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Genetic Transformation of Barley (*Hordeum vulgare* L.) to Engineer the Biosynthetic Pathway of Lysine and Threonine in the Endosperm

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Preface

You see things, and say why?
But I dream things that never were, and I say, why not?

George Bernard Shaw

Genetic engineering captured my attention long time ago. My postgraduate studies in the field of plant physiology encouraged me to prolong my studies in the level of plant genetic engineering as an attempt to understand more about this incredible amazing area of plant science.

This research study was done during the period from January 2002 to June 2006 under supervision of Professor Dr. Gerhard Wenzel (TUM) Technical University Munich, Institute of Plant Breeding, Center of Life and Food Sciences Wheihenstephan, and Dr. Martin Müller (LfL) Bavarian State Research Center for Agriculture at Freising. The experiments were carried out at the Institute of Crop Science and Plant Breeding (LfL) Gene Transfer Group.

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Really for achieving progress in science, it is not enough to have a place in a lab and financial support, for me as a foreign student the environment was most important. Therefore “Heartfelt Thanks” for the generous hospitality I found here in Germany.

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Special thank to all my family particularly my parents, my wife, my sweetheart daughters Shahd & Haidy and my friends for their kind help and encouragement.

Finally I am grateful to Egypt; people, land, Nile and the Egyptian government for providing the financial support for this work in the form of a four-year scholarship. Doubtless, this amazing opportunity has given me incredible inspiration for my future endeavors as a researcher and development as a person.

This thesis is dedicated to prophet MOHAMMAD (Prayer and Peace from ALLAH upon him)
The Last Messenger of God for his message of Knowledge, Freedom,
Justice, Peace and Mercy he brought to the whole Universe.

Contents

1. INTRODUCTION

1.1. General introduction

Human and monogastric animals cannot synthesize 10 out of the 20 amino acids and therefore need to obtain these from their diet. Cereal grains, rice, wheat, maize and barley, are the main source for human food and animal feed worldwide. However, cereal grains have poor nutritional quality because of their limited synthesis and accumulation of several essential amino acids, particularly lysine and threonine (Bright and Shewry, 1983).
Over 200,000 tons of lysine, produced by microbial fermentation are used annually to enrich cereal based diets for animals, resulting in increased costs of these feeds (Falco *et al.,* 1995, 1997). An increase in the lysine content of cereal grains such as maize or barley would reduce the need to supplement feed based on grains with crystalline lysine.

The aim of this work is to develop transgenic barley plants with elevated levels of lysine and threonine specifically in the grains through endosperm-specific expression of the mutated *E. coli lysC* and *dapA* genes using the *D-hordein* promoter of the barley *Hor3* gene. Alternatively plants should be produced with constitutively expressed genes using the ubiquitin promoter.
Towards this goal the following steps were planned and performed:

1. Establishing an efficient *Agrobacterium* transformation protocol for barley.
2. Construction of novel transformation vectors for expressing the *lysC* and *dapA* genes in barley grains.
3. Molecular and biochemical analysis of the transformants.

Since the middle of the last century, plant breeders began screening cereal grains of collections worldwide for high lysine content, but they found relatively little variation in kernel lysine. Munck *et al.* (1970) finally identified the high-lysine barley mutant “Hiproly” that contains 20-30 % more lysine than normal barley. Due to low yield and undesirable traits associated with the high lysine content trait this naturally mutant failed to give rise to any commercial varieties.

Another strategy to develop high lysine cereals was based on induced mutagenesis. This resulted in the mutant *opaque-2* (*o2*) maize (Mertz *et al.,* 1964), and the mutant Risϕ1508 of barley (Ingversen *et al.,* 1973). Unfortunately, the high lysine trait was negatively

correlated with other agronomic characteristics: in *o2* maize plants were susceptible to pathogens and the yield was lower than that of wild type plants (Azevedo *et al.*, 2003); the grains of Risϕ1508 were shrunken and gave a poor yield (Munck, 1992a, b). Therefore, these mutants have never been grown commercially, nor had been the character incorporated into any agronomically viable line. Thus, the contribution of mutation breeding methods towards achieving this aim was limited.

In higher plants and in many bacterial species, lysine is synthesized from aspartate via the aspartate-family biosynthetic pathway, which also leads to the synthesis of threonine, methionine and isoleucine (Figure 1). Both in plants and bacteria, lysine biosynthesis is controlled by two key enzymes, the first one, aspartate kinase (AK) which is feed-back inhibited by lysine and threonine, the second key enzyme, dihydrodipicolinate synthase (DHDPS) which is inhibited only by lysine (Bryan, 1980, Umbarger, 1978).

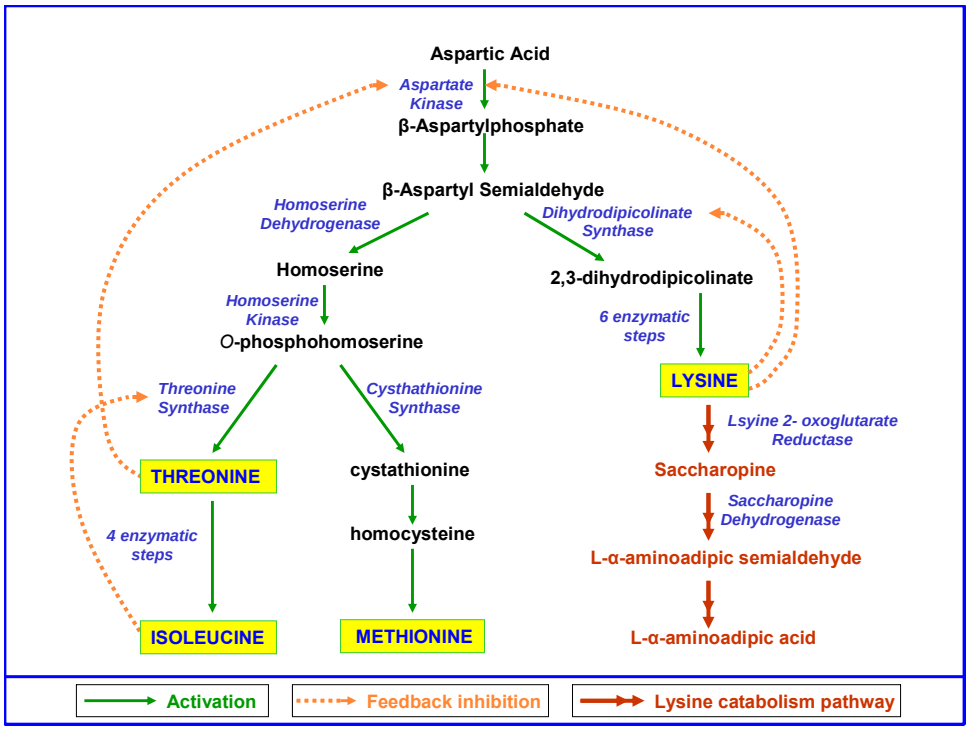

Figure 1: Aspartate family biosynthetic pathway, modified after Fornazier *et al.*, 2003

Recent studies have reported that the content of lysine and threonine can be increased by generating transgenic plants expressing feed-back insensitive bacterial AK and DHDPS enzymes (Ben-Tzvi *et al.*, 1996; Brinch-Pedersen *et al.*, 1996; Falco *et al.*, 1995, 1997; Galili, 1995; Karchi *et al.*, 1993; Kwon, *et al.*, 1995; Lee *et al.*, 2001; Shaul and Galili, 1992).

Constitutive transcription of mutated *E. coli lysC* and *dapA* genes, encoding feed-back insensitive AK and DHDPS, respectively, resulted in 14- and 15-fold increases in free lysine and threonine in the leaves of transgenic tobacco plants (Shaul and Galili, 1992).
In addition, seed-specific expression of the mutated *lysC* gene in tobacco seeds gave a 7-fold increase in the free threonine content and a 3-fold increase in methionine (Karchi *et al.,* 1993; Galili, 1995).
A dramatic increase (100-fold) in free lysine was obtained and the total lysine was doubled in seeds of canola plants transformed with the *dapA* gene of *Corynebacterium* (Falco *et al.,* 1995, 1997). Moreover, in soybean a co-expression of both genes resulted in a several hundred-fold increase in free lysine and up to 5-fold in total lysine (Falco *et al.,* 1995, 1997).

Brinch-Pedersen *et al.,* (1996) reported that, leaves of primary transgenic barley plants (T_0) expressing the mutated *E. coli lysC* and *dapA* genes exhibited a 14-fold increase of free lysine and a 8-fold increase in free methionine and a 50% reduction in free glutamine. In mature seeds of *dapA* transgenics, there was a 2-fold increase in free lysine, arginine, asparagine and a 50 % reduction in free proline, while no changes were observed in the seeds of the two *lysC* transgenic lines analyzed, compared to that of control seeds.

Based on these findings it is evident that generating a transgenic plant with a high-lysine production can be an effective tool and alternative method that overcomes the conventional breeding program limitations.

1.2. Transformation of barley (*Hordeum vulgare* L.)

Current transformation projects focus mainly on the quality of the barley grain for malting and feed (Brinch-Pedersen *et al.,* 1996; Nuutila *et al.,* 1999; Horvath *et al.,* 2000; Patel *et al.,* 2000) and on the disease resistance, mainly virus and fungal (Horvath *et al.,* 2003; Wang *et al.,* 2001). Moreover, barley grains can be used as a bioreactor to produce therapeutic proteins, which can for example be used directly in the detection of HIV antibodies in blood, which is more effective than the use of tobacco leaves and stems or potato (Schünmann *et al.,* 2002).

Cereals have been recalcitrant for transformation for a long time due to the lack of a suitable *Agrobacterium* transformation system. Only the development of an alternative gene delivery method has lead to a reliable establishment for a transformation system in cereals. For instance the biolistic system led to stable transformation of all cereal species, rice (Toriyama *et al.,* 1988; Zhang *et al.,*1988; Shimamoto *et al.,* 1989; Christou *et al.,* 1991), maize (Fromm *et al.,* 1990; Gordon-Kamm *et al.,* 1990), wheat (Vasil *et al.,* 1992; Weeks *et al.,* 1993), oat (Somers *et al.,*1992), and barley (Wan and Lemaux 1994). All that underlines that in the beginning *Agrobacterium* was not the suitable system to transform cereals, although a few studies provided evidence for *Agrobacterium*-mediated transformation of cereal tissues (Gould *et al.,* 1991; Raineri *et al.,* 1990).

Until recently *Agrobacterium*-mediated transformation was still restricted to only a few non-important species. The major breakthrough occurred in rice, for which two independent groups reported molecular and genetic evidence for its transformation using *Agrobacterium* as a vector (Chan *et al.,* 1992, 1993; Hiei *et al.,* 1994). Molecular analysis confirmed that the characteristic feature of T-DNA integration was basically similar to those in dicots (Hiei *et al.,* 1994). This stimulated further studies using *Agrobacterium* to transform other cereal crop species, which have been successful with maize (Ishida *et al.,* 1996) and wheat (Cheng *et al.,* 1997). Barley was the last insolent cereal plant to be transformed by *Agrobacterium*. In 1997 Sonia Tingay and colleagues reported the first successful attempt to produce fertile transgenic barley plants by co-cultivating immature embryos with *A. tumefaciens* carrying a binary vector for expressing the bacterial genes, *bar* and *uidA*.

There are a number of advantages of *Agrobacterium* transformation over particle bombardment, which refer to simplicity and the production of transgenic plants with a low copy number of the transgene and with simpler integration patterns. Therefore, the progress in developing the protocols to enhance the transfer of T-DNA from *Agrobacterium* to a plant cell represents the milestone in achieving reasonable efficiency in transformation of recalcitrant barley species, which is the prerequisite step to improve commercial barley species by genetic engineering (Lemaux *et al.,* 1999).

To establish a reproducible barley transformation system using *Agrobacterium* a high efficient regeneration procedure and an efficient *Agrobacterium* transformation protocol are required.

1.2.1. *In vitro* culture and regeneration as a prerequisite for transformation

Plants are characterized by *totipotency*. Each cell possesses and is potentially able to express the entire genetic program to form a fully fertile and complete plant body (Haberlandt, 1904). An efficient tissue culture system is a prerequisite for a cereal transformation system based on the introduction of DNA into totipotent plant cells. DNA can be introduced into isolated cells or protoplasts, explanted tissues, callus, or cell suspension cultures. However, the process is characteristically inefficient and only a proportion of cells in a target population will be transformed. These cells must be induced to proliferate at the expense of nontransformed cells, and this can be achieved by introducing a selectable marker gene and regenerating plants under an appropriate selective regime (Twyman *et al.,* 2002).

In barley, as in all cereals, a variety of explants have been successfully used for obtaining morphogenesis *in vitro* (Jähne-Gaertner and Lörz 1996). The most common are apical meristems (Weigel and Hughes 1985; Sharma *et al.,* 2004), mesocotyles (Jelaska *et al.,* 1984; Müller *et al.,* 1989), seedling segments (Becher *et al.,* 1992), mature embryos (Lupotto, 1984; Akula *et al.,* 1999), immature embryos (Thomas and Scott 1985; Chang *et al.,* 2003; Eudes *et al.,* 2003) and microspores (Jähne *et al.,* 1991; Li and Devaux 2001).

Although a variety of explants have been tested for establishing embryogenic callus cultures, immature embryos (IEs) are currently the most reliable and efficient regeneration system and target tissue for transformation (Wan and Lemaux1994; Tingay *et al.,* 1997;

Murray *et al.,* 2004; Travella *et al.,* 2005). However, the use of immature embryos is highly labour intensive for two reasons, firstly the isolation of the preferred target tissue the scutellum is only possible using a microscope, and secondly a regular cultivation of donor plants under controlled conditions is needed to obtain a sufficient number of immature embryos with the required physiological state. Alternative systems are continually sought or reevaluated to widen the selection of explants as targets for transformation.

Apart from the importance of explant choice, the genotype has a pronounced impact on cereal transformation. Model genotypes (or cultivars) known to be amenable in *in vitro* culture are being used almost exclusively, for example the variety "Golden Promise" of barley. Recently, efforts are being made to extend the transformation technology to elite genotypes, which are either agronomically important breeding lines or current commercial varieties (Cho *et al.,* 1998; Zhang *et al.,* 1999). But it is recognized that *in vitro* response is to a large extent genetically controlled and that culture requirements vary between genotypes. Hence, to develop broadly applicable culture systems for extending transformation capability across a range of germplasm, many attempts focused on the effect of culture medium components on the regeneration capacity (Zhang *et al.,* 1999; Chang *et al.,* 2003; Sharma *et al.,* 2004) and on the influence of the DNA delivery method on regeneration (Becker *et al.,* 1994; Koprek *et al.,* 1996; Rasco-Gaunt *et al.,* 1999a).

One of the crucial media components influencing the regeneration of cereals through somatic embryogenesis is the type of auxin. 2,4-dichlorophenoxyacetic acid (2,4-D) used together with or without cytokinins, such as zeatin or 6-benzylaminopurine (6-BAP), is described as the main auxin substance for inducing embryogenic callus. However, for barley numerous studies on the effect of 2,4-D, Dicamba (3,6-dichloro-O-anisic acid), Picloram (4-amino-3,5,6-trichloropicolinic acid) or 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) on the induction of embryogenic callus revealed that Dicamba significantly enhanced the regeneration through somatic embryogenesis (Lührs and Lörz, 1987; Castillo *et al.,*1998; Przetakiewicz *et al.,* 2003). Dicamba has been the most relevant auxin substance for *in vitro* culture in many barley transformation experiments (Wan and Lemaux 1994; Tingay *et al.,* 1997; Murray *et al.,* 2004; Travella *et al.,* 2005).

Immature embryos have a high potential to produce embryogenic callus which can develop to somatic embryos. Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular

connection with the original tissue (Von Arnold *et al.,* 2002). Somatic embryogenesis is an important tool for large scale vegetative propagation. Therefore, regeneration through somatic embryogenesis is preferred for an efficient transformation system. Somatic embryos can differentiate either directly from the explant without an intervening callus phase or indirectly after a callus phase. Explants from which direct embryogenesis is most likely to occur include microspores, ovules, zygotic embryos, somatic embryos and seedlings. Somatic embryogenesis is a multi-step regeneration process starting with proembryogenic masses (PEMs), followed by somatic embryo formation, maturation, and plant regeneration (Williams and Maheswaran, 1986). The induction of somatic embryogenesis implies the termination of a current gene expression pattern in the explant tissue, and its replacement with an embryogenic gene expression program. One possible mechanism for down regulation of current gene expression is DNA methylation, which is influenced by auxins (Lo Schiavo *et al.,* 1989).

1.2.2. Factors influencing *Agrobacterium*-mediated transformation

1.2.2.1. *Agrobacterium* strain and binary vector

Among many *Agrobacterium* strains, only a few have been used successfully in cereal transformation. Of importance is the supervirulence strain of A281 which has wide host-range and induces large tumors. Supervirulence is conferred by the Ti plasmid pTiBo542 (Jin *et al.,* 1987). The Plasmid pTiBo542 contains the *virG* and the 3' end of the *virB* operon in a region outside the T-DNA. Two systems are derived from pTiBo542:

a**)** the binary vectors utilizing the disarmed form of pTiBo542.

b**)** a super-binary vector carrying extra copies of *virB, virC* and *virG* from pTiBo542.

