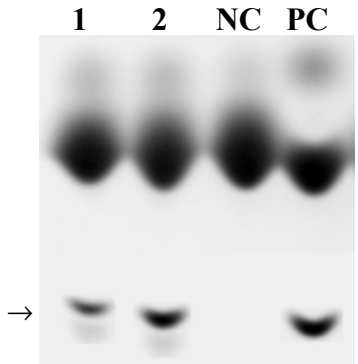


Figure 12. PAT activity in barley mesophyll protoplasts.
Lanes 1 and 2: protoplasts transformed with pUAOSsense and pUJIPsense, respectively; NC: negative control (non-transformed protoplasts); PC: positive control (transgenic tobacco carrying the *bar* gene); arrow: acetylated L-PPT.

Positive signals indicating PAT-activity were detected in samples originating from both the pUAOSsense- and the pUJIPsense-transformed protoplasts (Fig. 12).

4.2.2.2. Transient expression of JIP23 cDNA in barley mesophyll protoplasts

The appearance of AOS1 protein and JIP23 following transformation of barley protoplasts with the corresponding cDNA can certify that both sequences were integrated and expressed under the control of the *Ubi-1* promoter. Protoplasts were collected 2, 3 and 6 days after transformation with the pUJIPsense vector and used for Western-blot analysis. The highest amount of JIP23 was detected on the second day of cultivation followed by a decrease to an undetectable level until the 6th day of incubation (Fig. 13).

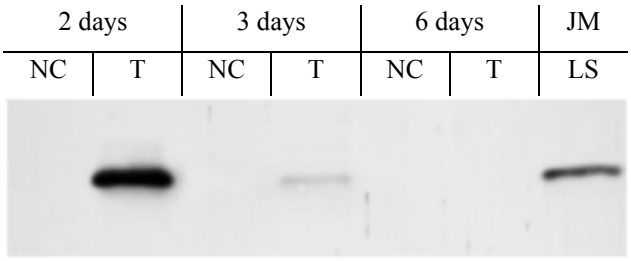


Figure 13. Accumulation of JIP23 in protoplasts transformed with pUJIPsense. Accumulation of JIP23 in nontransformed (NC) and transformed (T) protoplasts 2, 3 and 6 days after transformation compared with that occurring in barley leaf segments (LS) upon 24 h JM treatment.

The low amount of total protein isolated from protoplasts transformed with the pUAOSsense construct, did not allow the Western-blot analysis of AOS1.

4.2.3. Somatic embryogenesis of barley

To enable efficient stable transformation experiments, somatic embryogenesis was studied in the barley cultivar 'Salome' using isolated scutella without bombardment. For the induction of somatic embryogenesis, MS-based media of different hormone concentration were applied: scutella were placed on 91/4 medium containing 2 mg/l 2,4-D or on that with 3 mg/l 2,4-D (91/4m3). Embryogenic as well as hard and soft calli could be distinguished on the surface of scutella (data not shown). Embryogenic structures developed quickly and gave rise to fast-growing leaf/shoot primordia. After 2-4 subsequent transfers to media of lower hormone concentration, plants were regenerated on hormone-free MS medium (Table 16).

As many as 706 (78.5%) of the scutella induced at 2 mg/l and 110 (54.7%) of those induced at 3 mg/l 2,4-D concentration gave rise to hard calli or embryogenic structures which were transferred further in the regeneration process. In the first set of experiments 103 green and 21 albino plantlets were regenerated, corresponding to a regeneration rate of 11.5% and 2.3%, respectively. In the second set 29 green plantlets were found, corresponding to a regeneration rate of 14.4% (Table 16).

Table 16. Plant regeneration via somatic embryogenesis in barley scutella

Induction medium	Isolated scutella	Hard/embryogenic calli		Green plants		Albino plants	
	No.	No.	Responsivity	No.	Regeneration rate	No.	Regeneration rate
91/4	899	706	78.5%	103	11.5%	21	2.3%
91/4m3	201	110	54.7%	29	14.4%	0	0.0%

Responsivity and regeneration rate are expressed as % of the number of isolated scutella.

The size of the isolated scutella also influenced the development of calli. Small scutella (<1.0 mm) did not show any proliferation of the epidermal cells. Large scutella (>3.0 mm) showed swelling without callus formation on the adaxial side. Soft calli appearing on the abaxial side and germination of residual parts of shoot/root primordia were common features of this size of scutella. In contrast, scutella of medium size (1.5-2.5 mm) gave rise to all types of calli. Embryogenic

structures appeared on scutella of this size only, thus making them the best targets for stable transformation.

4.2.4. Optimization of the bombardment parameters

In order to achieve the highest transformation rate, the adjustment of the bombardment parameters for the given plant material is necessary. Due to the lack of a manual prepared exclusively for the PIG used in our experiments, the basic data for the initial steps of optimization were taken from two sources: (i) a transient assay with tomato cell suspensions bombarded with the same device (Stenzel, 1998) and (ii) a protocol for PIG which was successfully applied in the production of transgenic barley plants (Koprek, 1996). Additional information was taken from the original description of the PIG-system by Finer *et al.* (1992). First the parameters applied in the above-mentioned works were tested and later on adapted to our conditions.

The optimization was performed by the evaluation of the transient expression levels of the *uidA* and the *luc* genes, both under the control of the *Ubi-1* promoter. The genes were cloned in the pAHC25 and pAHC18 plasmids, respectively. In the first series of experiments, BMS maize suspension culture was used. Maize suspension cells lack endogenous or unspecific β-glucuronidase activity and exhibit strong *uidA* and *luc* expression. These experiments were taken as preliminary experiments and the results gave the basis for the subsequent optimizations with isolated barley scutella.

The parameters, which were necessary to be optimized, can be divided into two groups: (i) parameters adjusted directly on the PIG (He-pressure; filter unit/mesh, filter unit/target plate and mesh/target plate distances; vacuum pressure) and (ii) parameters connected with the bombardment method (gold amount per bombardment; type of macrocarrier; composition of the gold suspension), including the coating of the gold particles with DNA. According to the results of preliminary experiments (data not shown), each bombardment was performed at a vacuum pressure of 200 mbar.

Some additional factors like the age and plating of the target tissues also had strong influences on the activity of LUC and GUS. In order to make a proper evaluation possible, the experiments were evaluated individually instead of calculating mean values for each parameter. The sequences of the results from each experiment gave suitable information for the optimization of all bombardment parameters.

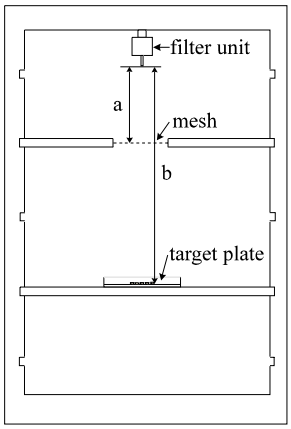


Figure 14. Distance parameters optimized in the PIG.
a: distance between filter unit and mesh; b: distance between filter unit and target plate.

4.2.4.1. Transient assays with BMS suspension cells

In these preliminary experiments summarized in Appendix 3 the parameters (distances, He-pressure, type of macro carrier) were optimized simultaneously. Assays for transient gene expression were performed two days after bombardment. For the LUC assay 300 mg cells grown in suspension were collected from the whole surface of the bombarded plates. In the histochemical GUS assays the total number of blue foci on the plates was counted (Fig. 15a).

Optimization of the helium pressure. The values of He-pressure (4.5, 5.5 and 6.5 bar) were chosen according to Koprek (1996). Transient assay results obtained at different pressures and at the same distance settings as secondary parameters were compared (Appendix 3a). In the experiments based on LUC assay, there was no significant difference in the efficiency of the three pressure values. Using histochemical GUS assay, however, 5.5 bar gave the best results in all but two experiments. Therefore, in the further experiments parameters were optimized at 5.5 bar He-pressure.

Optimization of the distances between the target tissues and the filter unit. In all experiments the wire mesh was placed into the center of the upper shelf. In this way target tissues were protected from the direct helium stream and gold particles were dispersed over the target plate. In order to determine the proper distances between the filter unit and the mesh as well as between the filter unit and the target plate (Fig. 14), transformation efficiencies obtained at constant He-pressure (5.5 bar) were compared (Appendix 3b).

Based on the LUC assay, in most cases the highest RLUs were obtained if the mesh was 1 cm and the target plate 7 cm from the filter unit. As this setting turned out to be inapplicable at scutella (see 4.2.4.2.), the second best setting (1/10 cm) was taken into consideration. In the experiments based on histochemical GUS-assay in all but one case the 4/13 cm mesh/plate distances gave the highest number of blue spots, thus contradicting to the results of the LUC-assays.

Optimization of the bombardment methods. Two bombardment methods were evaluated using the LUC assay: (i) the coated gold particles were suspended in ethanol and let dry on an aluminum foil disc (further: 'aluminum foil/ethanol' method) and (ii) the gold suspension in ethanol was dropped on a filter (further: 'filter/ethanol' method). The experiments were performed at 1/10 cm and 4/10 cm distances. The RLUs obtained with the 'aluminum foil/ethanol' method (12644 and 10625) were higher than those received after using the 'filter/ethanol' method (966 and 396).

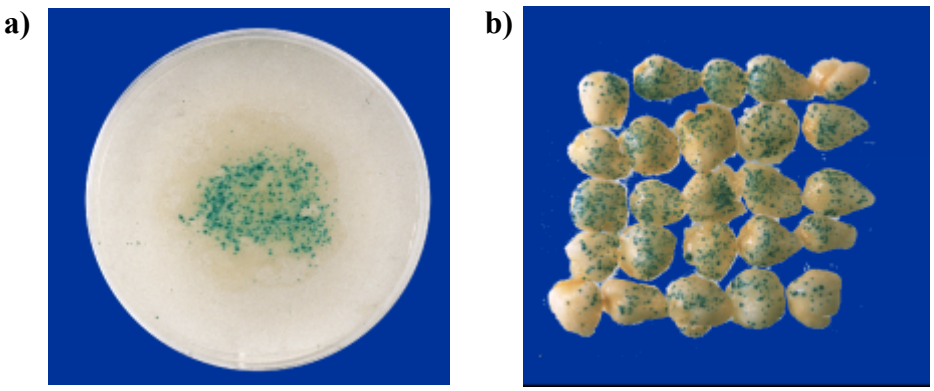


Figure 15. Histochemical GUS-assay in bombarded BMS suspension cells **(a)** and in barley scutella **(b)**.
Bombardments were performed with the 'aluminum foil/ethanol' method at 5.5 bar He-pressure and 4/10 cm mesh/target distances.

4.2.4.2. Transient assays with isolated scutella

The experiments were set up and evaluated like those performed with BMS suspensions. Twenty-five scutella of 1.5-3.0 mm size were bombarded on each plate. For the LUC assays 5 scutella were collected randomly from the whole surface of the bombarded plates. In the histochemical GUS assays the total number of blue foci per plate was counted and divided by the number of scutella (Fig. 15b).

Optimization of the helium pressure. The optimal He-pressure was determined with histochemical GUS-assay after bombardments by the 'aluminum foil/ethanol' method at two different distance settings. In the case of 4/10 cm mesh/target plate distance the highest number of blue spots per scutellum (164) was achieved at 5.5 bar, while at 7/13 cm distance 6.5 bar gave the best result (63). As the maximum number of the blue spots per scutellum was 2.5 fold higher in the first case, 5.5 bar He-pressure was considered to be the best setting, confirming the results obtained with BMS suspensions using the same assay (Appendix 3a).

Optimization of the distances between the target tissues and the filter unit. In all experiments the wire mesh was placed into the center of the upper shelf of the PIG. According to the optimization of the bombardment methods, only the results obtained with the 'aluminum foil/ethanol' method were taken into account for the determination of the optimal distance setting (Table 17).

Table 17. Optimization of the distance parameters with barley scutella

Distances a/b (cm)				
1/10	4/10	7/10	4/13	7/13
LUC activity (RLU/mg protein)				
3392	**38005**		6673	
6727	6477		**6958**	
GUS activity (blue spots/scutellum)				
43			**323**	
8	**15**		4	10
	128	103		15
	164			28
	146			20
	144			63

a: distance between filter unit and mesh; b: distance between filter unit and target plate. RLU/mg protein values were measured from 5 scutella selected randomly from 25 bombarded scutella/plate. 25-100 scutella/experiment were bombarded via the 'aluminum foil/ethanol' method, at 5.5 bar He-pressure. Blue spots/scutella were calculated from 25-125 scutella per experiment. Results exhibiting the highest LUC- or GUS-activity are printed bold in each experiment.

The two experiments based on LUC-assay did not result in a clear-cut answer. In the first experiment the 4/10 cm mesh/target plate setting gave more than five times higher RLUs than the second best one (4/13). In the second experiment the 4/13 setting gave the highest RLUs, however, all the RLUs received at different distances were in the same range (Table 17). The first experiment with histochemical GUS-assay confirmed the results of the BMS-based experiment where the 4/13 setting gave better results than the 1/10. In the next experiment, however, 4/10 proved to be better than 1/10 and 7/13 was better than 4/13. Therefore, in the further experiments the 4/10 and 7/13 settings were compared. In each case the 4/10 setting resulted in the highest number of blue spots per scutellum (Table 17).

Evaluation of the bombardment methods. The two bombardment methods ('aluminum foil/ethanol' and 'filter/ethanol') evaluated with BMS suspension cells were complemented with a third one in

the experiments with scutella. The coated gold particles were placed on an aluminum foil disc in a drop of the glycerol/$CaCl_2$/spermidine suspension as described by Koprek (1996). In each case this 'aluminum foil/glycerol' method gave the lowest expression rate in the histochemical GUS assays. In all but one experiment the 'aluminum foil/ethanol' method resulted in the highest transient expression rates in both the LUC- and the histochemical GUS-assays (Table 18).

Table 18. Optimization of the bombardment methods with barley scutella

A/E	F/E	A/E	F/E	A/G
LUC activity (RLU/mg protein)		GUS activity (blue spots/scutellum)		
38005	4650	**128**	54	2
31687	3392	**103**	65	6
8911	2880	15	**19**	0
6673	3089	**18**		0
		15	13	
		10	6	
		8	6	
		4	1	

A/E: 'aluminum foil/ethanol', F/E: 'filter/ethanol', A/G: 'aluminum foil/glycerol' (see text for details). RLU/mg protein values were measured from 5 scutella selected random from 25 bombarded scutella/plate. 25-100 scutella were bombarded per experiment. Blue spots/scutella values were calculated from 25-125 scutella per experiment. Results exhibiting the highest LUC- or GUS-activity are printed bold in each experiment.