Both systems have been successfully used for cereal transformation (Aldemita and Hodges,1996; Dong *et al.,* 1996; Hiei *et al.,* 1994; Ishida *et al.,* 1996; Tingay *et al.,* 1997). The hypervirulent *Agrobacterium* strain AGL1 (Lazo *et al.,* 1991) carries the hypervirulent, tumor-inducing plasmid pTiBo542, allowing optimal DNA transformation of many dicotyledonous and monocotyledonous plants (Tingay *et al.,* 1997; Wang *et al.,* 1997, 1998, 2000; Patel *et al.,* 2000; Travella *el al.,* 2005)

1.2.2.2. *Vir* gene expression

Inducers and inhibitors of virulence genes play an important role in *Agrobacterium*-mediated transformation in cereals (Sahi *et al.,* 1990). High expression of *vir* genes is mainly achieved by adding *vir* inducers such as acetosyringone (AS) during inoculation

and co-cultivation, which was firstly reported by Stachel *et al.,* (1985) in tobacco. Acetosyringone is shown in many experiments to be essential for monocot transformation: rice (Hiei *et al.,* 1994), wheat (Guo *et al.,* 1998), maize (Ishida *et al.,* 1996) and barley (Guo *et al.,* 1998). Although there are reports on transformed callus in the absence of this compound (Raineri *et al.,* 1990; Tingay *et al.,* 1997), it seems that the addition of AS is very helpful for cereal transformation.

Genetic engineering of *Agrobacterium* in order to stimulate *vir* gene induction or to increase vir protein activity has been achieved by modifying *virA* and *virG* genes to finally increase plant transformation efficiency (Gelvin and Liu, 1994; Gubba *et al.,* 1995; Hansen *et al.,* 1994; Liu *et al.,* 1992). This method may overcome the need of phenolic inducers and the problem of potential inhibitors released by some plants and represents a hopeful way to transform recalcitrant species.

1.2.2.3. Plant cultivars and target cells

Plant cultivars and target cells are important factors, which determine the regeneration capacity and the efficiency of *Agrobacterium* transformation. For example in barley "Golden Promise", and wheat "Bob White". The use of actively proliferating embryogenic cells i.e. cells of immature embryos or embryogenic callus derived from immature or mature embryos are described as the ideal cells for monocot plant transformation using *Agrobacterium* (Hiei *el al.,* 1994,1997; Patel *et al.,* 2000; Tingay *el al.,* 1997; Travella *et al.,* 2005).

1.2.2.4. Other factors

Great advances have been made over the past decade to increase the number of plant species that can be transformed and regenerated using *Agrobacterium.* However many important species or inbred lines remain highly recalcitrant to the *Agrobacterium*-mediated transformation. Many factors revealed significant differences in T-DNA delivery and regeneration including embryo size, duration of pre-culture, inoculation and co-cultivation, and the presence of acetosyringone and Silwet-L77 as a surfactant in the media (Wu *et al*., 2003).

Alteration of tissue culture conditions by the use of antioxidants during the transformation of rice, maize, and wheat has increased the probability of stably transforming cell types that can be regenerated (Enriquez-Obergon *et al*., 1999; Frame *et al*., 2002; Olhoft *et al*.,

2001; Olhoft and Somers 2001; Perl *et al*., 1996; Wu *et al*., 2003). The addition of a surfactant to the inoculation medium was responsible for the efficient transformation of wheat embryos and callus (Cheng *et al*., 1997; Wu *et al.,* 2003).

Another point to be added is that *A. tumefaciens* usually infects wounded cells in its natural hosts, thereby being attracted to phenolic compounds such as acetosyringone that induce expression of *vir* genes. Infection and T-DNA transfer are therefore stimulated by wounding tissues (e.g., by crushing or cutting) or by treatment with acetosyringone. Several monocot species have been infected by pretreatment of the target cells with the exudates from wounded dicot plants, such as potato (Schafer *et al*.,1987).

Moreover, the transformation of recalcitrant species such as barley (Tingay *et al*., 1997) and sunflower (Grayburn and Vick 1995) has been achieved by prewounding embryogenic or meristematic tissue with metal particles or glass beads, while sonication has facilitated the uptake of *Agrobacterium* by soybean tissue (Trick, 1997; Santarém *et al*., 1998).

The use of selectable marker genes like herbicide *(bar*) or antibiotic resistance (*hpt*) genes is a crucial factor for the production of transgenic plants. For barley most experiments were carried out with the *bar* gene but also hygromycin selection including a modified hygromycin (*hpt*) gene can be use as reported by Wang *et al.,* 1997, 1998, 2000; and Murray *et al.,* 2001.

1.3. Improving the nutritional quality of barley grains

1.3.1. The nutritional value of barley grains

Cereal grains contribute to more than 50% of the total world food calories. In addition to carbohydrate, these grains supply a large proportion of the protein required by man and animals. However, the nutritional value of cereal grains is low, because of their limited amounts of certain essential amino acids. Barley grains are deficient in lysine and to a lesser extent in threonine as shown in Table 1 (Bright and Shewry, 1983).

Table 1: The essential Amino Acids Composition of Cereal Grains, Compared with WHO Recommendation Values

Amino acids	Wheat	Maize	Rice	Barley	WHO* Recommendation values g/100g protein	Difference %
Cysteine	2.62	3.13	2.2	2.86	3.5 a	- 18.28
Methionine	1.34	2.04	2.2	1.70	—	—
Lysine	1.98	3.52	4.0	3.08	5.5	- 44.00
Isoleucine	3.61	3.62	4.7	3.64	4.5	- 19.11
Leucine	6.74	11.64	8.5	7.16	7.0	+ 2.28
Phenylalanine	5.07	4.88	5.4	5.48	6.0	- 8.66
Tyrosine	2.60	2.34	4.9	2.72	—	—
Threonine	2.74	3.93	3.8	3.27	4.0	- 18.25
Tryptophan	1.12	0.94	1.2	1.99	1.0	+ 99.00
Valine	3.68	4.89	7.0	4.58	5.0	- 8.40
N content (%) of sample	2.39	1.34	1.46	1.49		

WHO = The World Health Organization, a = value of Cysteine and Methionine together.
Source : Bright and Shewry, 1983

Cereal-based diets for livestock are routinely supplemented with lysine and threonine; these supplements are highly beneficial for livestock growth (Cuaron *et al.,* 1984; Fuller *et al.,* 1979). Thus, there is a need to increase the nutritional quality of cereal seed proteins, either, through increasing the synthesis of these deficient amino acids to enhance their incorporation into protein or to overproduce some of the storage proteins rich in lysine and threonine.

The need to increase lysine and threonine production is forced by a new calculation of recommendable human consumption. The 1985 FAO-WHO recommendation has been questioned by recent studies which indicate a requirement of about 5 % of lysine in used

protein (about 0.75g/kg of body weight daily), which is nearly three times the FAO-WHO recommendation (Young *et al.,* 1998). This high value may become soon the accepted recommendation, which will subsequently increase the world protein needs (Galili and Larkins, 1999).

1.3.2. Strategies to improve the nutritional quality of barley grains

Several approaches have been adopted for increasing the content of lysine and threonine in plants reflecting developments of plant breeding, biochemistry of amino acid metabolism, and genetic engineering.

1.3.2.1. Using classical breeding methods

For many years plant breeders have attempted to select or produce mutants to improve the nutritional quality of cereal grains (Figure 2).

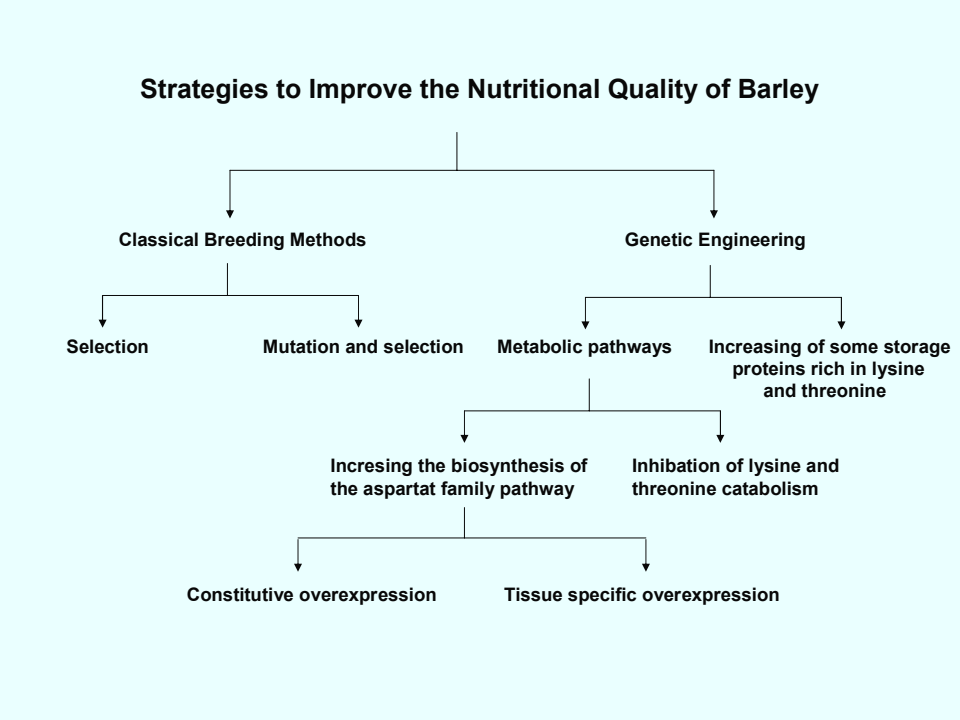

Figure 2: Different strategies to improve the nutritional quality of barley.

1.3.2.2. Using genetic engineering

There are two main approaches to achieve this aim as shown in Figure 2; firstly, by employing genes expressing high lysine proteins, and secondly, by employing genes to regulate the metabolism of lysine in plant cells.

1.3.2.2.1. Manipulation of seed storage protein genes

This approach takes advantage of natural, modified or synthetic genes expressing seed storage proteins rich in essential amino acids. Expression of seed storage protein genes has been reported in transgenic tobacco and petunia (Altenbach *et al.,* 1989; Beachy *et al.,* 1985; Ellis *et al.,* 1988; Hoffman *et al.,* 1988; Natio *et al.,* 1988; Sengupta-Gopalan *et al.,* 1985; Ueng *et al.,* 1988). Efforts have been undertaken to modify natural seed storage proteins by addition or replacement of amino acids to increase the lysine or methionine content of the protein (Krebbers *et al.,* 1993). Expression of these modified genes in seeds of tobacco, *Arabidopsis* or canola has been variable (De Clecq *et al.,* 1990). A number of sulfur-rich plant seed storage proteins have been identified and their corresponding genes isolated (Altenbach *et al.,* 1987; Higgins *et al.,* 1986; Kirihara *et al.,* 1988a, b; Pederson *et al.,* 1986). Expression of high methionine Brazil nut 2S albumin protein has been demonstrated in transgenic tobacco (Altenbach *et al.,* 1989), canola (Altenbach *et al.,* 1992), and soybean (Townsend and Thomas 1994) which significantly increased the total sulfur amino acid content of the seed. The major drawback in this strategy was in the immunogenic potential of foreign storage proteins. In case of the Brazil nut 2S albumin protein, the commercial development of soybean varieties containing this gene has been stopped due to allergy problems (Nordlee, *et al.,* 1996).

Although plant proteins enriched in lysine such as histone proteins (Liu *et al.,* 1993) and poplar bark storage protein have been isolated (Coleman *et al.,* 1992), no natural seed storage proteins enriched in lysine relative to the average lysine content of plant proteins have been identified. Instead the maize albumin elongation factor EF-1α contains 10% lysine which represents 90 % of the lysine found in endosperm proteins of two cultivars of maize (Habben *et al.,* 1995; Sun *et al*., 1997).

Torrent *et al.,* (1997) have constructed modified γ-zein genes by inserting synthetic oligonucleotides encoding Lys-rich sequences. Maize endosperms were transiently transformed by biolistic bombardment with Lys-rich γ-zein constructs under the control of both the 1.7 kb γ-zein (seed-specific) and the CaMV 35S promoters. The results of subcellular localization analyses and immunoelectron microscopy studies on isolated protein bodies demonstrated that modified γ-zeins accumulate within the endosperm and co-localize with endogenous α- and γ-zeins.

Yang *et al.,* (1989) developed a gene encoding polypeptides with elevated levels of lysine, methionine, tryptophan, threonine, and isoleucine in transgenic potato plants. Expression of *de novo* high-lysine/high methionine protein, containing 31% lysine and 20 % methionine (CP3-5) in mature seeds of transgenic tobacco plants, resulted in a significant increase in the total lysine content (Keeler *et al.,* 1997).

1.3.2.2.2. Engineering of the metabolic pathways of lysine and threonine

1.3.2.2.2.1. The influence of lysine catabolism

The lysine catabolism in plants (Figure 1) was initially studied in wheat, maize and barley in experiments using ^{14}C-lysine, with the radioactivity being incorporated into α-amino adipic acid and glutamate, indicating that this amino acid is oxidatively degraded to saccharopine (Brandt, 1975; Sodek and Wilson, 1970). Recent studies have indicated that lysine catabolism may play an important role for lysine accumulation in plants and for the control of the lysine content, particularly in seeds (Arruda *et al.,* 2000). The first enzyme in the lysine catabolic pathway is lysine 2-oxoglutarate reductase (LOR or LKR; lysine ketoglutarate reductase), which condenses lysine and 2-oxoglutarate into saccharopine. The second enzyme, saccharopine dehydrogenase (SDH), converts saccharopine into α-amino adipic acid semialdehyde and glutamate. Knockout of the bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase gene elevates lysine levels in *Arabidopsis* seeds (Zhu *et al.,* 2001).

In order to elucidate the relative significance of lysine synthesis and catabolism in determining the lysine level in plant seeds Zhu and Galili (2003) transformed wild type-*Arabidopsis* and an *Arabidopsis* knockout mutant for lysine catabolism with a bacterial feedback-insensitive dehydrodipicolinate synthase gene (DHDPS) under the control of a seed-specific promoter. Transgenic plants expressing the bacterial DHDPS, or the non transgenic knockout mutant, contain ~12-fold or ~5-fold higher levels, respectively, of seed free lysine than the wild-type control plants. However, the combination of these two traits caused a synergistic ~80-fold increase in seed free lysine level. Therefore, they concluded that these results imply that the expression of a feedback-insensitive DHPDS coupled with a reduction in lysine catabolism may be the preferred choice for increasing lysine accumulation in seeds of crop plants to remarkable high levels.

1.3.2.2.2.2. Increasing the biosynthesis of lysine and threonine

Constitutive overproduction of lysine and threonine in transgenic tobacco, *Arabidopsis*, maize and barley plants using the mutated *E. coli lysC* and *dapA* genes under the control of the cauliflower mosaic virus (CaMV) 35S promoter, resulted in increases of free lysine and threonine in the leaves of the transformed plants. Nevertheless, the accumulation of lysine and threonine in the seeds was limited (Ben-Tzvi *et al.,* 1996; Falco *et al.,* 1993; Glassman *et al.,* 1988, 1993; Kwon, *et al.,* 1995; Perl *et al.,*1992; Shaul and Galili 1992, 1993; Brinch-Pedersen *et al.,* 1996).

Therefore, in the subsequent studies seed specific expression of the bacterial AK and DHDPS was carried out using tissue specific promoters to overcome the associated limitations with constitutive expression of these genes. In this respect, a significant increase in free threonine and lysine in mature seeds of transgenic tobacco plants was obtained using a promoter derived from the gene encoding β–phaseolin (Karchi *et al.,* 1993, 1994). Similar promising results were obtained with transgenic canola and soybean plants which showed a higher than 50-fold increase in the seeds' free lysine, which increased the total seeds' lysine content (Falco *et al.,* 1995, 1997).

Recently, using a maize lysine-feedback-insensitive dihydrodipicolinate synthase gene under the control of the CaMV 35S and the rice glutelin (*GluB-1*) promoter for over-expression and seed-specific expression, respectively, Lee *et al.,* (2001) reported that the transgenic lines with the 35S promoter possess a higher content of free lysine in mature rice seeds than the lines transformed with the specific promoter (*GluB-1*). The level of free lysine in seeds of transgenic lines of the tissue-specific promoter was similar to that of the wild-type plants. From the comparison of DHPDS and lysine-ketoglutarate reductase (LKR) expression levels, they concluded that the presence of the foreign DHPDS gene driven by the *GluB-1* promoter leads to an increase of free lysine in the developing seeds which was accompanied by an increase of lysine ketoglutarate reductase (LKR) activity, resulting in enhanced lysine catabolism. However, over-expression of the mutant DHPDS gene in a constitutive manner overcame lysine catabolism and sustained a high level in mature rice seeds.

1.4. Work objectives

The present study has two aims:
Firstly, establishing an efficient system for *Agrobacterium tumefaciens*-mediated barley transformation. To develop this system, two *Agrobacterium* vectors are used, the binary vector pDM805 containing the *bar* gene for the bialaphos selection system and pWBVec10 containing the *hpt* gene for the hygromycin selection system.

Secondly, introducing the mutated *E. coli lysC* and *dapA* genes into the barley genome in order to promote synthesis and accumulation of lysine and threonine in the endosperm of barley grains. To achieve this aim, a set of novel transformation vectors are being constructed for expressing the target genes *lysC* and *dapA,* either under the control of the endosperm-specific promoter *D-hordein* of the barley *Hor3* gene or under the control of a constitutive promoter (*ubi1* or 35S). These vectors are used for producing primary transformants (T_0). Subsequently, molecular analysis is carried out to confirm the presence and integration of the transgenes in the barley genome. Biochemical analysis is performed to evaluate the expression of the integrated genes and their impact on the biosynthesis of lysine and threonine in T_0 transgenic plants.

2. MATERIALS AND METHODS

2.1. Chemicals, enzymes, kits and equipment

Chemicals

All chemicals used were with high purity (Analytical grade) and ordered from the following companies:

Sigma-Aldrich (Deisenhofen, Germany)

Merck (Darmstadt, Germany)

Roche (Switzerland)

Fluka (Switzerland)

Duchefa (Netherlands)

Roth (Karlsruhe, Germany)

Serva (Heidelberg, Germany)

Bio-Rad (USA)

Enzymes

Restriction Enzymes and DNA-modifying enzymes	(New England *BioLabs*)
RNase A	(Peqlab)
Ligase A	(New England *BioLabs*)
High fidelity *Taq* DNA polymerase	(Roche)
HotStar *Taq* DNA polymerase	(Qiagen)

Kits

DNA purification system	Wizard® *Plus* SV Minipres	(Promega)
Plasmid purification kits		(Qiagen)
QIA quick Gel Extraction Kit (50)		(Qiagen)
QIA quick PCR purification Kit (50)		(Qiagen)

Southern blot kits:	(New England *BioLabs*)
NEBlot® Phototope® Kit	
Phototop® –Star Detection Kit	

Equipment

Particle Bombardment (BioRad)

ChemiImager™ 5500 (Alpha Innotech Corporation)

Amino Acid Analyzer Biochrom 30 (ONKEN)

Photo Documentation system based on Olympus digital cameras and supported by Argus softwear (Version 3.3) (BioStep)

Vector NTI program (version 8)

Thermocycler (Gene Amp® PCR System 2700)

Electroporator (Easyject *prima* – EQUIBIO Limited)

Spectrophotometer (GeneQuant *pro* model 80-2109-98 Pharmacia Biotech)

Centrifuges: (Hettich -Model EBA12R) - (SIGMA - Model 3K15)

Freeze Dryer (Labconco)

Milli-Q *biocel* (MILLIPORE)

2.2. Bacterial strains and vectors

Escherichia coli

E. coli strain DH5α (Hanahan, 1983), was used for intermediate cloning steps with plasmid vectors pUC18, pUC19, pAK =pBluescript II SK+/35S-*lysC*, pDHDPS =pBluescript II SK+/ 35S-*dapA* (Brinch-Pedersen *et al.,* 1996), pUbi1/GUS and pD-Hor/GUS (Sørensen *et al.,* 1996).

Agrobacterium tumefaciens

Agrobacterium tumefaciens strain AGL1 (Lazo *et al*., 1991) was generally used in transformation experiments. *Agrobacterium* vector pDM805 (Figure 3) was used to optimize the transformation system using the reporter gene *uidA* and the selectable marker gene *bar* for bialaphos selection as described by Tingay *et al*., (1997). The transformation experiments based on the hygromycin selection system were carried out using pWBVec10 vector, (Wang *et al*., 1997, 1998) Figure 4.

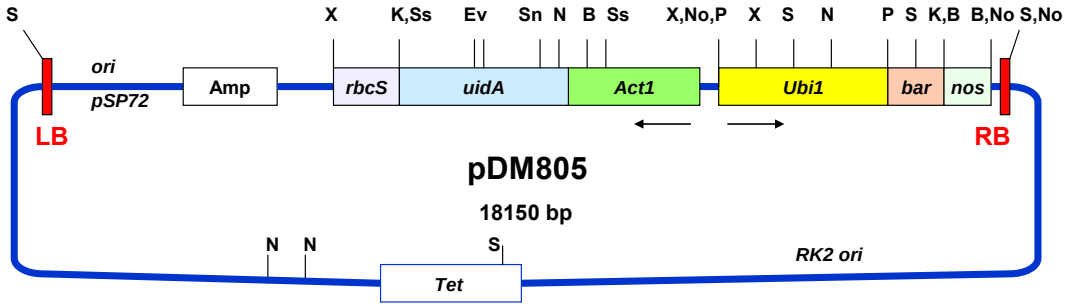

Figure 3: Structure and restriction map of the cereal transformation vector pDM805.

Abbreviations : *Act1*, promoter, first exon and intron of the rice actin 1 gene; *uidA*, coding region of the *E. coli β*-glucuronidase gene; *rbcS*, 3` transcript termination region of the rice rubisco gene; *ubi1*, promoter, first exon and first intron of the maize ubiquitin 1 gene; *bar*, coding region of *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene; *nos*, 3` transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene; *Amp*, ampicillin resistance gene of pSP72; LB, T-DNA left border sequence; RB, T-DNA right border sequence; *Tet*, tetracycline resistance gene .
Restriction sites are abbreviated as follows : B, BamHI; Ev, EcoRV, K, KpnI; N, NcoI; No, NotI; P, PstI; S,SalI; Sn,SnaBI Ss,SacI; X, XhoI

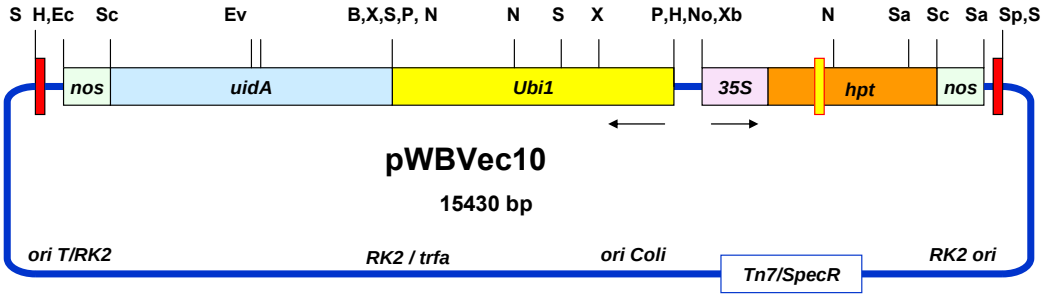

Figure 4: Structure and restriction map of the cereal transformation vector pWBVec10.

Abbreviations : *ubi1*, promoter, first exon and first intron of the maize ubiquitin 1 gene; *uidA*, coding region of the *E. coli β*-glucuronidase gene; *nos*, 3` transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene; 35S, CaMV 35S promoter; *hpt* coding region of *Streptomyces hygroscopicus* hygromycin B phosphotransferase gene; *CAT-1*, intron of castor bean catalase gene; LB, T-DNA left border sequence; RB, T-DNA right border sequence; *Tn7/SpecR*, spectinomycin resistance gene .
Restriction sites are abbreviated as follows : B, BamHI; Ec, EcoRI; Ev, EcoRV; H, HindIII, K, KpnI; N, NcoI; No, NotI; P, PstI; S,SalI; Sa, SacII, Sc, SacI, Sn,SnaBI; Ss,SacI; Sp, SphI, X, XhoI, Xb, XbaI

2.3. Molecular biology methods

2.3.1. Digestion, fragment separation, purification and ligation

Standard protocols were used as described in Sambrook and Russell (2001). Restriction enzymes from NEB were used in all digestion reactions. The restriction digested DNA was separated on 1.0 % agarose by electrophoresis at 80 volt and 50 mA for 1 hour. The DNA fragments were isolated by scalpel and purified using the gel extraction kit. Purified fragments were mixed with the opened vector in a reaction volume about 10 -20 µl containing 1x buffer of ligase A and 10 mM ATP, and incubated overnight at 16 °C.

2.3.2. Transformation of bacterial cells by electroporation

Preparation of competent cells of *E. coli* and *Agrobacterium tumefaciens*

The protocol for the preparation of *E. coli* DH5α electro-competent cells was used as described by Sambrook and Russell (2001). Preparation of the electroporation competent cells of *Agrobacterium tumefaciens* AGL1 started with the inoculation of 25ml MG/L medium (Garfinkel and Nester, 1980) Table 5, p. 24 containing the antibiotics rifampicin 20 mg/l and cabinicillin 100 mg/l with a single colony from fresh prepared colonies on selection medium. Subsequent steps to prepare the competent cells of *Agrobacterium* were performed using the of *E. coli* protocol.

Electroporation and selection of positive colonies

To transform the bacterial cells, 20-30 ng plasmid mixed with 40 µl of competent cells were transferred to an ice cold 2mm cuvette and electroporated at 2.5 kV and 400 Ω (Walkerpeach and Velten, 1994). After electroporation, 950 µl of SOC medium (Sambrook and Russell, 2001) was added and the tubes incubated at 37 °C for 1 hour in case of *E. coli* or at 28 °C for *Agrobacterium tumefaciens*. Petri dishes containing 25ml of selection medium were inoculated with 50-200 µl of the suspension cells and incubated overnight in case of *E. coli* or 2 days for *Agrobacterium* to select the positive colonies.

2.3.3. Preparation of DNA

Preparation of bacterial plasmid DNA

Plasmid DNA was isolated from *E. coli* using a promega kit. The *Agrobacterium* T-DNA plasmid was isolated using the Qiagen HiSpeed kit.

Isolation of plant genomic DNA

Genomic DNA was isolated from leaves of T_0 and T_1 transgenic plants using the Tris-CTAB method (Harwood *et al.,* 2000). Approximately 200 mg of leaf tissue was placed in a porcelain mortar and ground to powder under liquid nitrogen and then transferred to a 2.0 ml Eppendorf tube. 1 ml **(300 µl)*** of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2.0 % [w/v] hexadecetyltrimethylammonium bromide [CTAB]), was added and mixed thoroughly. The tubes were incubated at 65 °C for 60 min. After the sample cooled to room temperature, 800 µl **(225 µl)*** of chloroform/isoamyl (49:1) was added to each tube and samples were vortexed thoroughly. Tubes containing samples were centrifuged at 16,000 x g for 15 min. The aqueous phase of each sample was transferred to a fresh tube containing 750 µl **(200 µl)*** of chloroform/isoamyl (49:1), mixed and centrifuged again for 15 min. The aqueous phase was transferred to 1,5 ml tubes containing 400 µl **(180 µl)*** iso-propanol, mixed and centrifuged at 20,000 x g for 30 min. DNA pellets were washed with 500 µl **(100 µl)*** 70% ethanol and centrifuged again for 5 min, then ethanol was discarded and tubes dried for 10 min at room temperature. After the DNA was dried, 75 µl **(20 µl)*** of TE/RNase A solution (10 mM Tris-Cl, pH 8.0; 1mM EDTA, pH 8.0; 100 µg/ml RNase A) was added to each tube and incubated 30 min in a thermomixer at 50 °C/350 rpm to dissolve the DNA. The quality and concentration of the DNA was determined spectrophotometrically (A_{260}, A_{280}) and on a 1% agarose gel stained with ethidium bromide. DNA samples were stored at ─20 °C until use.

*** Amounts between brackets refer to the isolation of genomic DNA at micro scale for PCR screening**

2.3.4. PCR analysis

To investigate the presence of the transgene in the transgenic candidate plant, standard PCR reactions were performed using (100 ng) genomic DNA and specific primers in 50 µl reaction volume containing 1x PCR buffer (200 µM of each dNTP, 2.5 mM $MgCl_2$, 1 µM of each primer, and 2.5 units of Hotstar *Taq* DNA polymerase (Qiagen). The primer pairs and the size of the expected fragment are listed in Table 2. PCR products were analyzed by gel electrophoresis in 1 % agarose.

2.3.5. Southern blot analysis

Genomic DNA was isolated from leaf tissue of individual regenerated plants essentially as described in Harwood *et al*., 2000. 20-30 µg of genomic DNA was restricted with different restriction enzymes. The restricted DNA was size-fractionated by agarose gel electrophoresis and transferred to a positively charged membrane (Nytran® SuperCharge Schleicher&Schuell Bioscience) by using the TurboBloter (Rapid downward transfer system). Hybridization of membrane was performed according to the method outlined in the NEBlot® Phototope® Kit instruction manual with one modification: the probes were generated by PCR amplification using a specific primer set to amplify the biotinylated probe. The Phototop®–Star Detection Kit was used to detect the transgene sequences on hybridized filters. The emitted signals were detected by the ChemiImager™ 5500.

2.3.6. DNA sequencing

All sequences of either used genes or of constructed vector fragments have been verified by sequencing. Nucleotide sequencing was carried out by MWG-Biotech AG. These sequences were used in blast searches for similarities in the Gene Bank and in alignment analyses using the Vector NTI program.

Table 2: List of the primers used in PCR reactions

No.	Name	Sequence	Amplified fragment size (bp)
1	GUS Fwd	5'-CCT GTA GAA ACC CCA ACC CGT G-3'	1000
2	GUS Rev	5'-GAG CAT CTC TTC AGC GTA AGG G-3'	
3	HPT Fwd	5'-CTG AAC TCA CCG CGA CGT CTG-3'	1216
4	HPT Rev	5'-CTT TGC CCT CGG ACG AGT GC-3'	
5	Bar fwd Ting 97	5'-GGA TCT ACC ATG AGC CCA G-3'	357
6	Bar rev Ting 97	5'-TGC CTC CAG GGA CTT CAG-3'	
7	Gus fwd Ting 97	5'-TAG AAA CCC CAA CCC GTG AAA-3'	326
8	GUS rev Ting 97	5'-TGG CGT ATA GCC GCC CTG ATG-3'	
9	Ubi 1 Hind III fwd	5'-TCT CAA GCT TTG GGC TGC AGT GCA GCG TGA CCC GG-3'	2000
10	Ubi 1 BamHI rev	5'-CTC TGG ATC CTG CAG AAG TAA CAC CAA ACA ACA GGG-3'	
11	D-hor Hind III Fwd	5'-TCT CAA GCT TCT TCG AGT GCC CGC CGA TTT GC-3'	454
12	D-hor BamHI Rev	5'-CTC TGG ATC CCT CGG TGG ACT GTC AAT GAA TTG-3'	
13	Omega BamHI Fwd	5'-CTC TGG ATC CCA ACA ATT ACC AAC AAC AAA CAAC-3'	1540
14	AK XbaI Rev	5'-CAC ATC TAG ATA CTC AAA CAA ATT ACT ATGC-3'	
15	DHPS XbaI Rev	5'-CAC ATC TAG ACC CTA AAC TTT ACA GCA AAC CGG-3'	1840
16	OCS XbaI Fwd	5'-CAC ATC TAG ACT GCT TTA ATG AGA TAT GCG-3'	700
17	OCS SacI Rev	5'-CTC TGA GCT CCA TGT TGT CGC AAA ATT CGC-3'	
18	OCS rev190	5'-CAA TCA GTA AAT TGA ACG GAG AAT ATT-3'	508
19	Ak fwd997	5'-GGC ATA ATA TTT CGG TAG ACT TAA TCA-3'	
20	Ak rev284	5'-GTA ATG TTC TCC AGC AGA CGT TC-3'	487
21	TP rev210	5'-TAA TGT CAG TGT TGA CCT TCT TAA CTG-3'	202
22	DHPS fwd331	5'-AAG AAG GTT TGT ATC AGC ATT TCA AAG-3'	279
23	DHPS rev610	5'-CAC GCG TTA AGT TCC CTG TTG CC-3'	
24	HPT fwd168	5'-TTC TAC AAA GAT CGT TAT GTT TAT CGG-3'	271
25	HPT rev439	5'-CAT GTA GTG TAT TGA CCG ATT CCT T-3'	
26	HOR fwd17	5'-ATT TGC CAG CAA TGG CTA ACA GAC AC-3'	417
27	HOR rev434	5'-CTC GGT GGA CTG TCA ATG AAT TGA TC-3'	
28	virD2 fwd	5'-TCA AGT AAT CAT TCG CAT TGT GCC-3'	487
29	virD2 rev	5'-GCC GTG ACG AAG TGA AAT CTC-3'	
30	virG-1F	5'-GCC GAC AGC ACC CAG TTC AC-3'	380
31	virG-2R	5'-GCC GTA AGT TTC ACC TCA CC-3'	
32	virD-2C	5'-ATG CCC GAT CGA GCT CAA GT-3'	224
33	virD-2F	5'-TCG TCT GGC TGA CTT TCG TCA TAA-3'	

2.4. Barley transformation and regeneration

2.4.1. Growing conditions of donor plants

Plants of barley (*Hordeum vulgare* L.) spring cultivar Golden Promise were grown in growth chambers under a 16-h light/8-h dark period at 18 °C in light and 13 °C in dark. Light levels at head height were approximately 350-400 µE. All plants were cultivated in beat moss (2 liter pots) and fertilized with TriFerto fertilizer [NPK (12:12:17) +2 Mg] at the time of planting and then biweekly with 0.02 % Verdi (Peter's, 20-20-20) Wan and Lemaux (1994).

2.4.2. Barley immature embryo culture and regeneration

To test and establish the regeneration system, spikes were collected 15-21 days after anthesis and the developing barley seeds with immature embryos of about 1.5 to 2 mm in size were surface sterilized for 2 minutes in 70% ethanol, followed by 10 minutes in a solution of sodium hypochlorite containing 1% w/v chlorine, then rinsed one time in sterile distilled water (pH 3.0), and three times in sterile distilled water (pH 7.0). Immature embryos were dissected from young caryopses under a stereo binocular and the embryonic axis was removed with a sharp scalpel blade to obtain the intact scutellum tissue or bisected longitudinally to destroy the embryonic axis. After 2-3 days remaining embryonic tissue at the half scutellum tissue was removed. Embryogenic calli were derived from the scutellum tissue cultured on callus induction medium CIM (Table 3, p. 24) which was MS medium (Murashige and Skoog, 1962) supplemented with 30 g/l maltose, 1.0 mg/l thiamine-HCl, 0.25 g/l *myo*-inositol, 1.0g/l casein hydrolysate, 0.69 g/l proline, and 2.5 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) or 2.5 mg/l Dicamba (3,6-dichloro-O-anisic acid), pH 5.8 and solidified by 3.5 g/l Gelrite. Embryos were incubated during callus induction duration at 24 °C in the dark, and subcultured every two weeks.

Embryogenic calli were transferred to the regeneration medium (FHG medium) (Hunter, 1988) Table 4, supplemented with 1 mg/l 6-BAP (6-benzylaminopurine), pH 5.8 and solidified with 3.5 g/l Gelrite and incubated at 24 °C under fluorescent light (16 h/day). Regenerating plantlets were transferred to hormone-free CIM (half strength). After development of a root system, plantlets were transferred to soil and placed in growth cabinets set at 18°C, 16 h/day, 13 °C at night and 80 % humidity for 2 weeks, then 70 % until maturity.