The results of the optimization experiments are summarized in Table 19. For each parameter the values resulting in the highest transient expression levels are given. As the conclusion of the optimization experiments, in the last column the settings applied in the stable transformation of scutella are shown.

Table 19. Summary of the results of the optimization experiments.

Parameter	BMS-suspension		Barley scutella		Applied in stable transformation
	LUC-assay	GUS-assay	LUC-assay	GUS-assay	
He-pressure (bar)	4.5; 5.5; 6.5	5.5	-	5.5	5.5
Distances a/b (cm)	(1/7)*; 1/10	4/13	4/10; 4/13	4/10	4/10; 4/13
Bombardment method	A/E	-	A/E	A/E	A/E

a: distance between filter unit and mesh; b: distance between filter unit and target plate; A/E: 'aluminum foil/ethanol'. * Best setting but inapplicable in the bombardment of scutella.

4.2.5. Stable transformation experiments with barley scutella

Isolated scutella of the barley cultivar 'Salome' were bombarded with the novel plasmid constructs pUJIPsense, pUJIPantisense, pUAOSsense and pUAOSantisense. Scutella co-bombarded with pAHC25 and pAHC18 plasmids, but not used for transient assays, were also subjected to selection (Appendix 6e). The bombardment parameters in the stable transformation experiments were based on the results of the transient assays (Table 19). Vacuum was set to 200 mbar and He-pressure to 5.5 bar at each shot. Except for the bombardment of 275 scutella with pUJIPsense and that of 25 scutella with pAHC25/pAHC18, in each experiment the 'aluminum foil/ethanol' method was used. As the optimization of the mesh and target distances had not given a clear-cut result in the transient assays, all the possible combinations (except 1/13 cm) were applied. The majority of the scutella (19,067 pieces), however, was bombarded with mesh/filter unit and target plate/filter unit distances of 4 cm and 10 cm, respectively.

4.2.5.1. Somatic embryogenesis in the bombarded scutella

In the first set of experiments performed without osmotic treatment, the scutella were incubated on 91/4 or 91/4m3 medium for one day before bombardment. During this time intensive proliferation of the epidermal cells could be observed. At the transfer to the first selection medium three days after bombardment, early callus formation was visible on the edges of the most responsive scutella. These calli turned out to be highly embryogenic. They could be kept in culture for longer time and developed shoot/leaf primordia at higher frequency than other callus lines (data not shown). The development of the non-responsive scutella was arrested in the swollen stage. The color of these tissues turned to brown till the end of the first two weeks showing deleterious effect of the selection agent.

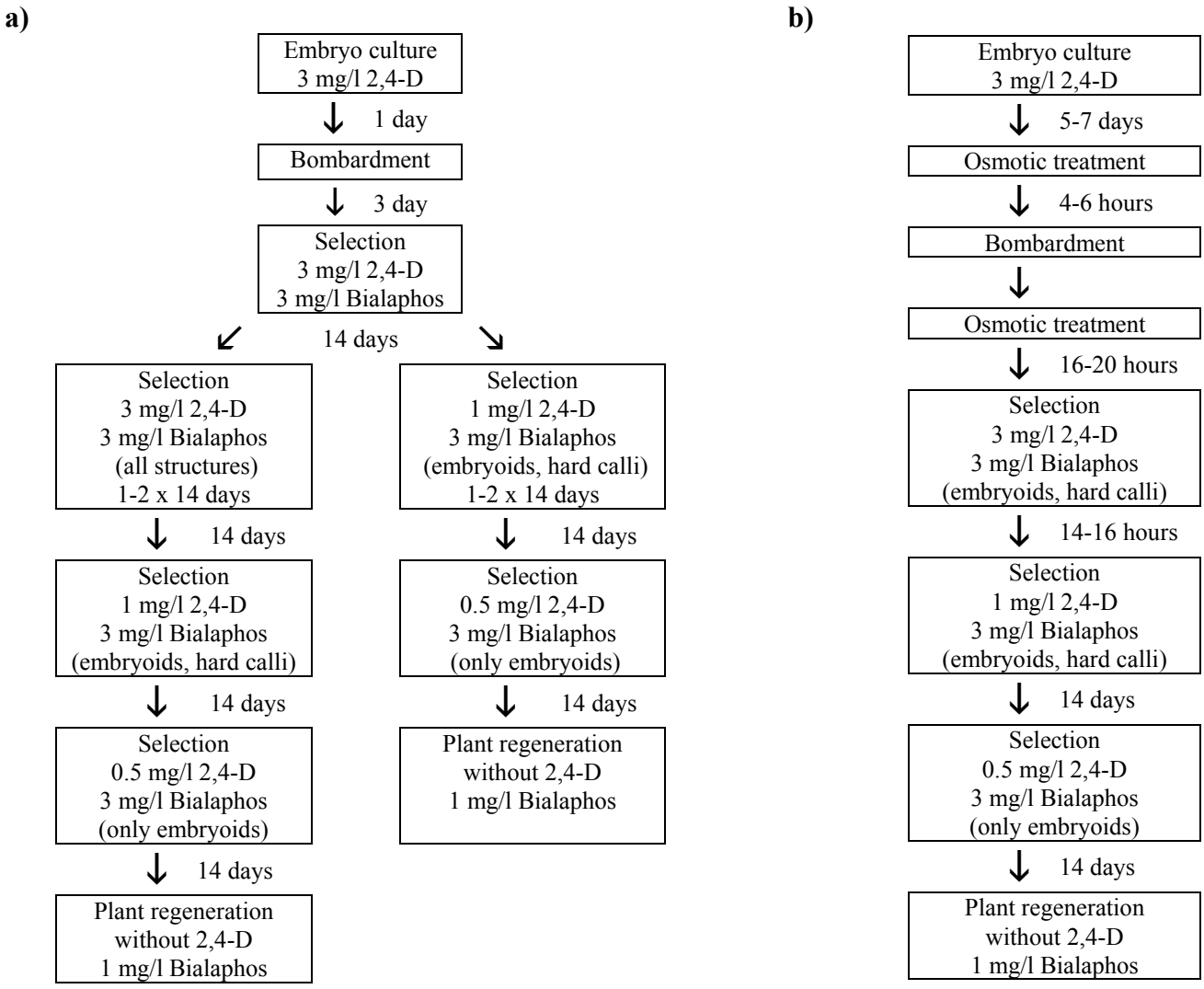


Figure 16. Selection strategies of bombarded barley scutella.
a) without osmotic treatment; b) with osmotic treatment. See text for details.

Appendix 6 and Figure 16 show the selection strategies applied. The developmental status and the number of structures (scutella, calli, embryoids) transferred to the next selection step varied among the experiments. Modified versions of two basic selection strategies were used: after the first selection step (i) only the developing hard and embryogenic calli were transferred to a medium of lower 2,4-D concentration or (ii) all the isolated scutella and the growing calli were transferred to a medium identical with the first one. In the case of (ii), the number of the transferred structures was reduced in a later step. Scutella not showing any response in the first two weeks, however, did not

produce any embryogenic structures in the later selection steps, either. The responsivity and the regeneration capacities were in the same range in the case of bombardments with pUAOSsense/antisense and pUJIPsense/antisense vectors without osmotic treatment, while these values were higher with pAHC25/pAHC18 (Table 20).

Somatic embryogenesis in scutella after osmotic treatment. In order to decrease the tissue damage caused by bombardment, the osmotic treatment of scutella was applied. Scutella were incubated on medium containing osmotica (mannitol and sorbitol) for 4-6 hours before and 16-20 hours after bombardment. Prior to osmotic treatment, scutella were cultured on induction medium for 5-7 days. Thus, the responsivity of the individual explants was visible relying upon the calli developed on the scutellar surface. The responsive scutella were transferred to induction medium supplemented with osmotica (sorbitol + mannitol), bombarded and subjected to selection (Fig. 16b). Excepting the experiments with pAHC25/pAHC18, the responsivity proved to be higher than without osmotic treatment. This referred to the regeneration rates of leaf- and shoot-like structures after bombardments with the pUJIPsense, pUJIPantisense and pUAOSantisense vectors as well. In contrast, bombardments with pUAOSsense and pAHC25/pAHC18 vectors did not result any regenerable embryoids (Table 20).

Table 20. Summary of the stable transformation experiments

	pUJIPsense	pUJIPantisense	pUAOSsense	pUAOSantisense	pAHC25/pAHC18
Without osmotic treatment					
Scutella	4600	6500	4850	6925	250
Calli*	684	724	443	898	51
Responsivity	15.0%	11.1%	9.1%	13%	20.4%
Shoots or leaves	2	1	4	2	5
Regeneration capacity	0.04%	0.02%	0.08%	0.03%	2.0%
With osmotic treatment					
Scutella	1440	1536	3152	192	480
Calli*	331	391	669	45	24
Responsivity	23.0%	25.5%	21.2%	23.4%	5.0%
Shoots or leaves	4	15	0	1	0
Regeneration capacity	0.3%	0.98%		0.5%	
TOTAL:					
Scutella	6040	8036	8002	7117	730
Calli*	1015	1115	1112	943	75
Responsivity	16.8%	13.9%	13.9%	13.2%	10.3%
Shoots or leaves	6	16	4	3	5
Regeneration capacity	0.1%	0.2%	0.05%	0.04%	0.7%

Number of bombarded scutella, number of hard/embryogenic calli and number of individual embryoids giving rise to shoots- or leaf-like structures of >2 mm summarized from Appendices 4-8. Responsivity: No. of hard or embryogenic calli/No. of scutella. Regeneration capacity: No. of shoots or leaves/No. of scutella.* Embryogenic and hard calli included.

4.2.5.2. Regeneration of putative transgenic plants

The number of shoot- and leaf-like structures transferred to regeneration medium was 6, 16, 4, 3 and 5 after transformations with the pUJIPsense, pUJIPantisense, pUAOSsense, pUAOSantisense and pAHC25 vectors, respectively (Table 20). In spite of the transfer to media supplemented with 0.5-5.0 mg/l zeatin to increase shoot development, most of the 2-10 mm long leaflets arrested in development and died after some weeks of culture.

Plantlets could be regenerated from three independent bombardments (Fig. 17).

- One plantlet was obtained from bombardment with the pUAOSsense plasmid. The 'aluminium foil/ethanol' method was used at 7/10 cm mesh-target tissue distances. Root development was induced on MS medium supplemented with 1.5 mg/l naphthalene acetic acid (NAA).

- One plantlet was obtained from bombardment with the pUJIPsense plasmid. The 'filter/ethanol' method was used at 7/13 cm mesh-target tissue distances. Root development was induced on MS medium supplemented with 1.5 mg/l NAA.

- One plantlet was obtained from co-bombardment with plasmids pAHC25/pAHC18 by the 'filter/ethanol' method. After the transient assays in the optimization experiments, remaining scutella were subjected to selection. The plantlet died before being transferred to rooting medium.

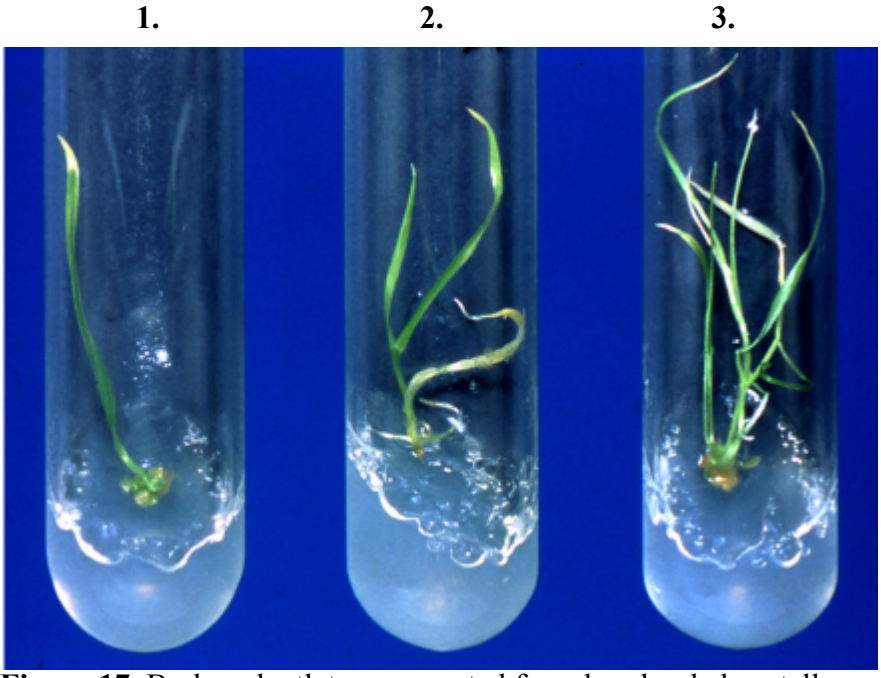
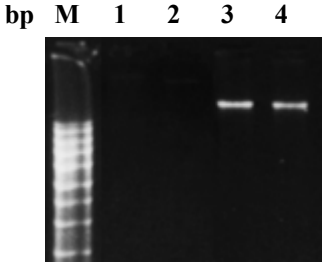


Figure 17. Barley plantlets regenerated from bombarded scutella.
Scutella were bombarded with the vectors (1) pUAOSsense, (2) pUJIPsense and (3) pAHC25/pAHC18.

4.2.6. Analysis of the putative transgenic plants

The regenerated plants derived from bombardments with the plasmids pUAOSsense and pUJIPsense were subjected to PCR analysis and PAT assay. These methods were expected to give the first information about the transgenic/non-transgenic nature of the regenerants.

Prior to perform the PCR with genomic DNA of the regenerated plants, the primers pat-12 and pat-22 were tested with the plasmids pUAOSsense and pUJIPsense used for transformation. These primers should amplify the region from 332 bp to 760 bp of the *pat* gene. The pat-12 and pat-22 primers were suitable to amplify this *pat* fragment of 430 bp in both the pUAOSsense and the pUJIPsense vectors (Fig. 18).