Table 3: composition of Barley callus induction medium (CIM)

Substances	Amount mg/l or Concentration µM
MS salts	4302.09
Myo-inositol	250
Casein hydrolysate	1000
L-proline	690
Maltose	30,000
Thiamin-HCl	1
$CuSO4 .5H_2O$	5 µM
Dicamba	2.5

Table 4 : Composition of barley regeneration medium (FHG)

Substances	Amount mg/l or Concentration µM
MS salts without NH_4NO_3	2652
NH_4NO_3	165
Myo-inositol	100
Glutamine	730
Maltose	62,000
Thiamin-HCl	1
$CuSO_4 .5H_2O$	5 µM
6-BAP (6-benzylaminopurine)	1

Table 5 : Composition of *Agrobacterium tumefaciens* medium (MG/L)

Substances	Amount mg/l
Manitol	5000
L-glutamic acid	1000
KH_2PO_4	250
NaCl	100
$MgSO_4$	100
Biotin	0.001
Tryptone	5000
Yeast extract	2500

2.4.3. Transformation protocols :

Agrobacterium tumefaciens-mediated transformation

Transgenic plants of barley (*Hordeum vulgare* L. cv. Golden Promise) were produced by co-cultivation of immature zygotic embryos with *Agrobacterium tumefaciens* strain AGL1 harboring a binary vector, which consists of a disarmed Ti plasmid (the supervirulent pTiBo542) and a T-DNA plasmid pDM805 (Tingay *et al.*, 1997) or pWBVec10 (Wang *et al.,* 1997, 1998). *Agrobacterium tumefaciens* culture was grown for 40 h at 28 °C from a single colony in MG/L medium (Garfinkel and Nester, 1980) Table 5, supplemented with 20 mg/l rifampicin, 100 mg/l carbenicillin, and 5 mg/l tetracycline in case of pDM805 or 100 mg/l spectinomycin in case of pWBVec10. A standard inoculum was prepared by adding 200 µl of culture (OD_{600} = 1.0) to 200 µl of 30 % sterile aqueous glycerol in Eppendorf tube and kept at room temperature for 2 h before being transferred to -80 °C. A full strength inoculum with approximately (2.8×10^9) bacterial cells/ml was obtained by growing the standard inoculum in 10 ml of MG/L medium for 16 h at 28 °C.

Immature embryos after isolation were immersed in a full strength *Agrobacterium* suspension (OD_{600} = 1.0) for 1 h, then transferred to a sterile Petri dish to remove the excess bacterial solution, and then immediately transferred, without rinsing, with the scutellar surface placed in contact to the callus induction medium. Plates were incubated at 24 °C in darkness for 2 or 3 days. After co-cultivation, embryos were transferred directly to callus induction medium (CIM) supplemented with 150 mg/l Timentin™ (Duchefa) and the selection agent (3 mg/l bialaphos; phosphino-thricylalanylalanine sodium or 50 mg/l hygromycin B). The selection process, as described by Wan and Lemaux (1994), occurred for up to 8 weeks. Resistant embryogenic callus lines were transferred to FHG medium (Hunter, 1988) supplemented with 1 mg/l 6-BAP, 3 mg/l bialaphos or 25 mg/l hygromycin B, and 75 mg/l Timentin, solidified with 3.5 g/l Gelrite, and incubated at 24 °C under florescent light (16 h/day). Regenerating plantlets were transferred to hormone-free half strength CIM supplemented with 75 mg/l Timentin and 1 mg/l bialaphos or 25 mg/l hygromycin B. After development of a root system, plantlets were transferred to soil in small pots and placed in a growth cabinet set at 18 °C with high humidity 80 % for 2 weeks and then were transferred to big pots and placed in a growth cabinet with 70 % humidity until maturity.

Transformation protocol using Particle Bombardment

Transient GUS expression experiments, were performed to test the activity of the endosperm-specific *D-hordein* promoter, used plasmids are shown in Figure 5, p. 27. The protocol was used as described by Knudsen and Müller (1991):

Developing caryopses (15-21 days after anthesis) were harvested and sterilized in 70 % ethanol for 2 min. The caryopses were rinsed twice in sterile water, the upper third of the grain apex cut off and the endosperm squeezed out. Five to ten isolated endosperms were placed on filter paper (Whatman No. 4; 2.5 cm) in a Petri dish on solid MS medium without hormones, and subjected to bombardment.

Preparation of gold particles for DNA Delivery

a) 60 mg of gold particles (1.5 µm) were suspended in 1.0 ml of ethanol (100%) by vortexing for 3 min, then centrifuged at 10000 rpm for 1 min.
b) The supernatant was removed and 1.0 ml of distilled sterile water was added.
c) The particles were resuspended and centrifuged at 10000 rpm for 1 min and the washing process was repeated.
d) Finally, the gold particles were resuspended in 1.0 ml of distilled sterile water, divided into 50 µl / Eppendorf tube, and stored at -20 °C.

Precipitation of DNA

a) Mixture of DNA-coating solution was prepared (50 µl of 2.5M $CaCl_2.2H_2O$ + 20 µl of 0.1 M spermidine free base + 5-15 µg DNA at concentration 1 µg/µl), and added to 50 µl gold suspension; after 3 min of vortexing the mixture was centrifuged at 10000 rpm for 10 seconds.
b) The supernatant was removed and 250 µl of ethanol (100%) was added and resuspended by vortexing, then centrifuged for 10 seconds and the supernatant was removed.
c) The coated gold particles were resuspended in 56 µl of ethanol (100%) by pipetting up and down, and then transferred to the macro carriers in 7 µl; about 6 macro carriers were prepared and allowed to dry for approximately 1 min.

Preparation of particle gun and shooting

The particle bombardment was prepared as follows:

1. The distance between the rupture disk and macro carrier was 8-10 mm, distance between macro carrier and stopping screen was 10 mm and the distance between the stopping plate and target tissues was 50 mm.
2. The internal parts of the unit were sterilized by spraying with 95% ethanol. The rupture disk holder, macro carriers and stopping screen were sterilized by soaking in ethanol for 5-10 min. The macro carriers and rupture discs (1100 psi) were sterilized for 1 min in 100% ethanol
3. The target dish was placed into the unit and the chamber was evacuated to 27.5 of Hg. When the pressure of helium reached (1100 psi) the rupture disc was destroyed, thereby accelerating the coated particles, which penetrated the endosperm tissues. The bombarded tissues were cultured on hormone free MS medium at 24 °C for 48 h. The histochemical assay for GUS activity was performed by soaking the endosperms in a solution of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (1.0 mM) for 24 h at 37 °C and the number of blue dots representing transformed cells or cell clusters was scored.

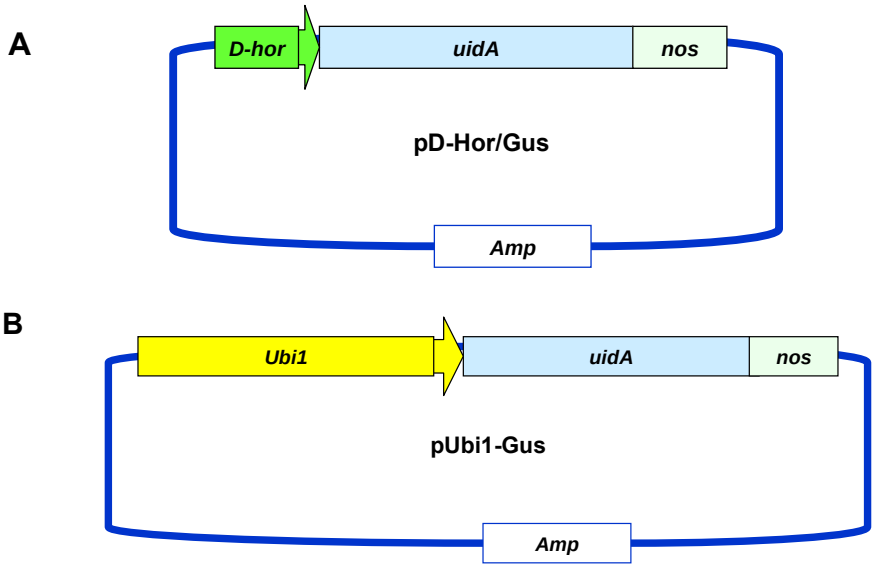

Figure 5: Plasmids for transient GUS expression; (A) pD-Hor/GUS for endosperm-specific expression, (B) pUbi1/GUS for constitutive expression.
Abbreviations: (*D-hor*), sequences of the promoter of the *D-Hor3* gene of barley; *uidA*, coding region of the *E. coli* β-glucuronidase gene; *nos*, 3` transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene; *ubi1*, promoter, first exon and first intron of the maize ubiquitin 1 gene; *Amp*, ampicillin resistance gene of pSP72.

Histochemical assay of GUS activity

Histochemical staining for GUS was performed using 5-bromo-4-chloro-3-indoxyl-*β*-D-glucuronic acid (X-gluc) (Jefferson *et al*., 1987). Samples were incubated overnight at 37 °C in GUS assay buffer. Best visualization of the developed blue color of GUS reaction in samples of leaf tissues was achieved by incubation overnight in 70% ethanol, to remove the chlorophyll.

Basta test

Transformed candidate plants were tested for their response to Basta (200g/l PPT; DL-Phosphinothricin, Hoechst AG, Frankfurt, Germany) by spraying with a 0.5 % (v/v) (1g/l PPT) phosphinothricin Basta solution plus 0.1 % Tween 20 (Cho *et al*., 1998). Plants were examined 7-14 days after herbicide application.

2.5. Construction of barley transformation vectors carrying the *dapA* and *lysC* genes

2.5.1. Vector design

For construction of the vectors for barley transformation, the starting plasmids pAK pDHDPS and pWBVec10 could not be used directly

1) because of the lack of suitable restriction sites.

2) and because of inconvenient orientation of the target and selection gene in the original starting plasmid pWBVec10.

To overcome these problems two new polylinkers were constructed: Poly19 and Poly8.

The polylinkers Poly19 and Poly8 were designed containing a set of 7 restriction sites (Figure 6&7).

The polylinker Poly19 60 bp (5'-CCA ACA AGC TTG CGG CCG CCC GCG GGA GCT CTC TAG AAC TAG TGA ATT CGG ATC CGC GCG-3') was inserted in pUC19 to produce pSMW5 (Figure 6, p. 30). This plasmid was used to change the restriction sites at the

end of the 35S/*hpt* gene (see vector pWBVec10, p. 18) from SacII—XbaI to be NotI—SpeI. It was now possible to insert the target gene close to the right border and the selection gene *hpt* to the left border of the T-DNA.

The 65 bp polylinker Poly8 (5'-CCA ACG GTA CCC CGC GGA AGC TTG GAT CCG AGC TCG CGG CCG CGG GGG ACT AGT GAA TTC GCG CG-3') was inserted in the T-DNA of pWBVec10 without *hpt* and *uidA* genes, to produce vector pSMW10 Figure 7, p. 31.

2.5.2. Fragments design

A set of primers was designed to insert new restriction sites in each fragment of the target genes using PCR. For gene construction three different fragments (F1 the promoter; F2 coding region of the gene; and F3 the terminator) were amplified; promoter, coding region of the gene, and the terminator as shown in (Figure 7).

The *D-hordein* promoter (*D-hor*) fragment (F1) was amplified using the forward primer D-hor HindIII Fwd and the reverse primer D-hor BamHI Rev (Table 2). PCR was performed with high fidelity *Taq* DNA polymerase (Roche) using plasmid pD-Hor/GUS as DNA template (Sørensen *et al*., 1996). To verify the new fragment, it was digested by HindIII and BamHI, and subcloned in a pUC19 vector. The nucleotide sequence was carried out by MWG-Biotech AG. The sequence was used for blast searches in the Gene Data Bank as well as alignment analysis was carried out with the Vector NTI program. The fragment was compared to the original sequence of the 434 bp *D-Hordein* promoter.

The dihydrodipicolinate synthase (DHDPS: *dapA*) coding sequence (fragment F2) including the (Ω) leader sequence from the tobacco mosaic virus mRNA and the transit peptide sequence (TP) coding for the chloroplast targeting sequence of the pea small subunit of ribulose 1,5-bisphosphate carboxylase (*rbcS*) were amplified by PCR as shown in Figure 6. By using Omega BamHI Fwd, DHPS XbaI Rev primers and the pDHDPS plasmid (Brinch-Pedersen *et al.,* 1996) as template, a 1140 bp fragment was defined. To obtain the intact DHDPS gene, the verified fragment of *dapA* was spliced to the *ocs* terminator and to the *D-hor* promoter fragment.

The *ocs* terminator fragment (F3) 700 bp was amplified using OCS XbaI Fwd and OCS SacI Rev primers.

The same procedure was performed with the aspartate kinase (AK: *lysC*) gene. Omega BamHI Fwd and AK XbaI primers were used to amplify a 1540 bp fragment. Alignment analyses were carried out to select the right fragments for constructing the new transformation vectors, which consist of the pSMW10 and the target genes either alone or in combination with the hygromycin B phosphotransferase gene (*hpt*).

The new transformation vectors (pSMW11—pSMW20 see results p. 71) were verified by restriction mapping and PCR analysis of the specific junctions using different specific primers.

Finally, the produced vectors were introduced into competent cells of the *Agrobacterium tumefaciens* AGL1 by electroporation. The isolated plasmid DNA from the *Agrobacterium* using Qiagen HiSpeed Kit was also verified by restriction mapping and PCR before preparing the standard inoculum with newly constructed vectors.

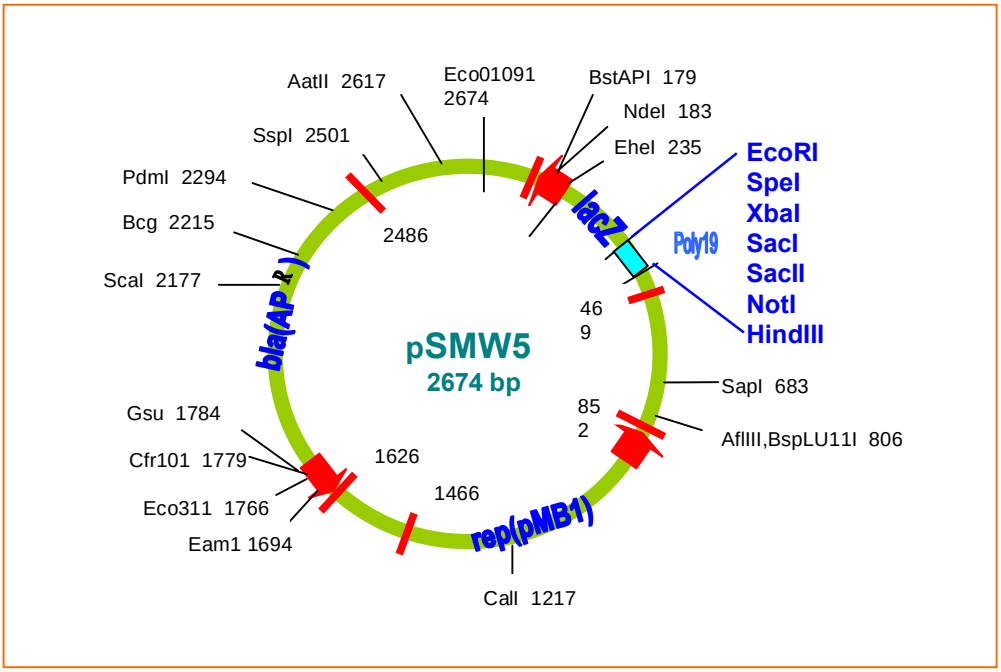

Figure 6: The pSMW5 vector = pUC19 + synthesized polylinker (Poly19).

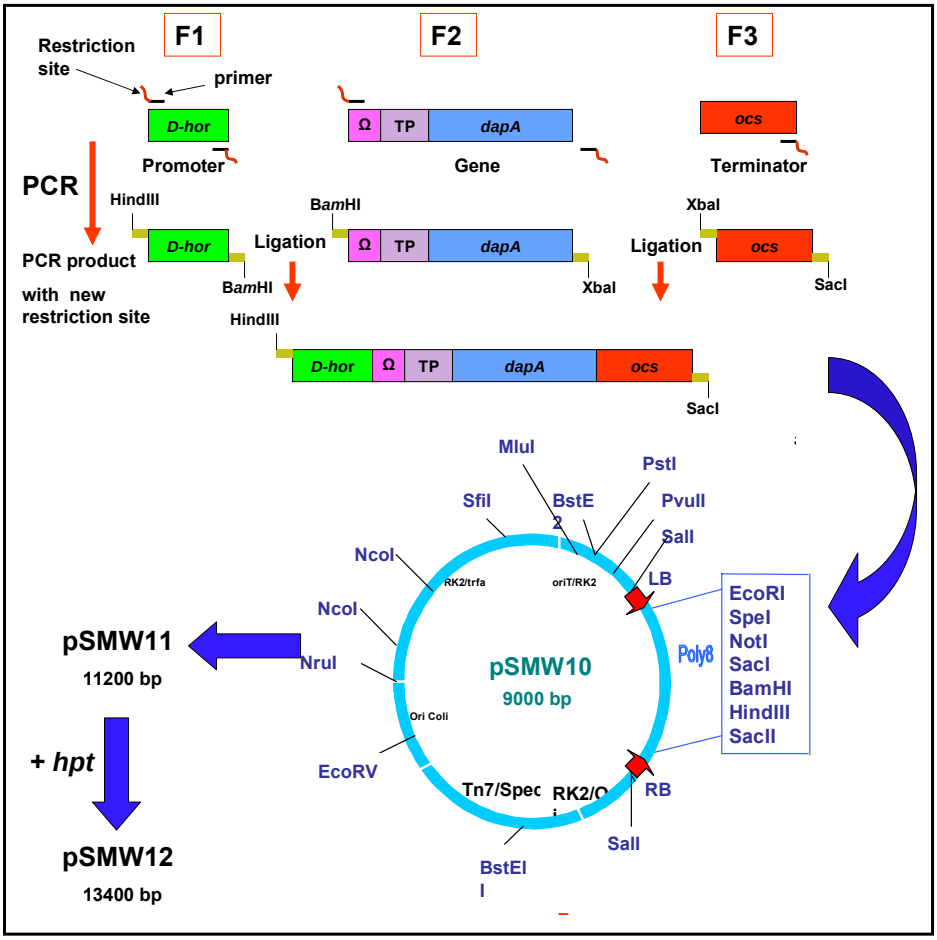

Figure 7: Cloning strategy of the *dapA* gene (DHDPS). Each fragment (F1, F2 and F3) was subcloned in plasmid pUC19. The intact gene was inserted into the *Agrobcterium* vector pSMW10 (pWBVec10 backbone + polylinker Poly8) to produce pSMW11 vector. The fragment *SpeI-NotI* of the selectable marker *hpt* gene was inserted into the pSMW11 to generate the vector pSMW12.