600
400

Figure 18. PCR analysis of the putative transgenic barley plants.
Lanes 1 and 2: plasmids pUAOSsense and pUJIPsense, respectively; lanes 3 and 4: plants from bombardment with pUAOSsense and pUJIPsense, respectively; M: molecular size marker (Smart Ladder).

Total DNA was isolated from the putative transgenic plants during both the *in vitro* and the *in vivo* culture. About 100 ng DNA of each of the pUAOSsense and pUAOSsense plantlets was subjected to PCR reaction. The reactions did not result in the desired fragment at any of the samples (Fig. 18). This suggests that the *pat* gene fragment was not present in the isolated DNA of the putative transgenic plants.

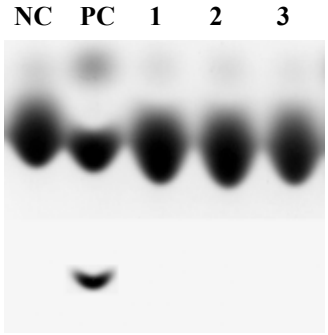


Figure 19. PAT assay of the putative transgenic barley plants.
Lanes 1 and 2: plants from bombardment with pUJIPsense; lane 3: plant bombarded with pUAOSsense; NC: negative barley control; PC: positive control of transgenic tobacco; arrow: acetylated L-PPT.

PAT assays were performed at the TU Braunschweig with proteins isolated from regenerated plants transferred to soil. None of the samples derived from plants bombarded with pUAOSsense and pUJIPsense exhibited PAT-activity (Fig. 19). The lack of the expression of the *pat* gene, confirming the result of the PCR, suggest that the regenerated plants do not contain the stably integrated *pat* transgene. Further assays (e.g. Southern-blots) on the integration of the *pat* gene as well as that of the AOS or JIP23 cDNAs were not planned in this part of the research program.

The results of this part of the current work can be summarized in the following points:

i. New plasmid vectors have been prepared, which contain the barley AOS1 or JIP23 cDNAs in sense or antisense orientation, under the control of the *Ubi-1* promoter. As selection marker, the *pat* gene under the control of the 35S promoter is integrated in the same construct.

ii. The functionality of the novel vector constructs have been revealed by the transient expression of the *pat* gene and that of the JIP23 cDNA in transformed barley mesophyll protoplasts.

iii. A plant regeneration system based on somatic embryogenesis from isolated scutella has been adapted for the barley cultivar ‘Salome’.

iv. Bombardment parameters have been optimized for the particle inflow gun. The experiments were based on the transient expression of the *luc* and *uidA* genes in BMS suspension cells as well as in isolated barley scutella.

v. Isolated scutella of the barley cv. ‘Salome’ have been bombarded with the plasmids described in (i) and according to the settings determined in (iv). Bombarded scutella have been selected and putative transgenic plants have been grown up using the regeneration system of (iii). Pre- and post-bombardment incubation of scutella on osmoticum-containing medium significantly improved responsivity and regeneration capacity. PCR and PAT-assays have been performed with the putative transgenic plants. Both tests gave negative results.

4.3. NEW SCIENTIFIC RESULTS

Based on the results of the microspore culture experiments, the following can be considered to be new scientific results:

i. The protocol for the regeneration of haploid/dihaploid plants from isolated microspores of triticale has been developed. The method was established in experiments with five genotypes and it is suitable for the application in plant breeding. Its further practical advantage is that through the microspore isolation method used here, the laboursome process of anther isolation can be avoided.

ii. The induction of haploid embryogenesis does not require exogenous hormone-supplement neither in triticale nor in barley microspore culture. It is the first description in unicellular systems of these two monocot species, that hormones are not essential exogenous factors required for the induction of haploid embryogenesis.

Based on the results of the vector preparation experiments, the following can be considered to be new scientific results:

i. Novel plasmid vector constructs have been prepared, which contain the barley AOS1 or JIP23 cDNAs in sense or antisense orientation, respectively, under the control of the *Ubi-1* promoter. Based on the same construct, vectors have been prepared, which contain a multiple cloning site (MCS) in sense or antisense orientation, respectively, downstream of the *Ubi-1* promoter. The cloning site includes restriction sites for at least ten enzymes. Thus, these vectors can be used for the cloning (and expression) of further genes/cDNAs.

ii. The functionality of the novel vector constructs in the genetic background of barley has been revealed by transient assays in transformed barley mesophyll protoplasts. Thus, it is suggested that the AOS and JIP23 sense and antisense vector constructs are suitable for the modification of endogenous jasmonate and JIP23 levels in barley an other cereals via genetic transformation.

5. DISCUSSION

5.1. INDUCTION OF HAPLOID EMBRYOGENESIS IN MICROSPORE CULTURE

The primary goals of this part of the thesis were (i) to establish the method of isolated microspore culture for triticale and (ii) to study the induction of embryogenesis and plant regeneration without exogenous hormone-supply in microspore cultures of triticale and barley. An identical microspore isolation protocol (microblending of spikes) and induction media of the same salt and vitamin composition (190-2) have been applied for both species.

5.1.1. Method of microspore isolation and culture in triticale

Up to date, haploid triticale plants have been generated via chromosome elimination technique (Inagaki and Hash, 1998, Wedzony et al., 1998) and via anther culture (Wang *et al.*, 1973; Bernard, 1980; Lukjanjuk and Ignatova, 1986; Martinez Garcia *et al.*, 1992; Karsai *et al.*, 1994; González and Jouve, 2000; Immonen and Robinson, 2000). Based upon preliminary studies in barley and wheat, the protocol of microspore culture has been established for triticale as well. Microspore embryogenesis could be induced and plants could be regenerated in cultures of directly isolated triticale microspores as well. Thus, microspore culture represents a novel way to induce triticale haploids and offers a comparable alternative to the previously established anther culture.

In triticale microspore culture, the induction of androgenesis was successful when microspores were in similar developmental stage as in anther cultures. This means the mid- to late-uninucleate stages which was optimal at the collection of donor spikes. During the pretreatment of spikes, however, microspores continued development and they were in the late-uninucleate to early-binucleate stages at isolation. Isolated microspores of this stage gave highly regenerable cultures in our experiments. In comparison, responsive cultures of microspores of the early-binucleate stage were not reported from anther cultures, moreover, no embryoid development was observed from microspores of this stage compared to the optimal late-uninucleate stage in triticale anther culture (Lukjanjuk and Ignatova, 1986). Microspores of the mid-uninucleate stage also give good response (Karsai and Bedő, 1997; Karsai *et al.*, 1994; González and Jouve, 2000) but microspores of earlier stages did not show any response in triticale anther culture (Lukjanjuk and Ignatova, 1986; Hassawi and Liang, 1990). A difference in the optimal microspore developmental stages has been observed in anther and microspore cultures of barley and wheat as well (Hoekstra *et al.*, 1992; Mordhorst and Lörz, 1993; Puolimatka *et al.*, 1996). Inside intact anthers, the prolonged development of microspores is supposed. This can explain that in anther culture an earlier microspore developmental stage is optimal compared to microspore culture (Hoekstra *et al.*, 1992).

The amount, viability and embryogenic capacity of microspores isolated via microblending of spikes were sufficient for the initiation of cultures. This isolation protocol is easy to perform due to the omission of the laboursome process of anther isolation. Microblending of spikes was earlier established for barley and wheat with great efficiency (Mejza *et al.*, 1993; Mordhorst and Lörz, 1993; Puolimatka *et al.*, 1996; Kasha *et al.*, 2001b). For detailed discussion about microblending, see 5.1.2. Separation of viable cells from dead ones by density gradient centrifugation was first suggested for protoplast preparation (Harms and Potrykus, 1978). In the present work, the simplified version of the method has been used. This protocol is based on mannitol and maltose gradient centrifugation (‘maltose-cushion’). It has been successfully adapted also for barley and wheat (Mordhorst and Lörz, 1993; Puolimatka *et al.*, 1996; Kasha *et al.*, 2001b). Using this method the ratio of viable microspores can be increased to 70-96%. This protocol can increase the number of viable microspores, and is recommended if their ratio is under 50% in the crude microspore suspension (Mordhorst and Lörz, 1993; Kasha *et al.*, 2001b).

Viable microspores of dense cytoplasm were successfully cultured in induction media of various hormone composition (hormone-free; 2,4-D + kinetin; PAA). Macro- and microelements as well as vitamins were those of the 190-2 medium. In triticale anther culture, 190-2 medium has been applied as regeneration medium so far (Wang and Hu, 1984; Karsai and Bedő, 1997). This corresponds to its original invention for regeneration in wheat anther culture (Zhuang and Jia, 1983). In wheat microspore culture, 190-2 has been successfully used as induction medium at one genotype: both embryoid and green plant regeneration rates exceeded those received with other media. In the other genotype, however, 190-2 proved to be inferior, thus suggesting a cultivar-specific response to different media (Puolimatka *et al.*, 1996).

In triticale microspore cultures, high levels of embryogenesis could be achieved in induction media without ovary-conditioning. Isolated triticale microspores induced in ovary-conditioned media did not give regenerable embryoids (data not shown). Ovary-conditioning is not necessary for the induction but for the maintenance of embryogenesis (Datta and Wenzel, 1987; Puolimatka et al., 1996). Nevertheless, it has been found to be essential in wheat microspore cultures (Mejza et al., 1993; Puolimatka et al., 1996; Hu and Kasha, 1997). In contrast, microspore cultures of barley result in plants at the same frequency without ovary-conditioning (Mordhorst and Lörz, 1993; Kasha *et al.*, 2001b). Ovaries are supposed to release "conditioning factors" such as PAA into the induction medium, thus promoting microspore embryogenesis (Ziauddin *et al.*, 1992; Mejza *et al.*, 1993; Hu and Kasha, 1997). Similarly, in non-regenerable microspore cultures of triticale the conditioning factors released by floating anthers were supposed to be required for the induction of androgenesis (Keller, 1991).

Each triticale genotype involved in microspore culture responded at a satisfactory level in the regeneration experiment. Most cultivars acting in the F_1 combinations ('Presto', 'Moniko', 'Tewo') have been previously tested in anther culture and were found to be responsive (Karsai *et al.*, 1994). These genotypes maintained their good tissue culture response in cross-combinations under microspore culture conditions. This corresponds to the highly inheritable nature of embryo induction and, independently from the previous one, of plant regeneration which was analysed in triticale anther culture (Balatero *et al.*, 1995).

Haploid embryogenesis was observed in the isolated microspore culture of triticale. Early steps of androgenesis (until the multicellular structure stage) have been earlier described in non-regenerable microspore cultures of triticale (Keller, 1991). This type of development has been observed in microspore culture of other cereals, such as barley, wheat and rye (Hoekstra *et al.*, 1992; Mejza *et al.*, 1993; Guo and Pulli, 1999). In haploid cultures of monocots, embryogenesis is the preferred way of development, pretending the symmetrical division of the microspores (Szakács and Barnabás, 1995). Embryoids can develop directly from dividing microspores (Guha and Maheshwari, 1966) or indirectly through a preceding callus-phase (Niizeki and Oono, 1968). A decisive role of the type and concentration of auxins on the developmental pathway has been observed in both somatic and haploid tissue cultures (Dudits *et al.*, 1991; Hoekstra *et al.*, 1996). For a detailed discussion of the roles of plant hormones, see 5.1.3.

From the ELSs of different genotypes, altogether 126 green plants were regenerated. The rate of albino regenerants, however, was high (>50%) in triticale microspore culture. In triticale anther culture high rates of albinism (up to 100%) can be usually observed, however this phenomenon is highly dependent on genotype and culture conditions (e.g. González *et al.*, 1997; Immonen and Robinson, 2000). The influence of the same factors on the rate of albinism was observed in barley microspore cultures (Kasha et al., 2001 and 5.1.2. of this work). Albinism is a typical problem in anther and microspore cultures of cereals and it has been explained with several possible reasons, effects of genotype and culture conditions being among them (for reviews, see Lukjanjuk and

Ignatova, 1986; Jähne and Lörz, 1995). Deletions in or degradation of the plastid genome are general phenomena in albino pollen plants (Day and Ellis, 1985; Carreda *et al.*, 2000).

The stomatal guard cell length and cytological examination of the 126 microspore-derived green plants indicated that the wast majority (90%) of the regenerants is haploid in triticale microspore culture. This high frequency of haploid regenerants is unique among haploid tissue cultures of cereals. In anther culture of triticale high frequencies (>60%) of spontaneous dihaploids could be achieved, but this phenomenon was strongly genotype-dependent (Immonen and Robinson, 2000). Microspore cultures of barley and wheat usually regenerate spontaneous dihaploid plants at a high frequency (>40%) as well (Ziauddin et al., 1992; Mordhorst and Lörz, 1993; Gustafson et al., 1995; Puolimatka et al., 1996). Aneuploidy also frequently occurs in microspore cultures (Hu and Kasha, 1997) and is especially common in anther cultures of triticale, due to its amphidiploid nature (Lukjanjuk and Ignatova, 1986; Immonen and Robinson, 2000).

5.1.2. Induction of androgenesis in isolated barley microspores using different culture media

Induction media of various composition have been used in barley microspore culture to date. Media were supplemented with plant hormones in each case (Table 1). Neither the induction media previously established for triticale nor the hormone-free induction of androgenesis has been studied in barley microspore culture to date.

The microblending-based isolation and culture method, originally invented by Mordhorst and Lörz (1993), has been successfully adapted to our conditions with the cultivars ‘Igri’ and ‘Kymppi’. Direct microblending of spikes is an effective method for initiating microspore cultures in barley, although the regeneration rates achieved are slightly lower than those received after maceration or microblending of anthers (Table 1). Recently, at cultivar ‘Kymppi’ microblending of spikes resulted in a poorer regeneration capacity than maceration by a teflon rod (Ritala *et al.*, 2001). This difference in the efficiency can be explained with the fact that microblended spikes contain anthers from the less-developed florets as well, while in other methods, anthers from selected parts of the spikes are processed (Mordhorst and Lörz, 1993). Nevertheless, the microblending of spikes offers a less laboursome method as the isolation of anthers is avoided. The protocol was originally invented for the model cultivar ‘Igri’ but it has also been adapted for other genotypes (Kasha *et al.*, 2001b). Microspores cultures produced by this method have been successfully used as target tissues for transformation (Jähne *et al.*, 1994; Leckband and Lörz, 1998; Scholz *et al.*, 2001).