2.6. Amino Acids Analysis

The concentrations of free amino acids in leaves of the transgenic plants were determined to investigate the expression of the transgenes (*dapA* and *lysC*). Leaves were ground in liquid nitrogen and dried by the freeze dryer. Dried tissue (100 mg/ replicate) was used to extract the free amino acids using a diluted HCl (0.2 M) EG, 1998. Each sample extraction was performed twice. Free amino acids concentration was determined by the amino acid analyzer for the samples in comparison to the standard of authentic substances Figure 8.

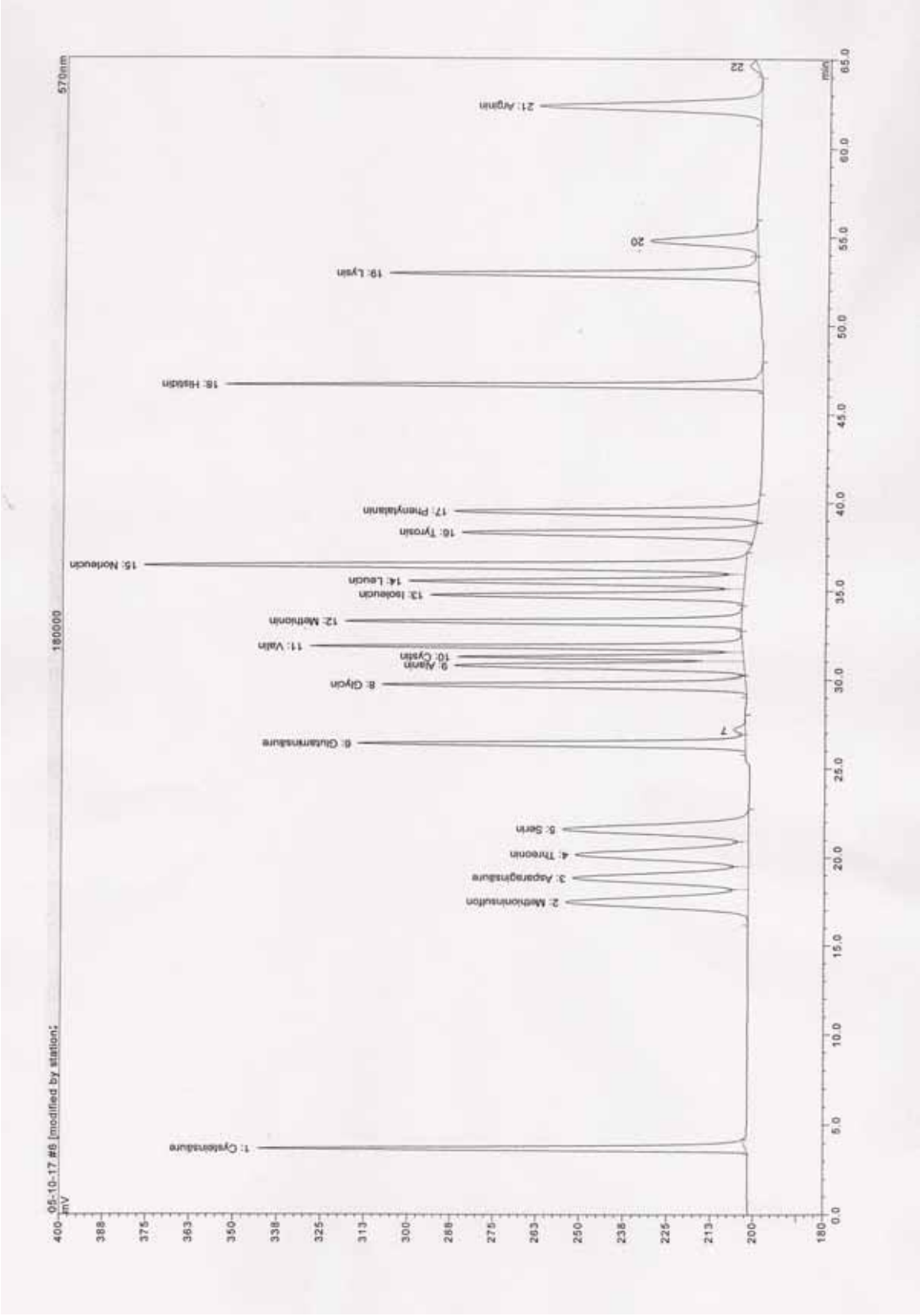

Figure 8: Chromatogram of standard free amino acids determined by the amino acid analyzer. Norleucine is used as internal standard.

3. RESULTS

3.1. Establishment of barley immature embryo culture and regeneration

A set of preliminary experiments was performed to determine the impact of different factors on the regeneration capacity.

Factors tested:

- type and concentration of auxin used in callus induction medium
- size of the immature embryo
- duration of the callus phase.

3.1.1. Impact of type and concentration of auxin on callus induction and regeneration capacity

To study the impact of the auxin type and the concentration on callus induction and regeneration efficiency, zygotic immature embryos (1.5-2 mm) were cut longitudinally and cultured on CIM supplemented with 2,4-D or Dicamba in five concentrations (0.0 , 1.5 , 2.5 , 3.5 and 4.5 mg/l) as shown in Table 6, p. 36.

The morphology of the calli derived from immature embryos cultured on CIM supplemented with Dicamba were compact and nodular. Many more somatic embryo like structures were observed and isolated (Figure 10 c,d &11, p. 34, 35) in comparison to 2,4-D, which led to the formation of a high proportion of soft and white calli. Only very few somatic embryos evolved (Figure 9, p. 34).
Whereas the optimum hormone concentration for callus growth was 2.5 mg/l for both hormones, Dicamba was superior in supporting the development of absolute numbers of regenerated plantlets (highest scored value: 4.76 plantlets/explant). 2,4-D was less than 50 % as efficient as Dicamba. This resulted in 95 % frequency of regeneration. With 2,4-D in comparison only 66 % embryogenic calli regenerated into proper plants.

On the basis of these results 2.5 mg/l Dicamba was used for subsequent experiments.

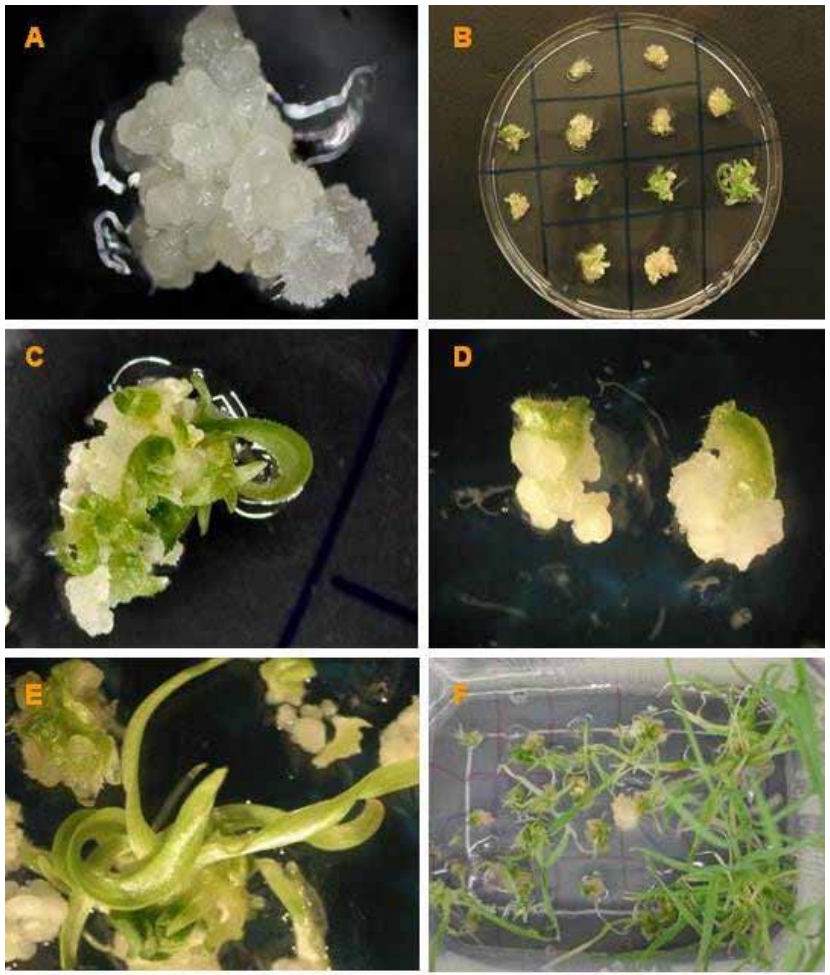

Figure 9: Callus induction and plant regeneration from barley immature embryos cultured on CIM supplemented with 2.5 mg/l 2,4-D. **(A)** Embryogenic callus after 21 days on CIM. **(B)** Shoot regeneration on FHG medium,14 days after subculture **(C)** Multiple shoots developing on FHG medium. **(D)** Shoots developed from callus tissues through shoot organogenesis **(E)** Shoots and plantlets after 4 weeks on FHG **(F)** Shoots and plantlets on root development medium in magenta box.

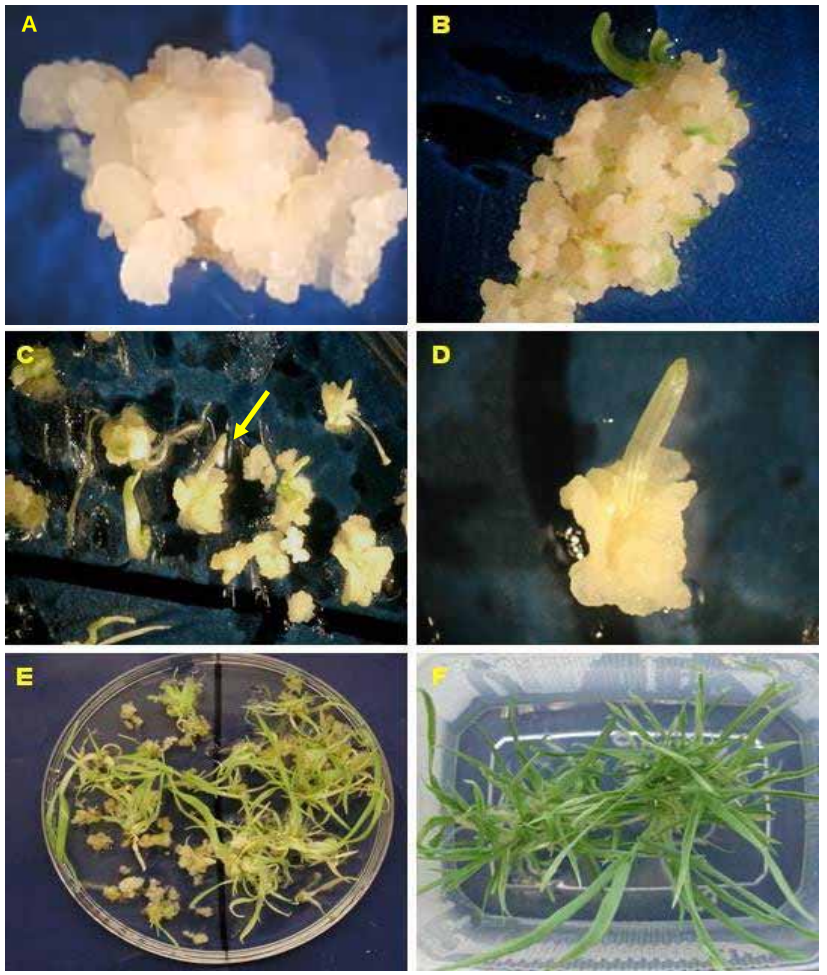

Figure 10: Callus induction and plant regeneration from barley immature embryos cultured on CIM supplemented with 2.5 mg/l Dicamba. **(A)** Embryogenic callus after 21 days on CIM. (B) Multiple shoots developing directly from embryogenic callus after transfer to regeneration medium FHG. **(C)** Germination and growth of somatic embryos on FHG, *arrow* points to a somatic embryo. **(D)** In focus the germinated somatic embryo from picuture C . **(E)** Regenerated green plantlets after 28 on FHG. **(F)** Regenerated plantlets after 21 days on rooting medium.

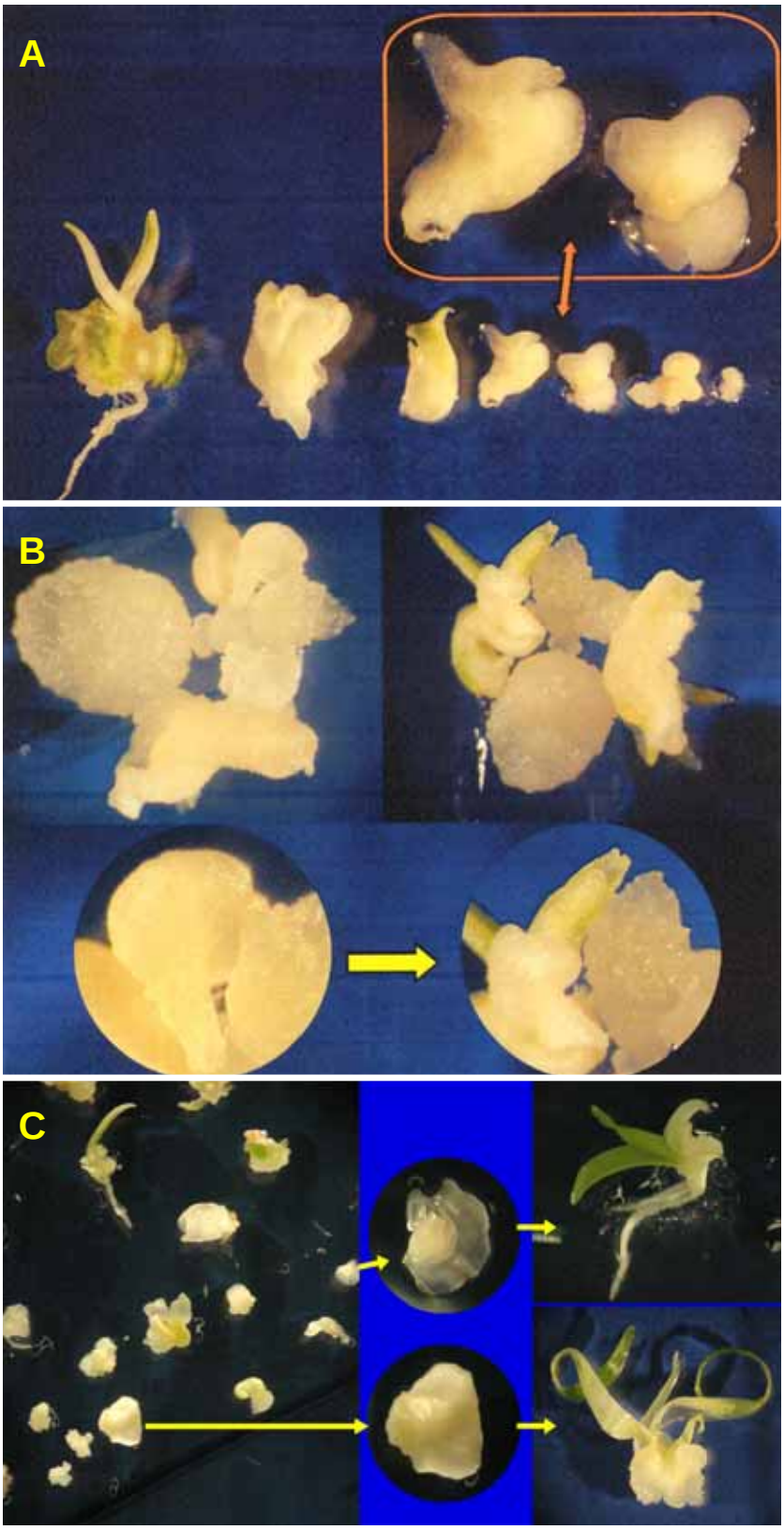

Figure 11: Somatic embryogenesis of barley (cv. Golden Promise). **(A)** Developmental stages of barley somatic embryos, from right to left, globular, heart, torpedo stage and germinated somatic embryo; in focus heart stage. **(B)** *(Left side)* some somatic embryos on callus induction medium supplemented with 2.5 mg/l Dicamba, *(right side: the germination)* 8 days after transfer of the somatic embryos to FHG medium; in focus, somatic embryo in heart shape and (*arrow points to the shape after germination*). **(C)** In focus, somatic embryos on FHG medium (*arrows point to the developed plantlets after germination*).

Table 6: Impact of type and concentration of auxin on callus induction and regeneration efficiency

Concentration mg/l	No. of explants (half embryos)	Callus quality % *	Average No. of regenerated plantlets/ explant	Frequency of regeneration (%)	Regeneration efficiency
2,4-D					
0.0	107	-	-	-	-
1.5	195	45.12	1.88	41.53	+
2.5	**188**	**74.46**	**1.96**	**65.79**	**++**
3.5	168	67.85	1.92	53.57	+
4.5	193	53.88	1.37	47.66	+
Dicamba					
0.0	123	-	-	-	-
1.5	214	61.68	2.29	76.16	++
2.5	**219**	**82.19**	**4.76**	**94.52**	**++++**
3.5	195	80.51	3.14	89.74	+++
4.5	178	56.17	1.97	61.24	+

Regeneration Efficiency with four levels (+ , ++, +++, ++++) the highest level ++++ = Highest regeneration % and High No. of regenerated plantlets /explant. Callus quality % = A score of 100 % the highest quality is given to shiny, compact, nodular slightly brown-colored callus, a score of about 25 % (the lowest quality) given to soft, friable, white callus, (Roussy *et al*., 2001).

3.1.2. Impact of immature embryo size on regeneration capacity

Three different sizes of immature embryos were isolated (12- 18 days post anthesis) and classified in three categories; small (1 mm) in length, medium (1.5 – 2 mm), and large (2.5 – 3 mm) (see Figure 12, p. 37). All explants were cultured on CIM supplemented with Dicamba (2.5 mg/l). Three independent experiments were carried out for each size. In total 529 half embryos small size, 509 half embryos medium size, and 701 half embryos large size were tested as shown in Table 7, p. 37. Embryogenic callus was observed more frequently in the calli derived from medium sized immature scutella than in the calli of small and large size. Explants of the large size produced a high ratio of non-embryogenic calli.

The highest regeneration efficiency 92.24 % in average, and highest number (4.10) of regenerated plantlets per explant was scored using the medium sized embryo. On the other hand, the lowest regeneration efficiency was recorded with large sized embryo, which

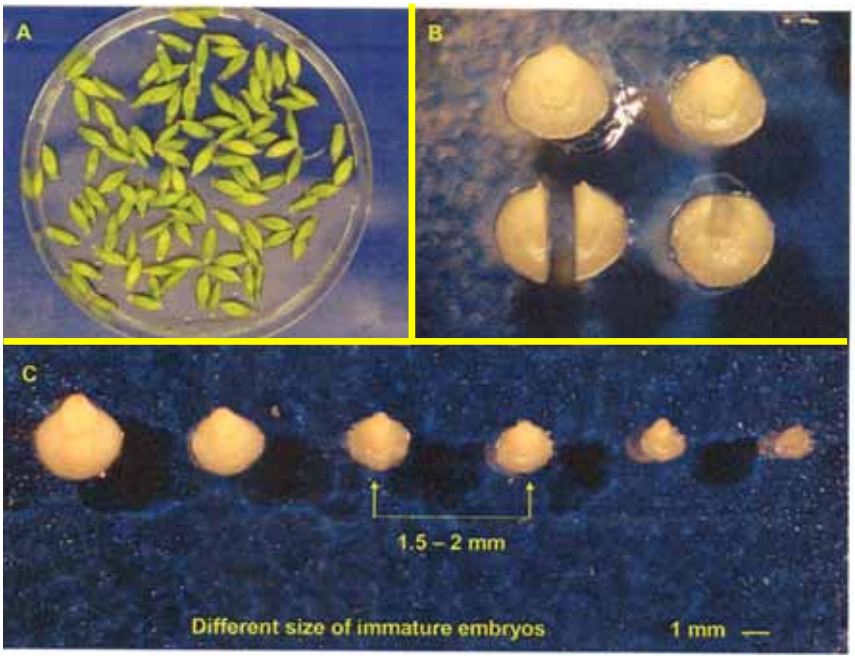

Figure 12: (A) Sterile developing barley grains, 21 days after anthesis. **(B)** Longitudinal cutting or removing the embryo axis. **(C)** Different size of barley immature embryos (arrows point to the medium size), (*left*) the large size and (*right*) the small size). *Bar* = 1mm

Table 7: Impact of immature embryo size on regeneration efficiency

Experiment No.	No. of explants (half embryos)	Callus quality %	Average No. of regenerated plantlets/explant	Frequency of regeneration (%)	Regeneration efficiency
Small size = 1 mm					
1	221	51.58	1.94	71.95	++
2	167	52.69	1.86	67.66	++
3	141	49.64	2.12	75.88	+
Medium size = 1.5 - 2 mm					
1	158	74.05	3.10	95.57	++++
2	133	76.69	4.63	91.73	++++
3	218	73.39	4.57	89.44	++++
Large size = 2.5 - 3 mm					
1	289	30.10	1.46	60.55	++
2	241	32.78	1.55	68.05	++
3	171	35.08	1.71	67.83	++

Regeneration Efficiency with four levels (+ , ++, +++, ++++) the highest level ++++ = Highest regeneration % and High No. of regenerated plantlets /explant. Callus quality % = A score of 100 % the highest quality is given to shiny, compact, nodular slightly brown-colored callus, a score of about 25 % (the lowest quality) given to soft, friable, white callus, (Roussy *et al*., 2001).

resulted in a relatively low regeneration percentage of 65.47 % on average, and a low number (1.57) of regenerated plantlets per explant. These results indicate that the size of the immature embryo is a critical factor for the regeneration of barley, and the size (1.5–2 mm) is a suitable parameter for immature embryo culture to achieve a high regeneration capacity for the barley plant.

3.1.3. Impact of callus induction period on regeneration capacity

Callus induction was studied throughout four different periods (2, 4, 6, and 8 weeks) Table 8, p. 39. Somatic embryos derived from embryogenic callus of scutellum tissue were observed after 18- 28 days from begin of cultivation. These somatic embryos germinated after 4 to 8 days when transferred to regeneration medium (Figure 11b,c p. 35). Therefore, the period from 18 to 28 days after culture initiation has been identified as a critical period for somatic embryogenesis in barely cv. Golden Promise.

The percentage of regeneration and the average number of plantlets/explant were affected by different callus induction durations. The highest frequency of regeneration (on average = 93.33%) was recorded with a 4-week treatment, although the highest number of plantlets/explant was recorded in a 6-week treatment. In addition, the highest regeneration efficiency was recorded when a 4-week callus induction duration was used, which produced the highest regeneration frequency with a satisfactory number of regenerated plantlets/explant.

3.1.4. Results of the barley regeneration experiments:

It can be summarized that a reproducible system was established for plant regeneration via somatic embryogenesis from immature scutella of barley (cv. Golden Promise) isolated from immature embryos (size 1.5 – 2 mm), cultured on callus induction medium (CIM) supplemented with 2.5 mg/l Dicamba for 4 weeks before transfer of the embryogenic calli to regeneration medium (FHG).

Table 8: Impact of callus induction period on regeneration efficiency

Experi-ment No.	No. of explants (half embryos)	Callus quality %	Average No. of regenerated plantlets/explant	Frequency of regeneration (%)	Regeneration efficiency
			2 weeks		
1	100	49.00	1.06	60.00	+
2	120	55.00	1.83	66.66	+
3	135	57.77	1.62	73.33	++
			4 weeks		
1	147	78.23	3.90	**93.20**	++++
2	126	78.57	4.06	**91.27**	++++
3	112	73.80	3.79	**95.54**	++++
			6 weeks		
1	142	80.98	**7.88**	82.39	+++
2	154	82.46	**6.46**	81.81	+++
3	106	80.18	**6.31**	86.79	+++
			8 weeks		
1	118	91.52	5.96	76.27	++
2	127	83.46	6.62	73.22	++
3	148	87.16	4.16	71.62	++

Regeneration Efficiency with four levels (+ , ++, +++, ++++) the highest level ++++ = Highest regeneration % and High No. of regenerated plantlets /explant. Callus quality % = A score of 100 % the highest quality is given to shiny, compact, nodular slightly brown-colored callus, a score of about 25 % (the lowest quality) given to soft, friable, white callus, (Roussy *et al*., 2001).

3.2. Establishment of *Agrobacterium tumefaciens*-mediated barley transformation using reporter genes and different selection regimes

To establish a reproducible and efficient protocol for *Agrobacterium tumefaciens*-mediated barley transformation, two different binary vectors in the AGL1 strain (Lazo *et al.,* 1991) harboring the supervirulent plasmid pTiBo542 were used.

Initially the binary vector pDM805 (Tingay *et al*., 1997) for the bialaphos selection system was employed. Results of transformation experiments using pDM805 indicated that under bialaphos selection it is possible to obtain transgenic calli with reasonable frequencies but only with very few calli producing plantlets. Consequently, the transformation rate was at low level. Therefore, aiming to achieve a better transformation rate, another selection system was chosen. The binary vector pWBVec10 (Wang *et al*., 1997,1998), containing the *uidA* and the *hpt* gene as selectable marker, which confers resistance for hygromycin B, was used in comparison to pDM805 to investigate the effect of the selection system on the transformation efficiency.

3.2.1. Transformation of barley immature embryos using the pDM805 vector and Bialaphos selection

At the outset of this study a set of a preliminary experiments based on transient expression of the *uidA* gene were conducted to investigate the influence of different factors on the efficiency of T-DNA transfer from *Agrobacterium* to barley cells. In these experiments transient GUS assay was performed after 7 days of inoculation. Transformation experiments followed to produce stable transgenic barley plants expressing *uidA* and *bar* genes, for evaluation of transformation efficiency.

3.2.1.1. Effect of different conditions on transient expression of the *uidA* gene

Factors which may affect the T-DNA delivery system were tested :

- inoculation and co-cultivation time
- presence of acetosyringone
- embryo size
- duration of preculture.

The effect of *Agrobacterium* inoculum density and inoculation time on transient GUS expression was studied (Table 9, p. 41). An inoculation time for 1 hour using a culture with the optical density of one (OD_{600} = 1) turned out to be the best inoculation condition. This

resulted in a high percentage of explants with GUS foci (64.15 %) and a high average number of GUS foci per explant (6.04) together with a good survival rate. Based on these results barley immature embryos were inoculated for one hour with *Agrobacterium* culture (OD_{600} = 1.0) for subsequent experiments.

Table 9: Effect of inoculum density and inoculation time on transient GUS expression

Inoculum density (OD_{600})	Time (hour)	No. of explants (half embryos) tested	Percentage of explants with GUS foci	Mean of GUS foci/ explant	Survival rate (%)
0.5	0.05	45	13.33	4.22	97.78
	0.5	62	22.58	5.09	95.16
	1.0	56	25.00	5.80	89.28
	2.0	57	35.09	6.14	96.49
	4.0	50	38.00	6.88	76.00
1.0	0.05	42	55.56	6.19	95.23
	0.5	57	66.07	5.47	94.73
	1.0	**49**	**64.15**	**6.04**	**87.76**
	2.0	55	58.82	7.23	78.18
	4.0	53	51.85	8.09	73.58
1.5	0.05	45	37.78	6.06	86.66
	0.5	47	42.55	11.34	85.10
	1.0	42	50.00	14.23	90.47
	2.0	51	52.94	9.12	78.43
	4.0	56	62.50	5.00	69.64
2.0	0.05	54	40.74	1.16	74.07
	0.5	56	37.05	2.36	67.85
	1.0	53	54.71	2.91	66.03
	2.0	51	41.17	3.17	68.63
	4.0	54	35.18	4.13	51.85
2.5	0.05	50	42.00	1.58	58.00
	0.5	46	58.69	2.87	63.04
	1.0	58	50.00	4.14	56.89
	2.0	55	36.36	2.01	43.63
	4.0	57	31.57	2.98	33.33

The effect of the *Agrobacterium*-barley co-cultivation period on transient GUS expression was determined (Table 10). Highest percentage of GUS expression (71.42 %) and highest number of GUS foci/explant (7.14) were recorded with 3 days of co-cultivation, while lowest GUS expression was scored with 0 or 5 days of co-cultivation period. Best survival rate, together with high numbers of GUS foci were observed with a 2 or 3 days co-cultivation period.

Table 10: Effect of co-cultivation period on transient GUS expression

Co-cultivation period (days)	No. of explants (half embryos) tested	Percentage of explants with GUS foci	Mean of GUS foci/ explant	Survival rate (%)
0	145	11.03	1.81	96.55
1	277	42.96	4.10	94.22
2	312	65.71	6.28	92.30
3	**259**	**71.42**	**7.14**	**85.32**
4	255	58.82	3.80	65.88
5	286	26.92	3.29	47.55

To investigate the effect of embryo size on transient GUS expression, barley immature embryos (12- 18 days post anthesis) were isolated and classified in four categories (Table 11). Highest percentage of GUS expression was observed using the size category 1.5-2 mm, while lowest percentage of GUS expression was observed using the size category 0.5 mm, which accompanied the lowest survival rate (26.39 %).

Table 11: Effect of embryo size on transient GUS expression

Embryo size (mm)	No. of explants (half embryos) tested	Percentage of explants with GUS foci	Mean of GUS foci/ explant	Survival rate (%)
0.5	197	22.33	1.17	26.39
1.0	218	48.62	5.51	74.31
1.5 – 2.0	**222**	**67.12**	**8.07**	**91.44**
2.5 – 3.0	230	37.39	1.93	83.04

Five different concentrations of acetosyringone (AS) were used to evaluate its impact on transient expression of the *uidA* gene. Barley immature embryos size (1.5-2mm) were inoculated for 1 hour in *Agrobacterium* cultures (OD = 1.0) containing different concentrations of acetosyringone. Explants (half embryos) of each treatment were co-cultivated for 3 days on CIM containing the same concentration of acetosyringone.
Results of transient GUS expression of different treatments in comparison to the control (without AS) showed that no positive effect was observed in the presence of acetosyringone (Table 12).

Table 12: Effect of the concentration of acetosyringone on transient GUS expression

AS concentrations µM	No. of explants (half embryos) tested	Percentage of explants with GUS foci	Mean of GUS foci/ explant	Survival rate (%)
0	271	61.25	6.87	92.98
50	265	62.26	6.42	94.34
100	283	61.84	5.31	92.22
200	266	58.27	5.80	90.98
400	289	56.40	3.68	85.81

Seven preculture periods were tested to determine the effect on transient GUS expression (Table 13, p. 44). Preculture of the explants for 3 days before inoculation with *Agrobacterium* resulted in the highest percentage of explants with GUS foci (83.93 %) and the highest average number of GUS foci per explant (11.73). The percentage of explants with GUS foci was increased from 61.53 to 83.94 %, and the average number of GUS foci/explant was about two folds of the control.

As a final result of the previous experiments it was concluded that the following conditions are necessary to achieve a high potential for T-DNA transfer from *Agrobacterium* to barley cells:

1- Preculture of barley immature embryos size (1.5-2 mm) for 3 days on CIM.
2- Inoculation for 1 hour in *Agrobacterium* culture (OD = 1.0)
3- Co-cultivation of explants with *Agrobacterium* for 3 days at 24 °C in the dark.

Table 13: Effect of the preculture period on transient GUS expression

3.2.1.2. Production of stable transgenic barley plants using pDM805

Transformation of barley immature embryos using *Agrobacterium* was illustrated (Figure 13, p. 45). Thirty-seven experiments were carried out using the pDM805 vector to produce fertile transgenic barley plants expressing *uidA* and *bar* genes (Table 14, p. 46 and 47). Data in Table 14 revealed that a high percentage of transgenic calli (tested by PCR) failed to regenerate (Figure 13 g, p. 45).

Nineteen successful experiments out of a total of 37 individual transformation experiments resulted in 80 independent lines (each line derived from one explant = half embryo). Forty positive lines for *uidA* and *bar* genes were selected from these 80 lines using GUS assay and Basta spray test, as well as the presence of *uidA* and *bar* genes in all produced lines were confirmed by PCR.

Analysis of individual T_0 positive plants showed that the presence of the *bar* gene was higher than that of the *gus* gene. This result suggests that this may be due to the presence of *bar* gene near to the right border of the vector pDM805. The gene located near to the right border has a higher potential to transfer and integrate into the genome of the target cell than the gene located near to the left border.

The transformation frequency ranged between 0.69 % and 3.13 %, with an average value of about 1.51 %. The transgenic plants derived from each line were very few and ranged between 1 and 3 plants/line, about 1.53 plants/line on average was calculated for the 19 positive experiments.