Both N24- and 190-2-based media were capable of inducing androgenesis in isolated microspores of both barley cultivars tested. N24 medium has a nitrogen-composition optimized exclusively for microspore cultures of the cultivar ‘Igri’ (Mordhorst and Lörz, 1993). The original 190-2 medium, however, was invented for regeneration in wheat anther culture (Zhuang and Jia, 1983). It has never been used in tissue cultures of barley. The green plant regeneration capacity of microspores was higher on the N24- than on the 190-2-based media. A possible reason for this difference can be the different nitrogen composition of these media (Table 15). While induction of androgenesis requires a stress signal provided by pretreatment, nitrogen content of the medium is responsible for embryogenic development and regeneration capacity of the microspores (Mordhorst and Lörz, 1993). This can explain the lower number of green plants regenerated from 190-0, in spite of the higher numbers of ELSs recorded on this medium compared to N24. The nitrate- and inorganic N-content, consequently the total N-content, of 190-2 medium is half of that calculated for N24. Ammonium content and inorganic/organic N-ratio, however, are within the optimal range. Optimized NO_3^-/NH_4^+-ratio has a significant impact on plant regeneration capacity of both haploid and somatic tissue cultures (Grimes and Hodges, 1990; Mordhorst and Lörz, 1993). A high level of nitrate and a small amount of ammonium or other forms of reduced nitrogen are essential for normal growth and development in haploid and somatic tissue cultures of both mono- and

dicotyledonous species (Halperin and Wetherell, 1965; Gamborg, 1970; Grimes and Hodges, 1990; Schmitz and Lörz, 1990). In the case of a NO_3^-/NH_4^+-ratio under 30:70, however, poor development of microspores was caused by the toxic effect of ammonium (Mordhorst and Lörz, 1993). High levels of ammonium-nitrate suppress embryoid formation and promote callus formation instead, leading to a low response and to a high percentage of albino regenerants (Olsen, 1987). The reduction of ammonium–nitrate content to 1/10 in MS medium led to an increased response of the cultured anthers (Clapham, 1973; Olsen, 1987).

190-2 medium was supplemented with 3 mM glutamine to increase the extremely low organic N-content (0.047 mM) of the original medium. Thus, organic N-content and inorganic/organic N-ratio were within the optimum range of the N24 medium (Mordhorst and Lörz, 1993). The role of glutamine in the promotion of embryogenesis has been early shown in pollen/microspore cultures of tobacco (Nitsch, 1977; Wernicke and Kohlenbach, 1977). Studies on its role in anther and microspore cultures of barley, however, gave contradictory results. In most cases, glutamine had a positive effect on embryogenesis and plant regeneration (Olsen, 1987; Hoekstra *et al.*, 1992; Mordhorst and Lörz, 1993). In contrast, no or inhibitory effect of glutamine have also been observed (Xu and Sunderland, 1981; Jähne *et al.*, 1994; Ritala *et al.*, 2001).

5.1.3. Hormone-free induction of androgenesis in microspore cultures of barley and triticale

Haploid embryoids were induced and green plantlets were regenerated from microspore cultures of both barley and triticale, without exogenous hormone-supplement in the induction and regeneration media. In barley, the regeneration rate achieved this way was significantly lower compared to the media containing hormones, for both cultivars tested. In contrast, a positive effect of hormone-free induction medium on embryoid yield has been detected in microspore culture of several triticale genotypes. Its impact on green and albino plant production, however, was not significant. Barley anthers responded similarly to induction media with or without hormone-supplement, but the hormone-free medium gave rise to calli of lower regeneration capacity (Cai *et al.*, 1992). This is in full concordance with the results reported here, and suggests that induction of androgenesis does not require hormones, but hormones are essential for efficient plant regeneration. In the microspore culture of some triticale genotypes, however, this theory was not confirmed, because regeneration capacity did not decrease in the absence of hormones. Moreover, in microspore cultures of the barley genotype ‘Jokioinen 1490’, green plants could be regenerated exclusively from microspores induced on hormone-free medium. The regeneration rate, however, was very low (<1%) (unpublished data). Similarly, the beneficial effect of hormone-free induction medium both on anther response and regeneration has been detected in oat anther culture (Kiviharju *et al.*, 1997).

The theory of hormone-free induction of androgenesis is further supported by the recently suggested role of stress as the single factor required for switching microspores from gametophytic to sporophytic pathway (for review, see Heberle-Bors, 1998). This proposal is based on the identical conditions required for the pretreatment of tobacco and wheat anthers/spikes prior to isolation of microspores (Touraev *et al.*, 1996a,b). Because the pretreatments applied in these species (starvation + heat shock) are partially different from those applied for barley and triticale in our studies (starvation + cold), the species-specifity of the required signal is suggested. This is, however, in contradiction with the above-mentioned general validity of the ‘starvation + heat shock signal’ model, although this pretreatment protocol has not been tested in our experiments.

Hormones commonly used in haploid cultures of barley are basically different from those used in triticale cultures. In barley, the cytokinin BAP is used most frequently, while in triticale the synthetic auxin 2,4-D, occasionally supplemented with the cytokinin-type kinetin, is the main source of hormones. In this sense, triticale cultures resemble those of wheat, also based on auxin-supplement (Puolimatka *et al.*, 1996, Hu and Kasha, 1997). The role of exogenously applied

hormones in haploid tissue cultures of cereals and the reasons for the species-specific preferences in their use are less understood.

The preferential use of cytokinins has been confirmed in our preliminary experiments. Here, auxin-based induction media (190-D/K and 190-PAA) did not result in plant regeneration from barley microspore cultures (data not shown). This preference for cytokinins is not confirmed by some other reports. In anther culture, high endogenous levels of auxins in barley anthers are supposed to lead to no or inhibitory effects of auxins added to the media. The removal of anther tissues, however, improved auxin (PAA)-sensibility of barley microspores (Ziauddin *et al.*, 1992). Moreover, 2,4-D has been found to be as effective as BAP in both anther and microspore cultures of barley (Hoekstra *et al.*, 1996). To achieve maximum embryogenesis and regeneration rates, however, the higher the contrentation of 2,4-D is the shorter the application time must be (Dudits et al., 1991; Hoekstra et al., 1996). The negative effect of high 2,4-D concentration for an extended period has been observed in anther cultures of barley, wheat and triticale (Marsolais and Kasha, 1985; Hassawi *et al.*, 1990).

According to these reports, the preference for cytokinins in the promotion of embryogeneis in barley haploid cultures seems to be overcome and the promoting role of 2,4-D can be extended over this species as well. Thus, the failure in the induction of regenerable embryoids in barley microspore cultures can be explained, most probably, with the unsuitable concentration of auxins added. The same concentrations, however, were successfully applied for wheat and triticale microspores. In triticale microspore cultures, the combination of 2,4-D + kinetin as hormone-supplement resulted in higher rates of green regenerants than PAA. Thus, the increased regeneration capacity reported as a result of PAA-addition to microspore cultures of barley and wheat (Ziauddin *et al.*, 1992; Hu *et al.*, 1995; Hu and Kasha, 1997), has not been confirmed here.

Androgenesis and plant regeneration are considered to be independent events of different genetic background (Knudsen *et al.*, 1989; Mordhorst and Lörz, 1993). Our results suggest that androgenesis and plant regeneration are independent events regarding hormone-requirement as well. Induction of androgenesis does not need exogenous hormone-supplement neither in barley nor in triticale microspore culture. The regeneration of plants, however, exhibits differences between genotypes and/or species regarding exogenous hormone-requirement: regeneration is hormone-dependent in barley and in some of the triticale genotypes tested, while it is independent of hormone-supplement in other triticale genotypes.

5.1.4. Future prospects

The easy-to-perform method for isolation and culture of isolated microspores of triticale could be used for generation of haploid plants from different genotypes, as described in this work. The doubled haploid lines received from different F_1 combinations were readily introduced into the traditional breeding process. This shows that the protocol can be involved in the production of new triticale cultivars. The avoiding of anther isolation offers a less laboursome alternative to the traditional anther culture.

Although transgenic triticale plants have been produced only by particle bombardment of scutellar tissues to date, promising results in barley suggest that isolated microspores of triticale could also be used as target tissues for transformation.

Hormone-free induction of embryogenesis in isolated microspores of two cereal species confirm that stress signals are necessary and sufficient for the induction of androgenesis as described earlier in wheat. The differences in regeneration capacity between species and genotypes, however, propose further studies on various pretreatments such as stress signals and on various hormone

combinations, involving a broader range of genotypes in the experiments. These results could clarify the need for a species- or genotype-specific stress signal which can have a promoting effect on regeneration capacity as well. Supposing, however, that embryogenesis and plant regeneration are independent events, studies on a specific hormone-requirement for regeneration seem to be also necessary.

5.2. PREPARATION OF NOVEL VECTOR CONSTRUCTS

The goal of the current work was to prepare novel plasmid vectors carrying cDNAs coding for AOS, the key-enzyme of JA-biosynthesis, or JIP23 in sense or antisense orientation, respectively. In addition, bombardment parameters were optimized for a particle inflow gun and the new constructs were used in a set of stable transformation experiments performed with 'Salome', the model cultivar of jasmonate studies. The plasmids and the results of the optimization experiments can be utilized in the production of transgenic barley plants which can mean the subject of further studies on the role of jasmonates in the development of barley and on the analysis of the function of JIP23 in this species.

5.2.1. The structure of the novel plasmid vectors

For the stable transformation of barley via particle bombardment, new vectors were prepared which carry (i) a cDNA coding for the gene of interest in sense or antisense orientation under the control of the *Ubi-1* promoter and (ii) the *pat* resistance gene under the control of the 35S promoter. Our novel constructs (pUAOSsense/antisense, pUJIPsense/antisense) are based on plasmids (pAHC20 and pWD26.41) which have already been used in barley transformation.

In addition to the vectors for the modification of AOS and JIP23 level, intermediate products of the vector preparation can also be utilized in the transformation of barley and other (primarly monocot) species. These vectors are derived from the penultimate stage of vector preparation when the AOS or JIP23 cDNA is still not inserted into the multiple cloning site (pUMCSsense and pUMCSantisense). The complete MCS contains restriction sites for at least ten enzymes thus enabling the cloning of a broad range of genes/cDNAs. Its utilization can be further broadened by the feature that fully identical constructs are available for the preparation of sense and antisense constructs. *Ubi-1* promoter-based expression vectors of similar structure have not been reported yet. From the basic set of *Ubi-1* promoter-based vectors prepared by Christensen and Quail (1996), pAHC17 contains a polylinker. This sequence offers restriction site for six enzymes, but no expression cassette containing a selection marker gene is included in the plasmid. The expression vectors pRT100 to 104 based on the 35S promoter also contain restriction sites in the same range (5-8 sites) as pAHC17 and our MCS (Töpfer *et al.*, 1987).

5.2.1.1. The promoters

The use of two different promoters to control a gene of interest and a resistance gene is a repeatedly performed approach to avoid or minimize transcriptional gene silencing (de Wilde *et al*., 2000). The co-transformation of the expression cassettes (i) *35S::resistance gene* and (ii) *promoter of various origin::transgene of interest* has already been successfully used for the generation of transgenic barley plants (Jähne *et al*., 1994; Ritala *et al*., 1994; Leckband and Lörz, 1998; Fang *et al.*, 2002). To date, the combination of 35S and *Ubi-1* promoters controlling different genes in the same plasmid has not been used in vectors for the particle bombardment of barley. Binary vectors of such construction, however, are efficiently used in the *Agrobacterium*-mediated transformation of barley (Horvath *et al*., 2000; Wang *et al*., 2001; Fang *et al.*, 2002).

In a transient expression system of barley, promoters of monocotyledonous origin (maize *Ubi-1*, maize-derived *Emu*, rice *Act-1*) exhibited higher promoter strength than the CaMV 35S promoter (Schledzewski and Mendel, 1994). Such a difference, however, has not been detected in stable transformation: transgenes controlled by the 35S or by the *Ubi-1* promoter were expressed with similar efficiency in the transgenic plants (Wan and Lemaux, 1994). In wheat biolistic transformation, the transformation efficiency was 0.8-1.1% if the resistance gene was under the control of the 35S promoter (Becker *et al.*, 1994, Nehra *et al.*, 1994) and it was in the same range (0.1-1.5%) in the case of a control by the *Ubi-1* promoter (Weeks *et al.*, 1993, Altpeter *et al.*, 1996a,b). Immature embryos/scutella of barley are usually co-bombarded with plasmids which integrate 35S and *Ubi-1* promoters separately. These results suggest that the combination of expression cassettes controlled by the *Ubi-1* and 35S promoters, respectively, in the same plasmid vector, like applied in our novel constructs, can be used for the generation of transgenic barley plants.

5.2.1.2. The *pat* resistance gene

The successful regeneration of plants containing stably integrated transgenes requires an appropriate selection system. Selection systems based on PAT are widely used in cereal transformation. From the genes encoding PAT, *bar* is the most widely used resistance gene for barley transformation (Table 3). The *pat* gene, which shows significant homology to *bar* (Wohlleben *et al.*, 1998), has also been successfully involved in the production of transgenic barley plants (Leckband and Lörz, 1998; Scholz *et al.*, 2001).

Selection of transgenic tissues expressing PAT in barley is usually performed on media supplemented with 3-5 mg/l bialaphos (for reviews, see Lemaux *et al.*, 1999). In our experiments a bialaphos concentration of 3 mg/l concentration was sufficient to select non-responsive scutella. These scutella exhibited necrosis after a few days on selective medium and usually died within two weeks of selection, similarly to an earlier report about the selection of non-responsive scutella of the cultivar 'Salome' (Koprek, 1996). The responsive scutella produced calli and embryogenic structures, thus confirming the suitability of the selection system.

5.2.1.3. The AOS cDNA

Constitutive overexpression or down-regulation of *AOS* by genetic transformation means one possible way to modify endogenous jasmonate level, as it was shown in dycotelodonous species (Harms *et al.*, 1995; Wang *et al.*, 1999a; Laudert *et al.*, 2000). The AOS cDNAs isolated from barley have not been used for homologous transformation so far, and no attempts have been made to analyze JA-function in transgenic monocotyledonous plants. Several features of jasmonates, however, support a transgenic approach for studying their role in monocots: (i) in barley high levels of JAs can be detected localized in organs and tissues stressed by water deficit (scutella, nodes, senescent flag leaf, husks) as well as in dividing tissues such as root tips and leaf bases (Hause, unpublished); (ii) products of JA-degradation have not been detected to date (Miersch, pers. comm.); (iii) JM, JA and its amino acid-conjugates can be involved in the signalling pathway simultaneously (Wasternack *et al.*, 1998). Thus, problems such as those caused by the influence of several factors (e.g. transport, metabolism, gene dosis) on the phenotypic expression of cytokinin-function do not occur here (Hewelt *et al.*, 1994; Faiss *et al.*, 1997). Furthermore, unlike in the transgenic manipulation of cytokinin- and auxin-biosynthesis performed to date (Tinland *et al.*, 1991; Hewelt *et al.*, 1994), the control of transgenes by constitutive promoters should not inhibit hormone-synthesis or the regeneration of specific organs.

To study the effects of jasmonate deficiency, a vector was constructed which contains the barley AOS1 cDNA in antisense orientation (pUAOSantisense). Down-regulation of endogenous hormone

levels by transformation with antisense genes/cDNAs coding for enzymes involved in their biosynthesis, is a well-established technique in several hormone classes (for review, see Hedden and Phillips, 2000). Besides the numerous examples for ACC antisense plants (Hamilton *et al.*, 1990; Oeller *et al.*, 1991), *Arabidopsis* plants with down-regulated IAA (Grsic *et al.*, 1998; Ouyang *et al.*, 2000) and gibberellin (Coles *et al.*, 1999) production also confirm the functionality of the antisense strategy. Similarly to our pUAOSantisense construct, the vectors applied in these studies contain homologous antisense sequences.

5.2.1.4. The JIP23 cDNA

In barley, putative functions of the most abundant jasmonate-induced protein, JIP23, have been described in early stages of development and in excised leaf segments (Lehmann *et al.*, 1995; Hause *et al.*, 1996). Because of the high tissue- and cell specificity of its expression and its occurrence in all barley cultivars (Hause *et al*., 1999), this protein may have an essential function. The constitutive overexpression of *JIP23* in barley should give detailed insights into functions of JIP23 in each developmental stage. Such an overexpression may override other effects of JIP23 formed during barley development. Constitutive overexpression may lead to altered photosynthetic capacity of leaves, altered responses upon abiotic stresses (drought, osmotic stress), and altered developmental processes such as germination. In a similar way, the suppression of *JIP23* gene expression by an antisense approach should also lead to phenotypical alterations giving hints to the function of JIP23 during germination and stress response. This is suggested by effects described for constitutive overexpression of barley *JIP23* in tobacco (Görschen *et al.*, 1997b). The amount of JIP23 correlated proportionally with the number of transgene integrations and was inversely correlated with the amounts of certain house-keeping proteins, mainly proteins involved in photosynthesis (e.g. subunits of RuBPCase). Transformation with antisense JIP23 cDNA, however, did not alter the protein pattern.

5.2.1.5. Transient expression of the transgenes in barley mesophyll protoplasts

To date, our novel vectors containing the AOS1 and JIP23 cDNAs have not been used in the homologous stable transformation of barley. To test their functionality within a transient gene expression system, barley mesophyll protoplasts were transformed with the sense constructs (pUAOSsense, pUJIPsense) by PEG-mediated gene-transfer. Barley protoplasts of various origin (cell suspensions, mesophyll, aleurone and endosperm cells) offer ideal target cells for transient expression systems. Except for some attempts with electroporation and microinjection, PEG-mediated gene-transfer is the usual way of introducing foreign genes into barley protoplasts (Table 3).

The PAT activity and the immunological detection of JIP23 indicated that both the resistance gene (*pat*) and the transgene (JIP23 cDNA) could be transiently expressed in barley. In the case of AOS1 Western-blot has not been prepared because of technical reasons, see above. Therefore, these vectors were regarded to be suitable for the stable transformation of barley as well.

5.2.2. Plant regeneration through somatic embryogenesis from barley scutella

Tissue cultures of high regeneration capacity are prerequisites for the generation of transgenic plants at high efficiency. Before starting the transformation we adapted an *in vitro* plant regeneration system based on somatic embryogenesis from isolated scutella of the barley cultivar ‘Salome’. In barley transformation the most frequently used target explants are immature embryos. Stably transformed plants could be generated after the particle bombardment of or *Agrobacterium*-mediated gene transfer into the adaxial (scutellar) side of excised immature embryos (Table 3). The

scutellum is of advantage compared to other explants due to its high regeneration capacity. This can also be seen in the relatively high regeneration rate of transgenic plants derived from scutella which can exceed 4% (Wan and Lemaux, 1994; Jensen *et al.*, 1995; Tingay *et al.*, 1997). Embryo axis disturbs the formation of embryogenic calli because of precocious germination and the production of non-embryogenic soft calli (Nonohay *et al.*, 1999). This could be avoided by complete removal of the embryo axis from the abaxial side of the scutella. Since any residual tissue of the axis can generate undesired structures, this laboursome method was essential for the preparation of scutella of the recalcitrant cultivar 'Salome' in our experiments. In barley transformation based on the highly responsive genotype 'Golden Promise', a simpler method, the longitudal bisection of embryos, is used (Wan and Lemaux, 1994).

Somatic embryoids were generated, without a callus-phase, on 'Salome' scutella. The regeneration rate of green plants, however, was very low (11.5-14.4%) in our experiments. This corresponds to earlier data where the induction frequency of embryogenic structures was 10% and it decreased to 9% in bombarded scutella (Koprek, 1996). In contrast, in the cultivar 'Golden Promise', the most frequently used genotype in barley transformation, induction frequencies of 83% and 80%, respectively, could be achieved in the same experiment.

Besides the effects of the genotype, somatic embryogenesis in cultured scutella can be influenced by (i) the growing conditions of the donor plants, (ii) the developmental state of the immature embryos and (iii) the *in vitro* culture parameters. Donor 'Salome' plants were grown in a fully controlled growth chamber and donor spikes were collected about 14 days post-anthesis. In general, higher and less variable green plant regeneration rates can be achieved from growth chamber-grown plants. These consistently high rates are prerequisites for an efficient transformation program (Dahleen, 1999). Somatic embryos and embryogenic calli could be observed only at immature embryos of 1.5-2.5 mm in size. Under growing conditions similar to ours, immature embryos of both narrower (1.5-1.8 mm; Koprek *et al.*, 1996) and broader (1.0-2.5 mm; Cho *et al.*, 1998) size ranges can be capable of plant regeneration through somatic embryogenesis, depending on genotype.

Somatic embryogenesis in isolated barley scutella was induced on MS-based media supplemented with casein-hydrolysate, maltose and 2 or 3 mg/l 2,4-D (91/4 and 91/4m3 medium, respectively). In scutellum cultures of 'Salome', 3 mg/l 2,4-D resulted in a lower number of hard and embryogenic calli. The plant regeneration rate, however, improved indicating a better (probably more embryogenic) callus quality (Table 16). Such an improved embryogenic capacity has also been observed by Schulze (pers. comm.), suggesting 3 mg/l 2,4-D as the optimal concentration in scutellum cultures of 'Salome'.

In scutellum cultures of 'Salome' a very low regeneration rate could be achieved, representing a remarkable disandvantage for stable transformation. The preferential use of this cultivar, however, was supported by the following facts:

i. There is a cultivar-specific difference in JIP patterns and in the JA- and stress-induced accumulation of JIP23 in barley (Herrmann *et al.*, 1989; Hause *et al.*, 1999). Additionally, JIP23 is supposed to be a reporter for a rise of endogenous jasmonates (Lehmann *et al.*, 1995; Hause *et al.*, 1996). In previous studies on the response of barley leaf segments upon JA-treatment, 'Salome' responded the most quickly and exhibited the strongest changes in comparison with other cultivars (Hause and Wasternack, pers. comm.). The cDNAs coding for AOS (Maucher *et al.*, 2000) and JIP23 (Hause, pers. comm.), respectively, were initially isolated from this cultivar, thus providing the proper probes for homologous transformation.

ii. Via bombardment of scutella from the cultivar ‘Salome’ with PIG, one fertile transgenic plant exhibiting PAT- and GUS-activity has been produced (Koprek, 1996). This suggests the suitability of this cultivar for stable genetic transformation.

5.2.3. Optimization of bombardment parameters for the use of PIG

The transformation protocol used here was based on the bombardment of isolated scutella by a particle inflow gun (PIG). The device had been previously used only for transient assays, among others, in barley leaves, onion epidermal cells and tomato suspension cells. To our knowledge, no stable transformation experiments had been performed with this PIG previously.

The conditions optimal for a high transient gene expression rate do not necessarily result in a high number of transgenic plants (Becker *et al.*, 1994; Altpeter *et al.*, 1996a; Koprek, 1996). Therefore, in generating stable transformants, the optimal ratio of maximal DNA transfer and minimal cell death caused by bombardment has to be find out (Klein and Jones, 1999). Transient transformation rates can be converted into stable transformation rates with a frequency of 1-9% (Klein *et al.*, 1988b; Russell *et al.*, 1992; Ritala *et al.*, 1993). Therefore, in the case of genotypes of low embryogenic potential, such as ‘Salome’ in our experiments, optimization must fulfill different requirements. On the one hand, optimized parameters have to lead to a transient expression strong enough to offer a reasonable regeneration potential of stable transformants. On the other hand, however, the few cells possessing regeneration potential must stay viable after bombardment. Transgenic barley plants have already been generated from isolated scutella of recalcitrant cultivars by bombardment with PIG. In the case of cultivars ‘Salome’ and ‘Femina’ the bombardment conditions offered by the PIG - especially the moderate gas-pressure - seemed to be essential (Koprek, 1996).

5.2.3.1. Bombardment method

Using PIG, DNA-coated microcarriers are usually accelerated directly in the He-stream (Finer *et al.*, 1992). This bombardment method was modified by the introduction of a macrocarrier (aluminium foil disc) to carry the particle-suspension in glycerol (Koprek, 1996). Bombardments with this ‘aluminium foil/glycerol’ method gave very low transient expression rates in our experiments. This might be be caused, at least partially, by the mesh which can dramatically decrease the velocity of gold particles suspended in the viscosic solution. The mesh, however, was essential to save scutella from being removed from the plate by the He-stream. In a different model of PIG without mesh, ‘aluminium foil/glycerol’ method was successfully used to generate transgenic barley plants from genotypes (e.g. ‘Salome’) exhibiting lower potential for the production of embryogenic calli (Koprek, 1996). Due to the lower gas blast and acoustic shock caused by the PIG, the upper layers of scutellar tissues were less injured than by PDS, the most widely used particle delivery system. Thus, their embryogenic capacity could be maintained.

The novel ‘aluminium foil/ethanol’ method invented by Pauk (pers. comm.) has been used with the greatest efficiency here. It resulted in higher levels of transient GUS and LUC expression than the ‘filter/ethanol’ method. The ‘aluminium foil/ethanol’ method resembles the working principle of PDS (see 3.2.4.2.) which enables a more effective acceleration of the microcarriers. At the same time, however, the target tissues are subjected to a moderate He-blast and acoustic shock, keeping these beneficial characteristics of the original PIG system. This method has already resulted in transgenic wheat plants (Pauk, unpublished) while the ‘filter/ethanol’ method was used only for transient expression assays (Hunold *et al.*, 1994; Stenzel, 1998).

5.2.3.2. Helium pressure and distance settings

The influence of helium pressure on transformation efficiency must be evaluated at a given distance between the microcarrier outlet (on filter unit) and the target tissue. Additionally, other factors such as bombardment method, the amount of particles per shot etc. have to be taken into consideration as well.

The optimal helium pressure of 5.5 bar determined in our experiments is the same as reported by Koprek (1996) by using a different bombardment method ('aluminium foil/glycerol' instead of 'aluminium foil/ethanol') and a different model of PIG. This pressure is in the range (4-7 bar) usually applied for PIGs (Hunold *et al.*, 1993; Vain *et al.*, 1993). We had to use, however, a longer filter unit/target plate distance as usual. The generally recommended 6 cm was completely inapplicable in our PIG because scutella were blown out of the plate by the gas stream, even if a mesh was used. Target plate/filter unit distances of 10 and 13 cm combined with 4 cm mesh/filter unit distance gave the highest transient expression efficiency. Mesh has always been inserted between the particle outlet and the target tissue (i) to reduce the size of the microprojectile aggregates, (ii) to help to spread particles over the target plate and (iii) to moderate the gas blast and the acoustic shock at the same time. These beneficial effects, however, were not tested in our experiments. Morover, they were not always visible as a significant increase in the number of transformation events in other bombardment studies (Finer *et al.*, 1992; Russell *et al.*, 1992; Sanford *et al.*, 1993; Hunold *et al.*, 1994).

5.2.3.3. Osmotic treatment of the target tissues

In a set of experiments scutella were incubated pre- and post-bombardment on medium containing osmotica (0.2 M mannitol + 0.2 M sorbitol). With the introduction of this osmotic treatment, the responsivity of the bombarded scutella of 'Salome' increased from 9-15% to 21-23%. There was also a significant improvement in the regeneration capacity which was 0-0.98% with and 0.02-0.08% without osmotic treatment (Table 20). In wheat, pre- and post bombardment treatment of embryos on media of 0.4 M osmoticum (mannitol + sorbitol) had a significant impact on the improvement of transformation efficiency (Altpeter *et al.*, 1996a). The osmotic treatment of the target tissues has been recently introduced in the biolistic transformation of embryogenic calli, shoot meristematic cultures and immature embryos of barley (Ahlandsberg *et al.*, 1999; Zhang *et al.*, 1999; Scholz *et al.*, 2001). Experiments to prove its positive effects, however, have not been performed. Osmotica cause plasmolysis which reduces cell damage by preventing extrusion of the protoplasm from the bombarded cells, thus leading to increase in transient gene expression as well as in the recovery of stably transformed clones (Vain *et al.*, 1993). Osmotic treatment may also act through inducing stress response that protects the cells from apoptotic reaction to particle penetration (Klein and Jones, 1999).