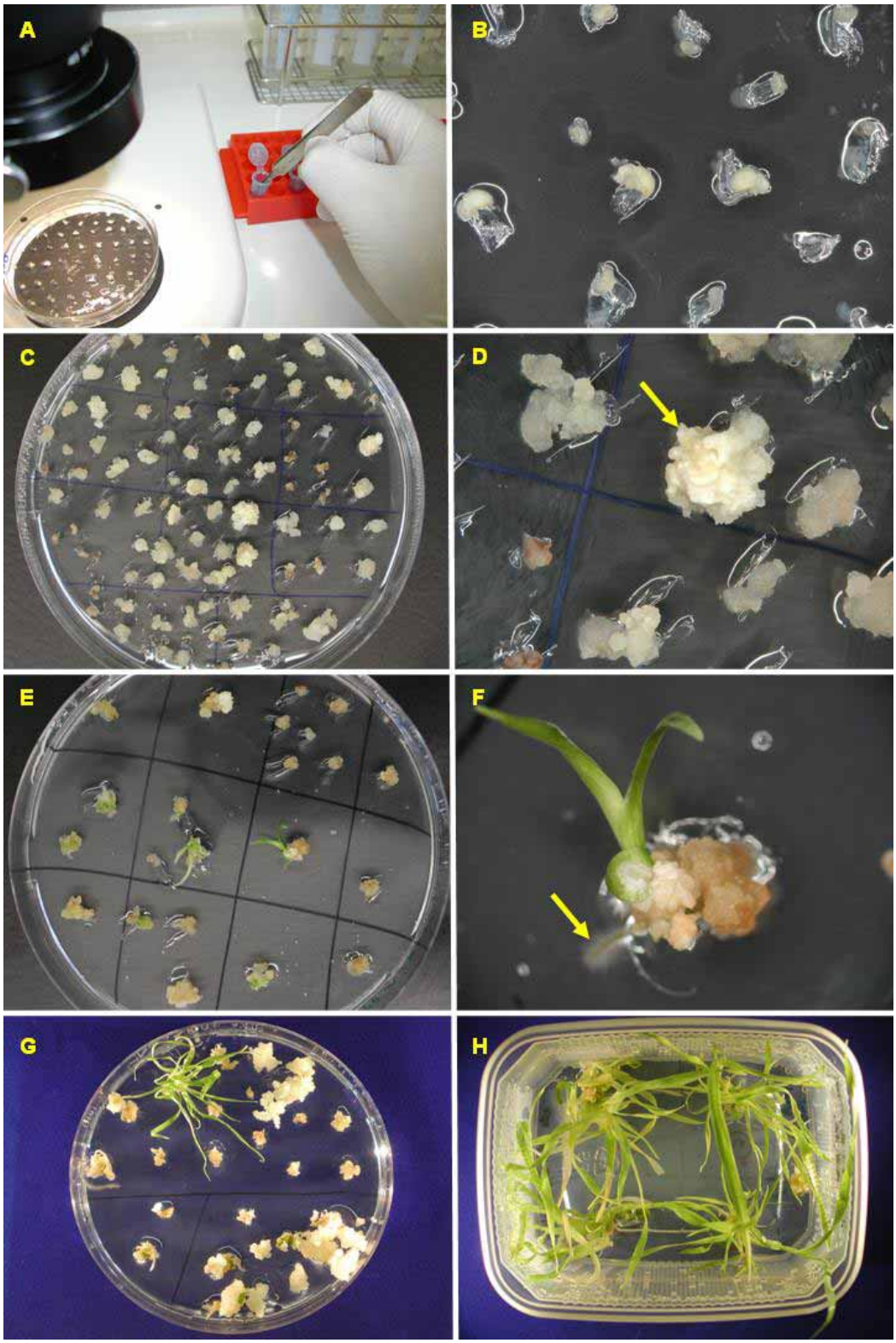

Figure 13: Production of transgenic barley plants using *Agrobacterium tumefaciens* (pDM805).
(A) Inoculation of barley half embryos with *Agrobacterium* in eppendorf tubes. **(B)** The explants after 3 days of co-cultivation. **(C)** Callus derived from the scutellum tissues after 4 weeks on CIM+ Bialaphos 3 mg/l + Timentin 150 mg/l. **(D)** Different types of callus on selection medium, *arrow* points to compact transgenic callus. **(E)** Regeneration of putative transformed plants after 2 weeks on FHG + Bialaphos 1 mg/l + Timentin 150 mg/l. **(F)** Regenerated plant, *arrow* points to the root of the developed plant from a somatic embryo. **(G)** A representative image for impact of Bialaphos on regeneration of transgenic calli of 3 independent lines after 4 weeks on FHG (only one line has green shoots). **(H)** Root developing of regenerated shoots, after 3 weeks on rooting medium.

Table 14: Transformation of barley immature embryos using AGL1 pDM805 (Bialaphos selection)

Experiment No.	No. of explants (half embryos)	No. of callus lines	No. of lines with green plantlets	No. of plantlets / line	Name of plantlets	Expression of marker genes		PCR Test *uidA* +	PCR Test *bar* +	Transformation frequencies %
						GUS +	Basta +			
P1	128	2	1	2	P1-L1 (1) P1-L1 (2)	-	L1 (1) L1 (2)	-	L1 (1) L1 (2)	0.78
P2	217	6	0	0	-	-	-	-	-	0
P3	102	8	0	0	-	-	-	-	-	0
P4	134	12	2	1 1	P4-L1 (1) P4-L2 (1)	L2 (1)	L1 (1) L2 (1)	L2 (1)	L1 (1) L2 (1)	1.49
P5	184	17	2	4 3	P5-L1 (1), (2), (3), (4) P5-L2 (1), (2), (3)	L1 (1), (2), (3), (4) L2 (1), (3)	L1 (1), (2), (3), (4) L2 (1), (2), (3)	L1 (1), (2), (3), (4) L2 (1), (2), (3)	L1 (1), (2), (3), (4) L2 (1), (2), (3)	1.08
P6	160	13	5	2 2 2 1 1	P6-L1 (1), (2) P6-L2 (1), (2) P6-L3 (1), (2) P6-L4 (1) P6-L5 (1)	L2 (2) L3 (1) L5 (1)	L1 (1), (2) L2 (1), (2) L3 (1), (2) L4 (1) L5 (1)	L2 (2) L3 (1), (2) L5 (1)	L1 (1), (2) L2 (1), (2) L3 (1), (2) L4 (1) L5 (1)	3.13
P7	170	9	3	1 1 1	P7-L1 (1) P7-L2 (1) P7-L3 (1)	L1 (1) L2 (1)	L1 (1) L2 (1) L3 (1)	L1 (1) L2 (1)	L1 (1) L2 (1) L3 (1)	1.76
P8	156	11	1	1	P8-L1 (1)	-	-	-	-	0
P9	126	6	1	1	P9-L1 (1)	-	-	-	-	0
P10	217	16	0	0	-	-	-	-	-	0
P11	132	12	2	1 1	P11-L1 (1) P11-L2 (1)	L1 (1) L2 (1)	L1 (1) L2 (1)	L1 (1) L2 (1)	L1 (1) L2 (1)	1.52
P12	154	3	1	1	P12-L1 (1)	-	-	-	-	0
P13	149	6	0	0	-	-	-	-	-	0
P14	179	18	2	1 1	P14-L1 (1) P14-L2 (1)	L1 (1)	L1 (1) L2 (1)	L1 (1)	L1 (1) L2 (1)	1.12
P15	200	26	0	0	-	-	-	-	-	0
P16	116	11	1	2	P16-L1 (1), P16-L1 (2)	-	L1 (1) L1 (2)	L1 (1)	L1 (1) L1 (2)	1.72
P17	123	9	5	2 1 1 1 1	P17-L1 (1) , (2) P17-L2 (1) P17-L3 (1) P17-L4 (1) P17-L5 (1)	-	L1 (2) L4 (1) L5 (1)	L5 (1)	L1 (2) L4 (1) L5 (1)	2.44
P18	202	17	2	1 1	P18-L1 (1) P18-L2 (1)	-	-	-	-	0
P19	172	11	4	2 1 1 1	P19-L1 (1), (2) P19-L2 (1) P19-L3 (1) P19-L4 (1)	-	-	-	-	0

P = Phosphinothricine (Bialaphos), L = line, individual numbers of regenerants given in parenthesis, Transformation frequency % = percentage of lines positive for at least one gene (each line derived from one explant) in relation to total No. of explants (half embryos) used.

Continued

Table 14: Transformation of barley immature embryos using AGL1 pDM805 (Bialaphos selection)

Experiment No.	No. of explants (half embryos)	No. of callus lines	No. of lines with green plantlets	No. of plantlets / line	Name of plantlets	Expression of marker genes		PCR Test *uidA* +	PCR Test *bar* +	Transformation frequencies %
						GUS +	Basta +			
P20	133	6	1	1	P20-L1 (1)	-	-	-	-	0
P21	182	7	2	1 1	P21-L1 (1) P21-L2 (1)	-	-	-	-	0
P22	288	14	2	1 1	P22-L1 (1) P22-L2 (1)	-	L1 (1) L2 (1)	-	L1 (1) L2 (1)	0.69
P23	217	21	5	1 1 1 1 1	P23-L1 (1) P23-L2 (1) P23-L3 (1) P23-L4 (1) P23-L5 (1)	-	-	-	-	0
P24	117	9	1	1	P24-L1 (1)	-	-	-	-	0
P25	73	14	9	3 8 lines x 1	P25-L1 (1), (2), (3) P25-L2 (1), L3 (1), L4 (1), L5 (1), L6 (1), L7 (1), L8 (1), L9 (1)	-	L1 (1) L5 (1)	-	L1 (1) L5 (1)	2.74
P26	171	19	11	2 lines x 3 2 lines x 2 7 lines x 1	P26-L1 (1), (2), (3) L2 (1), (2), (3) L3 (1), (2) L4 (1), (2) L5 (1) L6 (1) L7 (1) L8 (1) L9 (1) L10 (1) L11 (1)	L1 (1), (2), L1 (3) L3 (1)	L1 (1), (2), (3) L2 (1), (2), (3) L3 (1) L10 (1)	L1 (1), (2), (3) L2 (2) L3 (1)	L1 (1), (2), (3) L2 (1), (2), (3) L3 (1) L10 (1)	2.34
P27	139	16	5	4 4lines x 1	P27-L1 (1), (2), (3), (4) P27-L2 (1) L3 (1) L4 (1) L5 (1)	-	L1 (1) L1 (4)	-	L1 (1) L1 (4)	0.72
P28	95	12	8	2 lines x 2 6 lines x 1	P28-L1 (1), (2) L2 (1), (2) L3 (1) L4 (1) L5 (1) L6 (1) L7 (1) L8 (1)	-	L2 (1), (2) L8 (1)	-	L2 (1), (2) L8 (1)	2.10
P29	175	11	7	2 6 lines x 1	P29-L1 (1), (2) L2 (1) L3 (1) L4 (1) L5 (1) L6 (1) L7 (1)	L1 (1)	L1 (1) L4 (1)	L1 (1)	L1 (1) L4 (1)	1.14
P30	112	14	5	1	P30-L1 (1) L2 (1) L3 (1) L4 (1) L5 (1)	-	L5 (1)	-	L5 (1)	0.89
P31	202	19	1	2	P31-L1 (1), (2)	-	-	-	-	0
P32	197	6	0	0	-	-	-	-	-	0
P33	69	5	2	2	P33-L1 (1), (2) L2 (1), (2)	L2 (1)	L2 (1), (2)	L2 (1)	L2 (1), (2)	1.45
P34	111	15	4	4 2 1 1	P34-L1 (1), (2), (3), (4) L2 (1), (2) L3 (1) L4 (1)	L2 (1) L3 (1)	L1 (2), (3), (4) L2 (1) L3 (1)	L2 (1) L3 (1)	L1 (2), (3), (4) L2 (1) L3 (1)	2.70
P35	100	7	1	1	P35-L1 (1)	-	-	-	-	0
P36	137	11	8	3 lines x 2 5 lines x 1	P36-L1 (1), (2) L2 (1), (2) L3 (1), (2) L4(1) L5 (1) L6 (1) L7 (1) L8 (1)	-	-	-	-	0
P37	88	6	4	3 3 lines x 1	P37-L1 (1), (2), (3) L2 (1) L3 (1) L4 (1)	L1 (1), (2), (3)	L1 (1), (2), (3)	L1 (1), (2), (3)	L1 (1), (2), (3)	1.13

P = Phosphinothricine (Bialaphos), L = line, individual numbers of regenerants given in parenthesis, Transformation frequency % = percentage of lines positive for at least one gene (each line derived from one explant) in relation to total No. of explants (half embryos) used.

Post Regeneration Selection

Results of low transformation rate using pDM805 suggest that bialaphos may retard the regeneration process. Therefore, a set of new experiments (Post Regeneration Selection) was carried out to investigate this hypothesis; in these experiments immature half embryos were precultured for three days on CIM then inoculated with *Agrobacterium* culture of the binary vector pDM805 and co-cultivated for three days. After co-cultivation the explants were cultured on the selection medium CIM + 150 mg/l Timentin + 3 mg/l Bialaphos for 4 weeks and subcultured every 2 weeks.

Callus tissue of each line was subcultured on regeneration medium FHG + 150 mg/l Timentin (without Bialaphos) for 2 weeks. After regeneration each line was subjected to bialaphos selection (Figure 14). The FHG medium supplemented with 3 mg/l Bialaphos + 150 mg/l Timentin was used for this purpose. Two rounds of selection were performed on regeneration medium. The third round of selection was performed for the regenerated green plantlets of each line on the rooting medium ½ CIM + 5 mg/l Bialaphos + 150 mg/l Timentin.

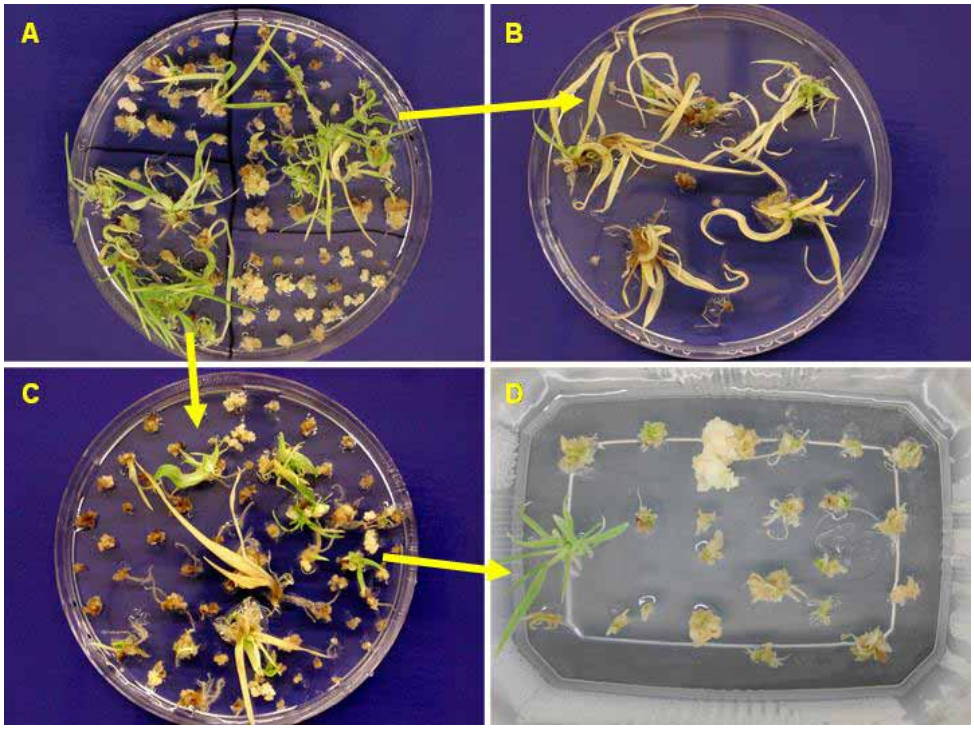

Figure 14: Post Regeneration Selection with Bialaphos: **(A)** 4 regenerated lines after 2 weeks without selection on FHG + Timentin 150 mg/l. **(B)** An example of Non-resistant line, all individual plantlets died after 2 weeks on selection medium FHG + Timentin 150 mg/l + Bialaphos 3 mg/l. **(C)** Selection of resistant plantlets (green) after the first round of selection 2 weeks on selection medium. **(D)** Selection of the resistant plants on rooting medium ½ CIM + Timentin 150 mg/l + Bialaphos 5 mg/l (the third round of selection) arrow points to the resistant green plant.

Four independent Post Regeneration Selection experiments were performed (Table 15). A total of 23 transgenic lines were generated from 582 half embryos with a transformation rate ranging between 1.12 % and 6.66 % and 3.95 % on average. This average value is more than 2.5-fold higher in comparison to the average value of the previous 37 experiments (Bialaphos selection) which gave an average of 1.51 %. Moreover, a total of 61 positive plants were detected in 23 lines with an average number of 2.65 plants/line which represents an increase of 73.20 % in comparison to the average number recorded for the 37 previous experiments (1.53 plants/line calculated for 19 positive experiments). Therefore, these results reveal that bialaphos has an inhibitory influence on the regeneration of barley (cv. Golden Promise) which leads to a reduction of the regeneration percentage of transgenic plants from transgenic callus tissues.

Table 15: Experiments of Post Regeneration Selection with Bialaphos

Experiment No.	No. of explants (half embryos)	No. of callus lines	No. of lines with green plantlets	No. of survived green plantlets / line	Name of survived green plantlets	Expression of marker genes		PCR Test *uidA* +	PCR Test *bar* +	Transformation frequencies %
						GUS +	Basta +			
PB1	150	112	67	1 line x 6 2 lines x 5 1 line x 4 2 lines x 3 4 lines x 1	PB1-L1 (1) -- (6) PB1-L2, L3 (1)-- (5) PB1-L4 (1)-- (4) PB1-L5, L6, (1)-- (3) PB1- L7, L8, L9, L10 (1)	L1 (1,2,6) L2, (2) L6, (2, 3) L8 (1)	L1 (1-6) L2, L3 (1-5) L4 (1-4) L5, L6, (1-3) L7, L8, L9, L10 (1)	L1 (1,2,6) L2, (2) L6, (2, 3) L8 (1)	L1 (1-6) L2, L3 (1-5) L4 (1-4) L5, L6, (1-3) L7, L8, L9, L10 (1)	6.66
PB2	146	84	36	1 line x 5 2 lines x 2 2 line x 1	PB2-L1 (1) -- (5) PB2-L2, L3 (1), (2) PB2-L4, L5 (1)	L1 (1,2,3) L2 (1,2) L5 (1)	L1 (1- 5) L2, L3 (1,2) L4, L5 (1)	L1 (1,2,3) L2 (1,2) L5 (1)	L1 (1- 5) L2, L3 (1,2) L4, L5 (1)	3.42
PB3	177	116	56	1 line x 3 1 line x 1	PB3-L1 (1), (2), (3) PB3-L2 (1)	-	L1 (1,2, 3) L2 (1)	-	L1 (1,2, 3) L2 (1)	1.12
PB4	109	97	64	1 lines x 5 1 line x 4 1 lines x 3 1 lines x 2 2 lines x 1	PB4-L1 (1) -- (5) PB4-L2 (1)-- (4) PB4-L3 (1)-- (3) PB4-L4 (1), (2) PB4-L5, L6 (1)	L1 (1, 2, 5) L2 (1) L3 (1,2,3) L6 (1)	L1 (1-5) L2 (1-4) L3 (1-3) L4 (1, 2) L5, L6 (1)	L1 (1, 2, 5) L2 (1) L3 (1,2,3) L6 (1)	L1 (1-5) L2 (1-4) L3 (1-3) L4 (1, 2) L5, L6 (1)	5.50

PB = Post-regeneration selection with Bialaphos, L = line, individual numbers of regenerants given in parenthesis, Transformation frequency % = percentage of lines positive for at least one gene (each line derived from one explant) in relation to total No. of explants (half embryos) used.