5.2.3.4. Changes in responsivity upon bombardment

Bombardment parameters resulting in the highest transient GUS and LUC activities have been determined with BMS suspension cells and barley scutella. The changes in the regeneration capacity caused by bombardment were checked after the application of these settings in stable transformation experiments. Without bombardment the induction rate of hard and embryogenic calli (responsivity) was 54.7-78.5% (Table 16). Upon bombardment with the novel vector constructs it decreased to 21.2-25.5% if osmotic treatment was used. The lack of osmotic treatment led to a more significant decrease to 9.1-15.0% (Table 20). In contrast, in earlier studies with 'Salome' the induction frequency of embryogenic structures (10-11%) did not decrease upon bombarded with PIG mand one transgenic plant could be regenerated, in spite of the low responsivity (Koprek,

1996). Since the responsivity reported here was in the same range (9.1-25.5%), our bombardment strategy was supposed to be capable of generating transgenic plants of 'Salome'.

5.2.4. Plant regeneration from bombarded scutella

In this thesis the results of the first application of the novel plasmid constructs in stable transformation experiments are reported. The AOS and JIP23 cDNAs cloned in the vector constructs were isolated from the barley cultivar 'Salome', the model genotype of jasmonate studies, thus, 'Salome' was chosen to be involved in the first transformation studies performed with the new vectors. Due to the very poor responsivity and regeneration capacity of this genotype, however, these experiments were taken as preliminary ones – preceding further bombardments using genotypes of high regeneration capacity.

Hard and embryogenic calli induced from bombarded scutella became necrotic after 4-6 weeks upon selection and usually died 5-6 weeks after bombardment. Surviving shoot primordia and green, potentially regenerative structures were incubated on regeneration medium in the light. However, their development was arrested and they did not show any response to neither hormones stimulating shoot development (zeatin, BAP) nor to NAA stimulating root development. The only exceptions from this were two putative transformants regenerated from scutella bombarded with pUAOSsense and pUJIPsense plasmids. These plantlets developed slowly and failed to generate roots. Although the lack of roots could indicate the non-transformed, 'escape' status (Altpeter, pers. comm.; Schulze, pers. comm.), the regeneration process was continued anyway. After several weeks of incubation on NAA-containing medium, roots had been generated and the plants were transferred to soil.

PCR and PAT-assays were found to be representative enough to show the presence of the *pat* sequence and the activity of the PAT enzyme in the putative transgenic regenerants. Both tests gave negative results. The detection of the integrated AOS1 and JIP23-cDNAs should have been performed only with the PAT-positive plants. This is the strategy generally used in stable transformation systems: co-transformation/co-expression frequency is determined in resistant transgenic plants only. The co-transformation frequency of transgenes was 100% in most transformation studies with barley, wheat, maize and oat (Becker *et al.*, 1994; Hagio *et al.*, 1995; Ishida *et al.*, 1996; Gless *et al.*, 1998). For barley lower co-transformation frequency of 84% and even lower co-expression frequencies of 33-48% have also been reported. In each case, however, the number of the resistant transgenic plants was taken 100% (Jähne *et al.*, 1994; Wan and Lemaux, 1994).

The failure in the generation of transgenic plants may be attributed to several factors such as (i) the low number of scutella giving rise to totipotent cells, (ii) the limited number of totipotent cells into which DNA can be introduced and (iii) the small number of totipotent cells being capable of generating fertile, green plants (Lemaux *et al.*, 1999). Moderate He-pressure, osmotic treatment and the incubation of bombarded scutella in an 'intermediate step', however, can significantly enhance the transformation efficiency in recalcitrant genotypes (Cho *et al.*, 1998). In our experiments, low He-pressure was provided by the PIG. This factor alone should be sufficient to improve transformation efficiency in 'Salome' (Koprek, 1996). Osmotic treatment improved the responsivity in our experiments too, but failed to increase transformation efficiency (see 4.2.5.1.). Bombarded scutella of the 'intermediate step' are incubated on a medium of modified composition (2,4-D + BAP, high cc. of $CuSO_4$) and are exposed to dim light. Under these conditions calli of higher embryogenic capacity are produced. Morover, the proliferation of small numbers of green, totipotent cells is also promoted. This way, regenerability may be improved because sufficient amounts of transformed tissues are available to give rise to plants (Cho *et al.*, 1998). In our experiments, green, potentially regenerative tissues were incubated on media containing cytokinin

(zeatin) and exposed to light. The green structures could be kept alive for several weeks, but failed to develop further.

5.2.5. Future prospects

The novel vector constructs of proven functionality are ready for use in transformation. The constructs carrying the AOS or JIP23 cDNAs can be used to produce transgenic plants for the studies on the role of jasmonates in the development of barley as well as the analysis of function of JIP23. According to the results of the preliminary stable transformation studies, however, the use of a barley genotype of better regeneration capacity, such as cultivar 'Golden Promise', seems to be essential. This cultivar has established protocols for the PDS-1000/He particle bombardment system which could be adapted for the current transformation studies. Recently, for the transformation of barley *Agrobacterium*-mediated gene-transfer has been found to give better results than the traditional biolistic protocol (Altpeter, pers. comm.). Supported by published results as well, this method could also be introduced. In this case, however, new vector constructs should be designed, maintaining the order of promoters and transgenes of the current ones.

The novel vector constructs containing the full-length multiple cloning site offer a broad range of usage in the future. The various genes/cDNAs cloned in the MCS enable the involvement of the constructs in different transformation studies based on particle bombardment.

6. SUMMARY

I. Induction of haploid embryogenesis in isolated microspore cultures of triticale and barley under hormone-free conditions.

This part of the thesis is dealing with different aspects of microspore embryogenesis. Anther culture, one of the techniques based on androgenesis, is commonly used to accelerate the breeding process of triticale and other cereals today. Isolated microspore culture, however, has some additional advantages: it offers a unicellular system of synchronized haploid cells, which are excellent targets for transformation and for studies on the biochemical and molecular background of embryogenesis. In contrast to anther culture, microspore culture of triticale has not been reported to date.

1. We established the protocol for isolated microspore culture of triticale introducing five genotypes ('Presto' and 'Tewo x Moniko', 'Presto x Moniko', 'Presto x Novisadi', 'Novisadi x Moniko' F_1) and three induction media of different hormone-composition (hormone-free; 2,4-D + kinetin; PAA) into the experiments. The microblending of cold-pretreated spikes could be efficiently used for the isolation of microspores. The concentration of viable microspores was increased by density-gradient centrifugation. Microspores of the late-uninucleate to early-binucleate stages exhibited the capacity for division and gave rise to embryoids in induction media both with and without hormone-supplement. Plant regeneration was performed on hormone-free regeneration medium, regardless of the induction medium applied. Altogether 126 plants have been regenerated and transferred into soil.

2. In microspore cultures of triticale 90% of the regenerants were haploid which is in contrast with microspore cultures of other cereals, where usually spontaneous dihaploid plants can be regenerated at high percentage. Following colchicine-treatment, the proportion of fully or partially fertile triticale plants varied between 43% and 67%, excepting 'Presto x Novisadi' F_1 where no fertile plants were obtained. The fertile plants offered a proper material to be introduced into the traditional breeding process.

3. Hormone-free induction medium had a positive impact on embryoid production from 'Novisadi x Moniko' F_1 triticale microspores. Its effect on green and albino plant regeneration, however, was not significant. Induction media supplemented with hormones had no significant effect on any of the three parameters (embryoid, green and albino plant production).

In microspore cultures of the barley cultivars 'Igri' and 'Kymppi' androgenesis could be induced in media both with and without exogenous hormone-supplement. Here, however, the regeneration capacity of embryoids was significantly lower in hormone-free media. These data suggest that androgenesis and plant regeneration are independent events with respect to hormone-requirement as well. Cold pretreatment of spikes provides the signal which initiates and promotes embryogenesis in isolated microspores of both triticale and barley. The regeneration of plants, however, exhibits differences between genotypes and/or species regarding exogenous hormone-requirement.

4. The induction medium successfully used in triticale microspore culture has been tested also in barley micropore culture. Here, this medium gave a significantly lower regeneration rate in comparison with a medium of optimized nitrogen-composition. This difference was irrespective of the hormone-content of media and might be caused by the significantly lower (50%) inorganic N-content of the triticale-medium. Barley cultivars responded similarly to the suboptimal medium, while there was a significant difference in their response to a medium of optimized composition.

Our results suggest that some critical factors of androgenesis (e.g. stress required for the induction of embryogenesis) are of general validity irrespective of species and genotype, while others (e.g. hormone-requirement of plant regeneration, optimal composition of media) are specific for species and genotype.

II. Preparation of novel vector constructs to alter endogenous levels of jasmonates and JIP23 via genetic transformation of barley.

Barley can be regarded as a model system for studying the role of the jasmonates and jasmonate-induced proteins in monocots. While studies on jasmonate modulation by transgenic approaches have already been performed in dicotyledonous species, no corresponding data are available for monocots. Most of our current knowledge on the role of jasmonates in monocots is based on treatment with JAs or on stress-induced JA-formation. The comprehensive goal of our research program was to apply a transgenic approach for the studies on the role of jasmonates in the development of barley.

1. As one part of the main program and the goal of the current work, novel plasmid vectors have been prepared. Two of them carry the cDNA coding for AOS1 from barley in sense and in antisense orientation, respectively, under the control of the *Ubi-1* promoter. Additionally, the vector contains the *pat* selection marker gene under the control of the 35S promoter. Since AOS has regulatory role in JA-biosynthesis, modulation of endogenous jasmonate levels can be expected in AOS-transformants.

2. JIP23 is the most abundant jasmonate-induced protein of barley. Therefore, the analysis of its function was also attempted by a transgenic approach. Corresponding vectors were constructed by inserting a cDNA coding for JIP23 of barley in sense or antisense orientation, respectively, into the basic vector construct identical to that established for AOS.

3. As an intermediate product of vector preparation, two vector constructs were produced which carry a multiple cloning site (MCS) in sense or antisense orientation, downstream of the *Ubi-1* promoter. Additionally, the vector contains the *pat* selection marker gene under the control of the 35S promoter. The MCS includes restriction sites for at least 10 enzymes, thus enabling the cloning (and expression) of a wide range of genes/cDNAs .

4. The novel vector constructs were shown to be functionally active on the basis of the transient expression of the *pat* gene and that of the JIP23 cDNA in transformed barley mesophyll protoplasts.

5. Preliminary stable transformation experiments were planned with the barley cultivar ‘Salome’, the model genotype of jasmonate studies. Previously, a plant regeneration system based on somatic embryogenesis had been adapted for the barley cultivar ‘Salome’. Scutella of 1.5-2.5 mm diameter were optimal for the generation of embryogenic structures on media containing 2,4-D. The plant regeneration capacity was very low (10-15%).

6. Prior to stable transformation, bombardment parameters were optimized by transient assays in maize suspension cells and barley scutella bombarded by a particle inflow gun. A He-pressure of 5.5 bar and a target distance of 10-13 cm gave the highest transient *β*-glucuronidase and luciferase expression rates. Above the target plate a wire mesh has been applied to reduce the size of the microprojectile aggregates and to moderate gas blast and acoustic shock. The best transient expression results were received if coated gold particles were placed on the surface of an aluminium foil disc upon bombardment.

7. Scutella of cultivar ‘Salome’ were bombarded with the novel vector constructs, as their first use in stable transformation experiments. Bombarded scutella were exposed to selection on media containing 3 mg/l bialaphos. This concentration was sufficient to select non-responsive scutella and allowed the responsive ones to produce calli or embryogenic structures. Responsivity and regeneration capacity of ‘Salome’ scutella could be significantly increased by pre- and post-bombardment incubation of scutella on osmotica-containing medium.

8. Two putative transgenic lines have been received from the preliminary stable transformation experiments. These originated from bombardments with plasmids carrying the AOS1 and the JIP23 cDNAs in sense orientation, respectively. PCR-analysis and PAT-assay, however, revealed that the *pat* sequence was not present and was not expressed in these plants.

According to the results detailed above, functionable vector constructs are available for the production of transgenic barley plants exhibiting altered jasmonate or JIP23 levels. Similarly, a wide range of vectors and subsequently transgenic plants can be generated by the use of the vector construct containing an extensive MCS.

In addition, bombardment parameters were optimized for a particle inflow gun, thus facilitating the usage of the device in further studies based on the particle bombardment of scutella. Similarly, the first set of stable transformation experiments with the new vector constructs give useful information for further experiments, regarding the choice of donor genotype, bombardment and selection method.

7. ÖSSZEFOGLALÁS

Dolgozatomban két, egymástól független kutatási programban elért, a növénybiotechnológia különböző területeit érintő eredményeimről számolok be.

I. Haploid embriogenezis indukciója tritikále és árpa mikrospóra-tenyészetében, hormonmentes körülmények között.

E program célja a mikrospóra-embriogenezis különböző kérdéseinek vizsgálata volt. Az androgenezisen alapuló módszerek közül a portoktenyésztés napjainkban általánosan alkalmazott eljárás mind a tritikále, mind az árpa nemesítésében. A fajta-előállítás folyamatának lerövidítése által szerzett előny mellett az izolált mikrospórák tenyészetei a növénybiotechnológia szempontjából figyelemreméltó jellemzőkkel is rendelkeznek. Szinkronizált fejlődésű sejtekből álló egysejtes rendszer lévén a mikrospóra-tenyészetek kitűnő alapanyagot jelentenek a transzformáció, valamint az embriogenezis biokémiai és molekuláris hátterének tanulmányozása számára. Míg a tritikále portoktenyésztése egy, napjainkban a fajtaelőállítás gyakorlatában is alkalmazott technológia, tritikále mikrospórák eredményes tenyésztéséről az első beszámolók a jelen dolgozatban ismertetett eredményeink alapján készültek.

1. Munkánk során kidolgoztuk a tritikále mikrospóra tenyésztésének módszerét. A kísérleteket öt genotípussal (Presto valamint Tewo x Moniko, Presto x Moniko, Presto x Novisadi, Novisadi x Moniko F_1) és három, különböző hormon-összetételű (hormonmentes; 2,4-D + kinetin; PAA) indukciós tápoldattal végeztük. A tápoldatok makro-, mikroelem- és vitamin-összetétele megegyezett a 190-2 táptalajéval. A hidegben (4 °C, 14-21 nap) előkezelt kalászok homogenizátorban történő feldolgozása hatékony eljárásnak bizonyult a mikrospórák izolálására. Az életképes mikrospórák arányát sűrűség-gradiens („maltóz párna") centrifugálással növeltük meg. A késői egy-sejtmagvas és korai két-sejtmagvas fejlődési állapotú mikrospórák osztódása mind hormonmentes, mind hormontartalmú tápoldatban embrioidok fejlődését eredményezte. Az embrioidok további fejlődését szilárd indukciós táptalajon biztosítotuk. A növényregenerálás hormonmentes regeneráló táptalajon (190-2) történt. Összesen 126 növényt regeneráltunk és ültettünk talajba.

2. A tritikále mikrospóra-tenyészetekből kapott regeneránsok 90%-a haploid volt, ami ellentétben áll az egyéb gabonafélék mikrospóra-tenyészeteiben kapott spontán dihaploid növények nagy arányával. A kolchicin-kezelést követően, az egyes genotípusoktól függően, a teljesen illetve részlegesen fertilis tritikále növények aránya 43% és 57% között változott, kivéve a Presto x Novisadi F_1-et, melyből nem kaptunk fertilis növényt. A felnevelt növények a hagyományos nemesítés számára megfelelő alapanyagot jelenthetnek.

3. A hormonmentes indukciós tápoldat pozitív hatással volt a Novisadi x Moniko F_1 tritikále mikrospóra-tenyészetek embrioid-kihozatalára, a zöld és albinó növények regenerációját, azonban nem befolyásolta szignifikánsan. A hormonokkal (2,4-D + kinetin illetve PAA) kiegészített indukciós táptalajok sem az embrioid-indukcióra, sem az albinó és zöld növények regenerációjára nem voltak szignifikáns hatással.

Az Igri és Kymppi árpafajták mikrospóra-tenyészetében az androgenezis indukciója mind hormontartalmú, mind hormonmentes N24A2,7G3 illetve 190-2 alapú tápoldatokban lehetséges volt. A hormonmentes tápoldatban kapott embrioidok regenerációs kapacitása, azonban szignifikánsan alacsonyabb volt, függetlenül a tápoldat só- és vitamin-összetételétől. Adataink alapján, az embriogenezis és a növényregeneráció egymástól független eseményként való értékelése az eltérő hormonszükséglettel is igazolható. A kalászok hideg előkezelése jelenti az embriogenezis indukciójához szükséges szignált mind a tritikále, mind az árpa mikrospóra-tenyészetében. A

növényregenerációban, azonban, már különbségek tapasztalhatók az egyes fajok és/vagy genotípusok hormon-szükségletét illetően.

4. A tritikálében sikerrel alkalmazott hormonmentes indukciós tápoldatot (190-0) árpa mikrospóra-tenyészetben is teszteltük. Itt, azonban, ez a tápoldat szignifikánsan alacsonyabb szintű regenerációt eredményezett, mint egy optimalizált nitrogén-összetételű tápoldat (N24A2,7G3). Ez a különbség független volt a tápoldatok hormontartalmától, és feltételezhetően a tritikále-táptalaj szignifikánsan (50%) alacsonyabb szervetlen nitrogén-tartalmával áll összefüggésben. Az Igri fajta válaszadása szignifikánsan jobb volt a Kymppi-énél az optimalizált összetételű tápoldatban, míg a hormonmentes tápoldatban a két genotípus válaszadása hasonló szinten volt.

Eredményeink, tehát, azt mutatják, hogy az androgenezis egyes meghatározó tényezői (pl. az indukciót kiváltó stressz) fajtól és genotípustól függetlenül általános érvényűek, míg mások (pl. a növényregeneráció hormon-igénye és a táptalajok optimális összetétele) fajra illetve genotípusra specifikusak.

II. Új vektorok építése az endogén jazmonát és JIP23 szint megváltoztatására az árpa genetikai transzformációja útján.

Az árpa a jazmonátok valamint a jazmonátok által indukált fehérjék egyszikűekben betöltött szerepének tanulmányozására használt modellnövény. Míg a jazmonátok endogén szintjének transzgénikus úton való módosítását kétszikűekben többször is leírták, egyszikűekben hasonló eredményekről nem számoltak be. A jazmonátok egyszikűekben játszott szerepéről szerzett információink jazmonát-kezeléseken illetve stressz által indukált jazmonát-szintézisen alapulnak. Kutatási programunk átfogó céljaként, a jazmonátok árpában betöltött szerepét transzgénikus növényekben kívántuk vizsgálni.

1. A jelen dolgozathoz kapcsolódó munkák fő céljaként új plazmid vektorokat állítottunk elő. A két vektor az árpa AOS1 cDNS-ét tartalmazza szensz illetve antiszensz helyzetben, az *Ubi-1* promoter szabályozása alatt. A *pat* szelekciós markergén a *35S* promóter irányítása alatt található a vektorokban. Az AOS jazmonát-szintézisben betöltött szabályozó szerepéből adódóan a plazmid felhasználásával előállított transzgénikus növényekben az endogén jazmonát szint megváltozását vártuk.

2. A JIP23 a legnagyobb mennyiségben előforduló jazmonátok által indukált fehérje árpában. Hatásmechanizmusának tanulmányozására szintén a transzformáción alapuló megoldást választottuk. A szükséges vektorok alapjául az AOS-transzformációnál is használt konstrukció szolgált, melybe az árpa JIP23 cDNS-t szensz illetve antiszensz helyzetben építettük be.

3. A vektorépítés köztes termékeként létrehoztunk egy olyan konstrukciót, mely egy többszörös klónozóhelyet (multiple cloning site, MCS) tartalmaz szensz vagy antiszensz helyzetben, az *Ubi-1* promótertől 3’ irányban. A konstrukció szintén magában foglalja a 35S promóter irányítása alatt lévő *pat* gént tartalmazó expressziós kazettát. A MCS legalább tíz restrikciós endonukleáz hasítási helyét tartalmazza, így a vektor különböző gének/cDNS-ek klónozására (és kifejeztetésére) alkalmas.

4. Az új vektorok funkcionálisan aktívnak bizonyultak a *pat* gén és a JIP23 cDNS árpa mezofillum protoplasztokban mutatott tranziens expressziója alapján.

5. Az új vektorok stabil transzformációban történő felhasználására előkísérleteket terveztünk a jazmonát-kísérletek modell genotípusa, a Salome árpafajta felhasználásával. Ezt megelőzően, az éretlen embriókból izolált scutellumokból szomatikus embriogenezis útján történő növényregenerálás rendszerét alkalmaztuk erre a genotípusra. 2,4-D-tartalmú MS táptalajon a 1,5-

2,5 mm átmérőjű scutellumokat voltak legalkalmasabbak embriogén kalluszok indukciójára. A Salome fajta ebben a rendszerben igen alacsony regenerációs hányadost (10-15%) mutatott.

6. A stabil transzformációs kísérletek megkezdése előtt a génbelövés paramétereit génpuskával (particle inflow gun) belőtt kukorica szuszpenziós sejtekben és árpa szkutellumokban, a *bar* és a *luc* riportergének tranziens expressziója alapján optimalizáltuk. 5,5 bar He-nyomás valamint a célszövet és gyorsító egység közti 10-13 cm távolság eredményezte a legmagasabb szintű expressziót. A célszövet fölé helyezett drótszövet a mikrohordozókból képződött aggregátumok méretét, valamint a gáznyomás által okozott akusztikus sokkot csökkentette. A legmagasabb szintű tranziens expressziót eredményező belövési módszernél a DNS-sel bevont aranyrészecskéket egy alufólia-korong felületére etanolban vittük fel.

7. Az új vektorok stabil transzformációs programban történő első felhasználásaként, a Salome árpafajta szkutellumait lőttük be a korábban optimalizált paraméterek figyelembe vételével. A belőtt szkutellumokat 3 mg/l bialaphos-t tartalmazó táptalajon szelektáltuk. Ez a herbicid koncentráció elpusztította a nem-reagáló szkutellumokat, ugyanakkor lehetővé tette kalluszok illetve embriogén struktúrák fejlődését a reszponzív szöveteken. A Salome fajta szkutellumainak belövés előtt és után ozmotikumokat tartalmazó táptalajon való inkubálása jelentős mértékben javította a reszponzivitást és a regenerálóképességet.

8. A stabil transzformációs előkísérletekből összesen kettő, feltételezetten transzgénikus vonalat állítottunk elő. Ezek az AOS1 illetve a JIP23 cDNS-t szensz helyzetben tartalmazó plazmidokkal történt belövésekből származtak. PCR- és PAT-analízis bizonyította, hogy a *pat* gén nem volt jelen, illetve nem fejeződött ki a regenerált növényekben.

A fenti eredmények alapján, tehát, működőképes vektorok állnak rendelkezésre módosított jazmonát illetve JIP23 szintet mutató transzgénikus árpa növények előállítására. Emellett, vektorok - és velük transzgénikus növények - széles köre állítható elő a többszörös klónozóhelyet tartalmazó vektorok felhasználásával.

A fentiek mellett, a belövési paraméterek optimalizálása megkönnyíti a kísérletekben használt génpuska alkalmazását további, szkutellumok belövésén alapuló kísérletekben. Hasonlóképpen, az új vektorokkal végzett első stabil transzformációs kísérletek eredményei hasznos információkkal szolgálhatnak további kísérletekben a donor genotípus, valamint a belövési és szelekciós módszerek megválasztásához.

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9. APPENDIX

Appendix 1. Stomatal guard cell length and fertility of plants regenerated from microspore cultures of the triticale genotypes 'Presto' (a), 'Tewo x Moniko' F_1 (b), 'Presto x Novisadi' F_1 (c), 'Presto x Moniko' F_1 (d) and 'Novisadi x Moniko' F_1 (e)

a) 'Presto'

No. of plant	Length of stomatal guard cells* (μm)	Ploidy level*	No. of spikes**	Total seed set**
1.	29	n	3	40
2.	27	n	4	90
3.	30	n	3	36
4.	27	n	4	81
5.	31	n	-	-
6.	30	n	-	-
7.	31	n	1	21
8.	30	n	1	17
9.	36	2n	19	409
10.	31	n	-	-
11.	29	n	-	-
12.	38	2n	-	-
13.	32	n	-	-
14.	24	n	-	-
15.	32	mixoploid	5	115
16.	28	n	12	233
17.	28	n	-	-
18.	32	n	-	-
Total:			52	1042

Data determined before (*) and after (**) colchicine-treatment of haploid and mixoploid plants. Length of stomatal guard cells is the average of ten cells. Spikes of plants 9 and 16 were derived from divided tillers.

b) 'Tewo x Moniko' F_1

No. of plant	Length of stomatal guard cells* (μm)	Ploidy level*	No. of spikes**	Total seed set**
1.	29	n	-	-
2.	29	n	1	1
3.	30	n	3	49
4.	30	n	-	-
5.	28	n	1	3
6.	29	n	-	-
7.	29	n	4	101
8.	29	n	1	20
9.	27	n	-	-
10.	30	n	4	10
11.	38	mixoploid	4	82
12.	32	n	1	3
Total:			15	269

Data determined before (*) and after (**) colchicine-treatment of haploid and mixoploid plants. Length of stomatal guard cells is the average of ten cells.

c) 'Presto x Novisadi' F_1

No. of plant	Length of stomatal guard cells* (µm)	Ploidy level*	No. of spikes**	Total seed set**
1.	25	n	-	-
2.	25	n	-	-
3.	29	n	-	-
4.	22	n	-	-
5.	28	n	-	-
6.	30	n	-	-
Total:			0	0

Data determined before (*) and after (**) colchicine-treatment of haploid and mixoploid plants. Length of stomatal guard cells is the average of ten cells.

d) 'Presto x Moniko' F_1

No. of plant	Length of stomatal guard cells* (µm)	Ploidy level*	No. of spikes**	Total seed set**
1.	30	n	2	9
2.	27	n	1	29
3.	28	n	-	-
4.	31	n	-	-
5.	30	n	-	-
6.	27	n	2	16
7.	26	n	-	-
8.	25	n	2	29
9.	21	n	1	0
10.	32	n	-	-
11.	32	n	1	33
12.	26	n	-	-
13.	28	n	-	-
14.	27	n	-	-
15.	27	n	-	-
16.	27	n	1	1
17.	30	n	3	64
18.	25	n	4	35
19.	24	n	-	-
Total:			17	216

Data determined before (*) and after (**) colchicine-treatment of haploid and mixoploid plants. Length of stomatal guard cells is the average of ten cells.

e) 'Novisadi x Moniko' F_1

No. of plant	Length of stomatal guard cells* (µm)	Ploidy level*	No. of spikes**	Total seed set**
1.	26	n	-	-
2.	31	n	-	-
3.	29	n	3	40
4.	25	n	-	-
5.	26	n	-	-
6.	29	n	3	53
7.	32	n	-	-
8.	29	n	-	-
9.	26	n	-	-
10.	27	n	1	33
11.	25	n	-	-
12.	25	n	-	-
13.	27	n	2	30
14.	31	n	-	-
15.	29	n	1	1
16.	29	n	4	100
17.	38	2n	3	52
18.	27	n	-	-
19.	30	n	-	-
20.	30	n	-	-
21.	30	n	-	-
22.	28	n	6	126
23.	28	n	-	-
24.	29	n	3	73
25.	37	mixoploid	-	-
26.	29	n	2	23
27.	29	n	2	37
28.	29	n	8	248
29.	38	2n	3	49
30.	30	n	4	26
31.	30	n	3	99
32.	30	n	4	51
33.	35	2n	4	84
34.	31	n	1	1
35.	30	n	-	-
36.	30	n	-	-
37.	35	mixoploid	4	88
38.	36	mixoploid	-	-
39.	39	mixoploid	3	31
40.	39	2n	4	79
41.	34	n	-	-
42.	29	n	-	-
43.	25	n	1	31
44.	26	n	-	-
45.	30	n	3	59
46.	29	n	-	-
47.	29	n	2	29
48.	29	n	4	78
49.	38	mixoploid	-	-
50.	29	n	-	-
51.	22	n	-	-
52.	25	n	-	-

Appendix 1e continued.

53.	26	n	-	-
54.	22	n	-	-
55.	34	n	-	-
56.	34	n	2	47
57.	23	n	-	-
58.	26	n	2	59
59.	35	n	-	-
60.	32	n	3	37
61.	29	n	1	1
62.	28	n	3	83
63.	24	n	-	-
64.	32	n	-	-
65.	28	n	-	-
66.	32	n	2	81
67.	31	n	-	-
68.	26	n	-	-
69.	28	n	-	-
70.	30	n	-	-
71.	32	n	-	-
Total:			91	1829

Data determined before (*) and after (**) colchicine-treatment of haploid and mixoploid plants. Length of stomatal guard cells is the average of ten cells.

Appendix 2. Composition of basic media used in microspore cultures of barley and triticale

Component	**N24***	**190-2****
Macro elements (mgL^{-1})		
NH_4Cl	144	
$(NH_4)_2SO_4$		200
KNO_3	2457	1000
$Ca(NO_3)_2 \times 4H_2O$		100
$CaCl_2 \times 2H_2O$	450	
$MgSO_4 \times 7H_2O$	350	200
KH_2PO_4	200	300
KCl		40
Na_2EDTA	37	37
$FeSO_4 \times 7H_2O$	28	28
Micro elements (mgL^{-1})		
$MnSO_4 \times H_2O$	25.00	6.10
$ZnSO_4 \times H_2O$	7.50	3.00
H_3BO_3	5.00	3.00
KI	0.75	0.50
$Na_2MoO_4 \times 2H_2O$	0.25	
$CuSO_4 \times 5H_2O$	0.025	
$CoCl_2 \times 6H_2O$	0.025	
Vitamins (mgL^{-1})		
Myoinositol	100	100
Nicotinic acid	1	0.5
Pyridoxine HCl	1	0.5
Thiamine HCl	10	1
Amino acids (mgL^{-1})		
Glycine		2
Glutamine	222	

* Mordhorst and Lörz, 1993

** Zhuang and Jia, 1983

Appendix 3. Optimization of (a) He-pressure and (b) distance parameters of bombardment with BMS suspension cells

a) Optimization of He-pressure

He-pressure (bar)			He-pressure (bar)		
4.5	5.5	6.5	4.5	5.5	6.5
LUC activity (RLU/mg protein)			GUS activity (blue spots/plate)		
335	**967**		5	1	**24**
413	240		12	**74**	20
238	68		62	**75**	1
318	**382**		37	**127**	19
5303	3199	2247	3	**25**	
3428	1635	3078	10	**12**	
6421	296	5561	12	**16**	
3581	789	**4039**	**21**	20	
4194	**6274**	4297		**245**	192
5217	**11225**	5325		**215**	156
3933	4957	**5768**		**226**	176
458	1044	**6629**		**272**	183

RLU/mg protein values are calculated as the average of 2 bombarded plates. Number of blue spots were calculated as the average of 1-4 plates. Plates were bombarded via the 'filter/ethanol' method. Results exhibiting the highest LUC- or GUS-activity are printed bold in each experiment.

b) Optimization of the distance parameters

Distances a/b (cm)				Distances a/b (cm)			
1/7	1/10	4/10	4/13	1/7	1/10	4/10	4/13
LUC activity (RLU/mg protein)				GUS activity (blue spots/plate)			
967	240	68	382	1	74	75	**127**
3199	1635	296	789	**25**	12	16	20
6274	**11225**	4957	1044	245	215	226	**272**
				14	20	18	**35**

a: distance between filter unit and mesh; b: distance between filter unit and target plate. RLU/mg protein values are calculated as the average of 2 bombarded plates. Number of blue spots were calculated as the average of up to 4 plates. Plates were bombarded via the 'filter/ethanol' method, at 5.5 bar He-pressure. Results exhibiting the highest LUC- or GUS-activity are printed bold in each experiment.

Appendix 4. Solutions used in isolation and transformation of mesophyll protoplasts of barley

CPW salts (mgL^{-1})

KH_2PO_4	27.2
KNO_3	101.0
$MgSO_4x7H_2O$	246.0
KI	0.16
$CuSO_4x5H_2O$	0.025
$CaCl_2x2H_2O$	1480

Washing solution (**WLW**)

Mannitol	0.68 M
CPW salts	
pH 5.8	
800±20 mOsmol/kg H_2O	

Ca/PEG

$Ca(NO_3)_2x4H_2O$	0.1 M
Mannitol	0.4 M
PEG 4000	40%
pH 7-9	

Enzyme solution (**EW**)

Cellulase (Ono-zuka R 10)	2%
Pectinase	0.02%
Mannitol	0.57 M
CPW salts	
pH 5.8	
800±20 mOsmol/kg H_2O	

MaMg/G

Mannitol	0.6 M
$MgCl_2x6H_2O$	15 mM
MES	0.1%
pH 5.8	

Culture medium (**T/G**) (mgL^{-1})

Macro elements*	
½ conc. MS macro elements	
FeNaEDTA	36.70
Micro elements**	
$ZnSO_4$	1
H_3BO_3	1
$MnSO_4x4H_2O$	0.1
$CuSO_4x5H_2O$	0.03
$AlCl_3$	0.03
$NiCl_2x6H_2O$	0.03
KI	0.01
Vitamins***	
myo-Inositol	100
Ca-Panthotenate	1
Nicotinic acid	1
Pyridoxine	1
Thiamine	1
Biotin	0.01
Hormones	
NAA	1.5
BAP	0.5
IAA	0.5
ZEA	0.5
Other organic compounds	
L-Glutamine	375
Sucrose	20 g/l
Mannitol	105.5 g/l
pH 5.8	
800±20 mOsmol/kg H_2O	

* Murashige and Skoog (1962)
** Heller (1953), occasionally replaced with micro elements of B5 medium (Gamborg *et al.,* 1968)
*** Morel and Wetmore (1951)

Appendix 5. Media applied in plant cell and tissue cultures

	91/4 (mgL^{-1})	**L32** (mgL^{-1})
Macro elements	MS macro elements*	
Micro elements	MS micro elements*	
Vitamins	MS vitamins*	
Hormones		
2,4-D	2.0	1.0
Other organic compounds		
Caseine hydrolysate	500	-
Synth. coconut-milk	-	5 ml/l
Maltose	60 gL^{-1}	-
Sucrose	-	30 gL^{-1}
	pH 5.8	pH 5.8

* Based on Murashige and Skoog (1962)

** Prepared as 200x stock solution

Synthetic coconut-milk for L32	(mgL^{-1})**
L-Alanine	29.7
γ-Amino-butyric acid	26.0
L-Arginine HCl	3.1
L-Asparagine	3.8
L-Aspartic acid	1.7
L-Glutamine	0.3
L-Glutamic acid	15.7
Glycine	2.7
L-Histidine	0.05
L-Leucine	5.0
L-Lysine	2.0
L-Methionine	0.05
L-Phenylalanine	0.05
L-Proline	1.9
L-Serine	12.8
L-Threonine	4.1
L-Tyrosine	0.05
L-Valine	2.3
L-Cysteine	3.0
Hydroxyproline	1.25
Ascorbic acid	0.1
Biotin	0.01
Folic acid	1.0
Ca-Panthotenate	0.1
Riboflavin	0.1
Cobalamine	0.0015
Choline	10.0

Appendix 6. Selection strategies in the stable transformation experiments

a) Particle bombardments with the pUJIPsense vector

	Scutella on the 1. selection medium		Transferred hard or embryogenic calli			No. of shoots*
	No. of scutella	Medium	No. of calli	Medium	Selection step	
Without osmotic treatment						
	400	91/4bi	33	91/4bi	2. (i)	
	875	91/4bi	76	91/4m1bi	2. (i)	1
	1275	91/4bi	213	91/4m3bi	3. (ii)	1
	600	91/4m3bi	127	91/4m3bi	3. (ii)	
	850	91/4m3bi	223	91/4m3bi	2. (i)	
	600	91/4m3bi	12	91/4m1bi	3. (ii)	
SUM:	4600		684			2
With osmotic treatment						
	864	91/4m3	252	91/4m3bi	2.	4
	576	91/4m3bi, 91/4m3	79	91/4m3bi	3.	
SUM:	1440		331			4

* No. of individual embryoids giving rise to shoot- or leaf-like structures >2 mm.
(i) and (ii) refer to the basic selection methods detailed in 4.2.5.1.

b) Particle bombardments with the pUJIPantisense vector

	Scutella on the 1. selection medium		Transferred hard or embryogenic calli			No. of shoots*
	No. of scutella	Medium	No. of calli	Medium	Selection step	
Without osmotic treatment						
	775	91/4bi	49	91/4m1bi	2. (i)	
	1250	91/4bi	299	91/4m3bi	3. (ii)	1
	1350	91/4m3bi	288	91/4m3bi	2. (i)	
	2050	91/4m3bi	66	91/4m3bi	3. (ii)	
	1075	91/4m3bi	22	91/4m1bi	3. (ii)	
SUM:	6500		724			1
With osmotic treatment						
	1535	91/4m3bi, 91/4m3	391	91/4m3bi	2.	15
SUM:	1535		391			15

* No. of individual embryoids giving rise to shoot- or leaf-like structures >2 mm.
(i) and (ii) refer to the basic selection methods detailed in 4.2.5.1

c) Particle bombardments with the pUAOSsense vector

	Scutella on the 1. Selection medium		Transferred hard or embryogenic calli			No. of shoots*
	No. of scutella	Medium	No. of calli	Medium	Selection step	
Without osmotic treatment						
	450	91/4bi	71	91/4m1bi	2. (i)	4
	250	91/4bi	13	91/4m3bi	3. (ii)	
	1100	91/4m3bi	213	91/4m3bi	2. (i)	
	3050	91/4m3bi	146	91/4m3bi	3. (ii)	
SUM:	4850		443			4
With osmotic treatment						
	2096	91/4m3	321	91/4m3bi	2.	
	1056	91/4m3bi	348	91/4m3bi	2.	
SUM:	3152		669			0

*No. of individual embryoids giving rise to shoot- or leaf-like structures >2 mm.
(i) and (ii) refer to the basic selection methods detailed in 4.2.5.1

d) Particle bombardments with the pUAOSantisense vector

	Scutella on the 1. Selection medium		Transferred hard or embryogenic calli			No. of shoots*
	No. of scutella	Medium	No. of calli	Medium	Selection step	
Without osmotic treatment						
	125	91/4bi	20	91/4m1bi	2. (i)	
	250	91/4bi	31	91/4m3bi	3. (ii)	
	2350	91/4m3bi	663	91/4m3bi	2. (i)	2
	2700	91/4m3bi	159	91/4m3bi	3. (ii)	
	1500	91/4m3bi	25	91/4m1bi	3. (ii)	
SUM:	6925		898			2
With osmotic treatment						
	192	91/4m3	45	91/4m3bi	2.	1
SUM:	192		45			1

*No. of individual embryoids giving rise to shoot- or leaf-like structures >2 mm.
(i) and (ii) refer to the basic selection methods detailed in 4.2.5.1

e) Particle bombardments with the pAHC25 vector

	Scutella on the 1. Selection medium		Transferred hard or embryogenic calli			No. of shoots*
	No. of scutella	Medium	No. of calli	Medium	Selection step	
Without osmotic treatment						
	50	91/4bi	28	91/4m1bi	2. (i)	2
	100	91/4m3bi	9	91/4m3bi	2. (i)	
	100	91/4m3bi	14	91/4m1bi	2. (i)	3
SUM:	250		51			
With osmotic treatment						
	480	91/4m3, 91/4m3bi	24	91/4m1bi	3.	
SUM:	480		24			

*No. of individual embryoids giving rise to shoot- or leaf-like structures >2 mm.
(i) refers to the basic selection methods detailed in 4.2.5.1.

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SUMMARY

MICROSPORE CULTURE AND GENETIC TRANSFORMATION STUDIES IN BARLEY AND TRITICALE

MONOSTORI TAMÁS

Microspore cultures are optimal objects for biotechnological and plant breeding studies of practical aspect. Besides, the decisive factors of haploid embryogenesis can be studied here in a synchronized unicellular system. In the first part of the current work, the protocol for the regeneration of haploid/dihaploid plants from isolated microspores of triticale is described. The method was established in experiments with five genotypes and it is suitable for the application in plant breeding. Its further practical advantage is that through the microspore isolation method used here (microblending of spikes), the laboursome process of anther isolation can be avoided. In further studies, it was observed that the induction of haploid embryogenesis does not require exogenous hormone-supplement neither in triticale nor in barley microspore culture. It is the first description in unicellular systems of these two monocot species, that hormones are not essential exogenous factors required for the induction of haploid embryogenesis. Thus, these results suggest that some critical factors of androgenesis (e.g. stress required for the induction of embryogenesis) are of general validity irrespective of species and genotype, while others (e.g. hormone-requirement of plant regeneration, optimal composition of media) are specific for species and genotype.

Barley can be regarded as a model system of studies on the role of the jasmonates and jasmonate-induced proteins (JIPs) in monocots. Our current knowledge on the role of jasmonates and JIPs in monocots is based on treatment with JAs or on stress-induced JA-formation. In the second part of the current work, the prerequisites were prepared for further transgenic studies on the role of jasmonates in the development of barley. Novel plasmid vector constructs have been prepared, which contain the barley AOS1 or JIP23 cDNAs in sense or antisense orientation, respectively, under the control of the *Ubi-1* promoter. Based on the same construct, vectors have been prepared, which contain a multiple cloning site (MCS) in sense or antisense orientation, respectively, downstream of the *Ubi-1* promoter. The cloning site includes restriction sites for at least ten enzymes. Thus, these vectors can be used for the cloning (and expression) of further genes/cDNAs. The functionality of the novel vector constructs has been revealed by the transient expression of the *pat* resistance marker gene and that of the JIP23 cDNS in barley mesophyll protoplasts transformed with the sense vector constructs. Thus, it is suggested that the AOS and JIP23 sense and antisense

vector constructs are suitable for the modification of endogenous jasmonate and JIP23 levels in barley an other cereals via genetic transformation.