Analysis of the T_0 transgenic plants expressing *uidA* and *bar* genes

Histochemical Assay of GUS Activity

A representative part of embryogenic calli tissues of each transformation experiment was tested for GUS activity 4 weeks after *Agrobacterium* inoculation and before transfer of the calli to the regeneration medium FHG (Figure 15). After shoot and root regeneration GUS assay of leaf samples of the (T_0) transgenic plants was performed to select the positive GUS lines (Figure 16, p. 51).

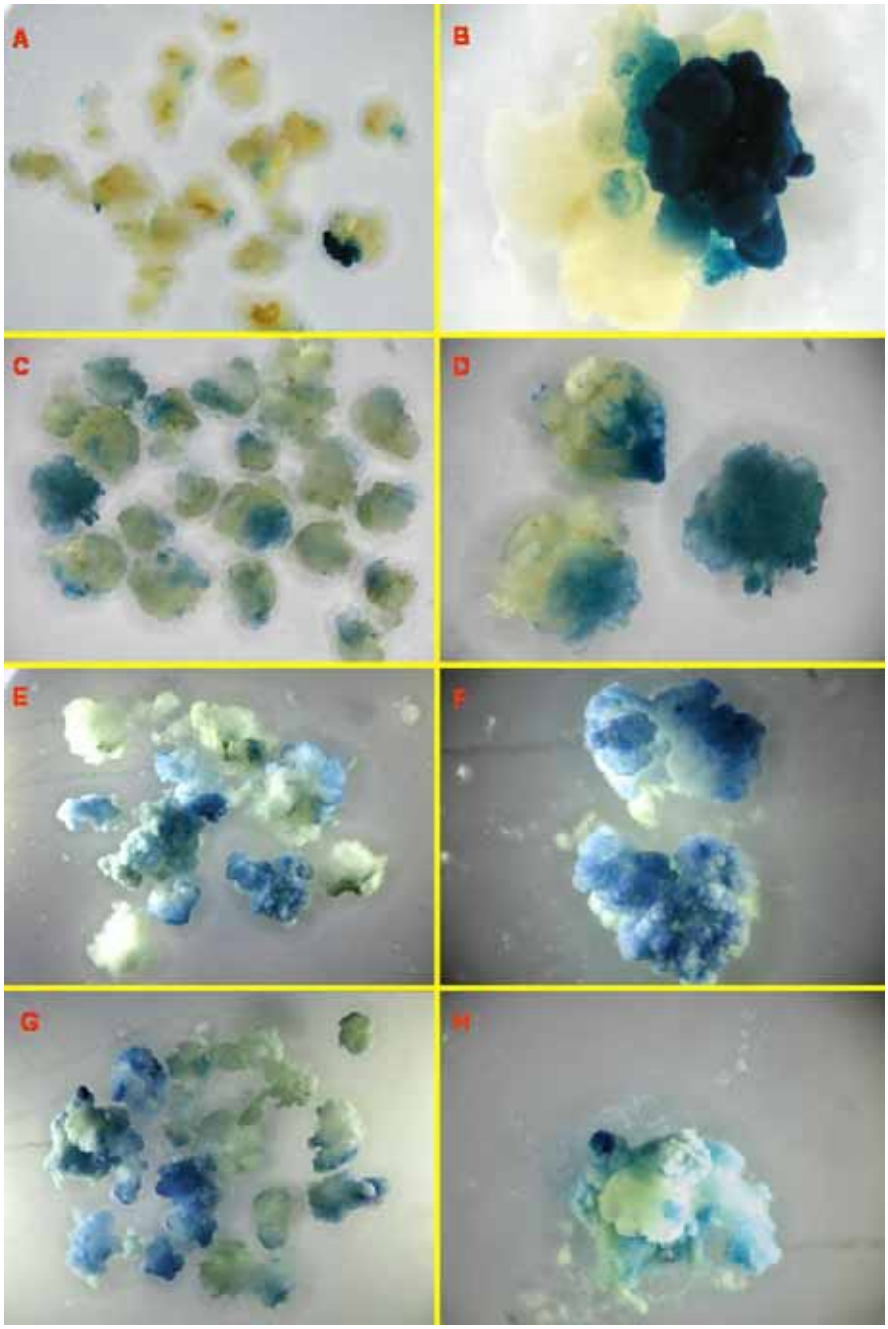

Figure 15: Evidence of stable transformation in calli tissues expressing GUS after 4 weeks of inoculation of barley immature half embryos with *Agrobacterium*. **(A,B)** without preculture. **(C-H)** Transgenic calli of 3 independent experiments, explants were precultured for 3 days before inoculation. On right side *(in focus)* calli expressing GUS.

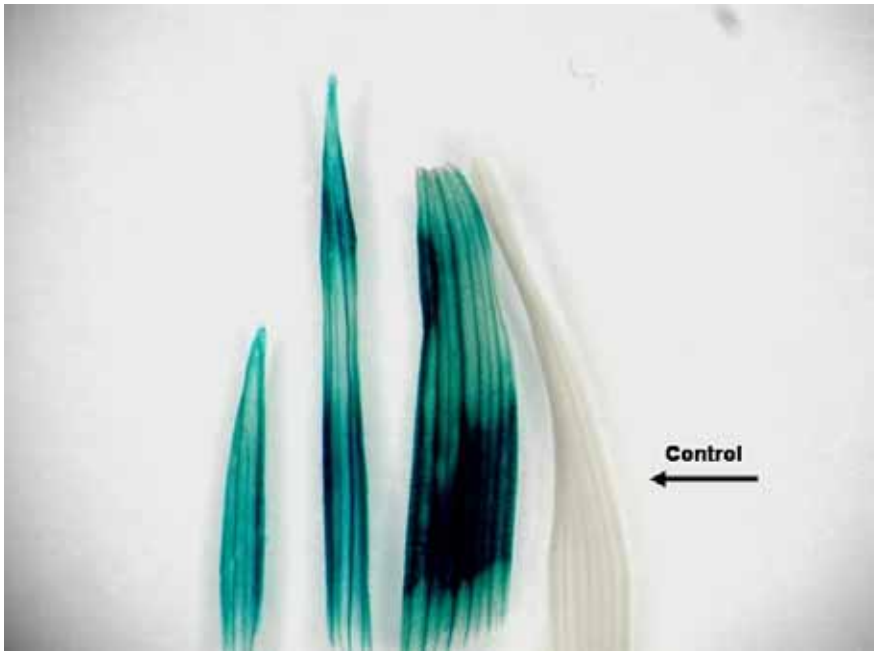

Figure 16: GUS assay of leaf samples from T_0 transgenic plants (*right* leaf of the control)

Expression of the *bar* gene (Basta test)

Expression of the *bar* gene was confirmed by leaf-painting using Basta herbicide (1g/l PPT) solution. After 7 days, PPT resistance was determined and the resistant plants were selected according to the percentage of necrosis suffered from leaf surface painted with the herbicide solution (Figure 17).

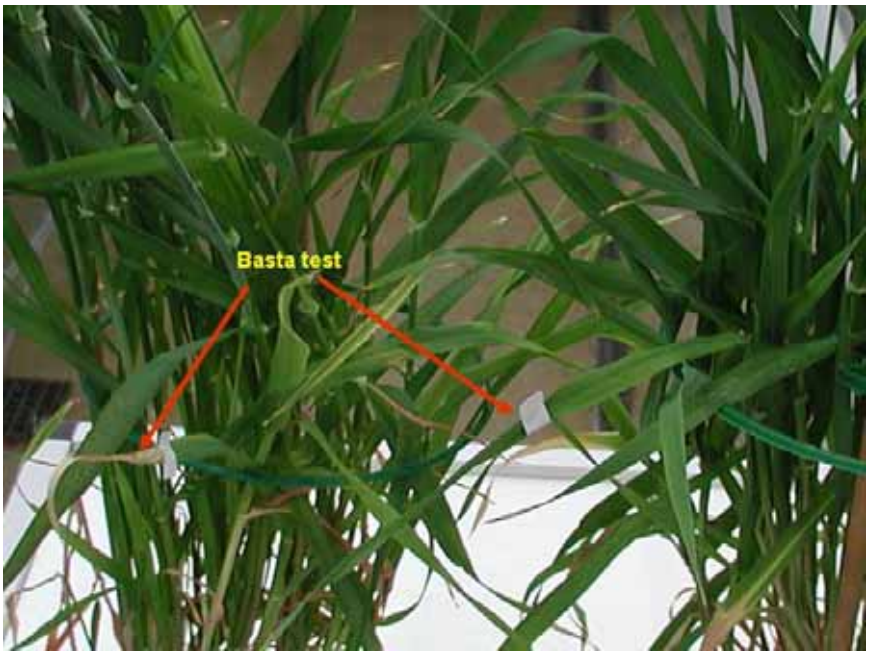

Figure 17: Expression of *bar* as confirmed by leaf-painting using PPT solution (1 g/l) after 7 days. Non-transformed plant (*left*) and T_0 transformed plant (*right*).

Expression of *uidA* and *bar* genes in T_1 generation

GUS assay of leaf samples of T_1 plants was performed to select plants expressing the *uidA* gene as an evidence for the expression of the *uidA* gene in the T_1 plants. A demonstration of GUS expression in three T_1 transgenic plants are shown (Figure 18).

Plants of the T_1 generation; 2-week old seedlings of the progeny of T_0 transgenic plants were sprayed three times with Basta solution (1g/l PPT), after 7 days resistant plants were selected (Figure 19).

Figure 18: Histochemical GUS assay of T_1 generation, three positives seedlings (*left*), and control plant (non-transgenic) (*right*).

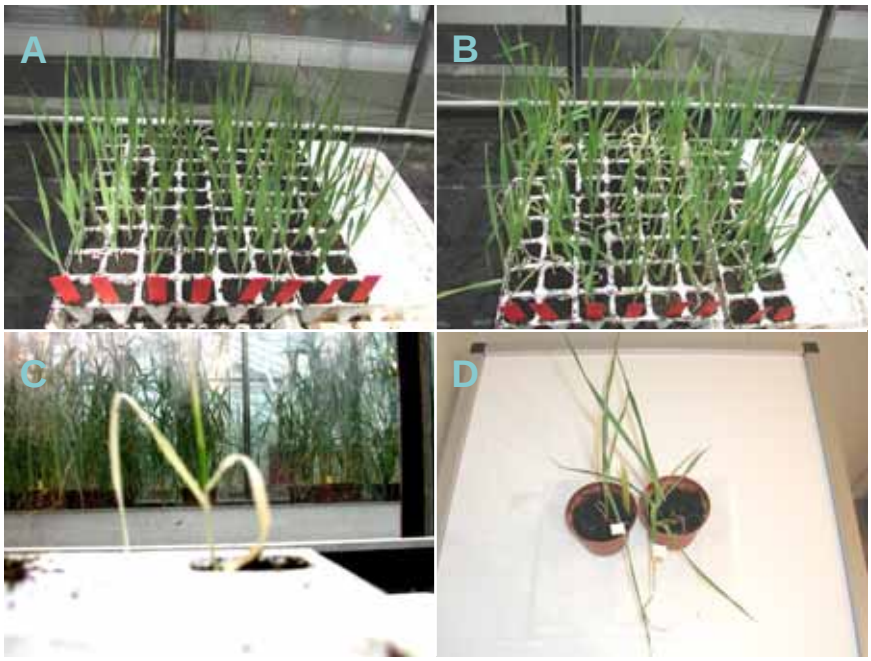

Figure 19: (A) The seedlings were sprayed using PPT solution (1 g/l) to determine the inheritance of the *bar* gene in T_1 generation,. **(B)** After 7 days the non-resistant plants were dead. **(C)** Non-transgenic plant. **(D)** 7 days after leaf-painting resistant plant(*left*), non-resistant plant(*right*).

Inheritance of *uidA* and *bar* genes in T_1 plants

Histochemical GUS assay and Basta test were performed to demonstrate the inheritance of *bar* and *uidA* genes in 60 plants of the T_1 generation of 6 transgenic plants representing 6 (T_0) individual lines (Table 16). PCR reactions were performed to confirm the presence of *uidA* and *bar* genes in the genome of the T_1 plants (Figure 20). The obtained data revealed that both genes were inherited in the T_1 generation and followed the Mendelian pattern (3:1) for single gene segregation.

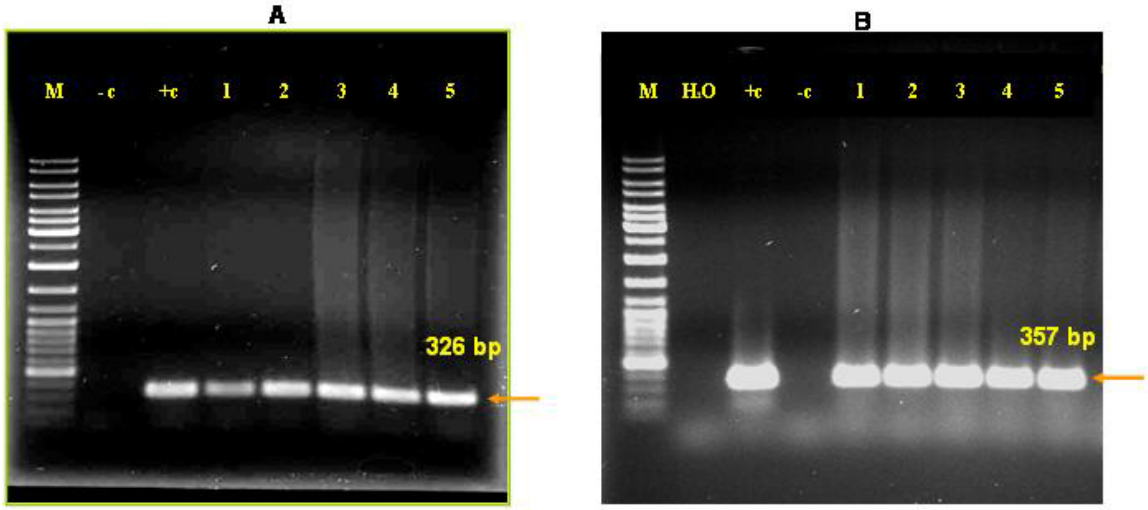

Figure 20: PCR analysis of genomic DNA from leaf tissues of control and T_1 plants. (A) Genomic DNA from non-transgenic control (-c), the plasmid pDM805 (+c), and five T_1 plants of individual T_0 transformed lines were used in PCR reactions with the primer set GUS Ting 97 to amplify the 326 bp *uid A* fragment (*arrow*). (B) The primer set Bar Ting 97 to amplify the 357 bp internal *bar* fragment (*arrow*).

Table 16: Segregation of the *uidA* and *bar* genes in the T_1 generation

Name of T_0 Transgenic plant	No. of T_1 plants tested by GUS assay, Basta test, and PCR	Segregation of *uidA* and *bar* genes (+) : (-)
P4-L2 (1)	12	7 : 5
P6-L3 (1)	9	7 : 2
P11-L1 (1)	13	12 : 1
P29-L1 (1)	12	9 : 3
P33-L2 (1)	9	5 : 4
P37-L1 (2)	5	5 : 0
Total	60	45 : 15 = (3:1)

(+) : Number of tested plants positive for both genes
(-) : Number of tested plants negative for both genes

Results of the transformation experiments using the binary vector pDM805 and Bialaphos selection can be summarized in the following points:

1- It is possible to transform barley immature embryos, although with low transformation efficiency.
2- It is possible to obtain transgenic calli lines with high rate, but with low regeneration capacity. This observation suggests that Bialaphos may has a negative impact on the regeneration process.
3- It is possible to enhance the transformation rate through optimizing the protocol of Post Regeneration Selection with bialaphos.
4- The transgenes can be inherited to the next generation.
5- Optimization treatments reveal that preculture has a positive influence on the transformation rate.

3.2.2. Transformation of barley immature embryos using the pWBVec10 vector and hygromycin selection

3.2.2.1. Optimization experiments

Due to low transformation efficiency of the Bialaphos selection system a second binary vector system AGL1 pWBVec10 (Wang *et al*., 1997,1998) with the *hpt* gene for hygromycin selection was tested.

All experiments were carried out using immature embryos with 1.5-2mm in size, inoculated for 1 hour with *Agrobacterium* culture (OD = 1.0), co-cultivated for 3 days on CIM, then selected on CIM + Timentin 150 mg/l + Hygromycin B 50 mg/l for 4 weeks and subcultured every 2 weeks on fresh medium. Then, the embryogenic callus lines were transferred to the regeneration medium FHG + Timentin 150 mg/l + Hygromycin B 25 mg/l.

On the basis of the results of the optimization experiments with pDM805, only the effect of the preculture period on the transient GUS expression was investigated. The obtained data in Table 17, p. 56 confirmed the positive influence of a 3 days preculture treatment on transient GUS expression, either by using intact or half scutellum of barley immature embryos. Preculture of half scutellum of immature embryos enhanced the percentage of explants with GUS foci, which recorded on average 76.38 % in comparison to 62.43 % for the control (without preculture). Interestingly in case of direct inoculation without preculture the distribution of GUS foci was only observed on the upper side of the scutellum close to the groove of the embryo axis. Applying preculture treatment caused an increase of GUS foci on the upper side of the scutellum, additionally the GUS foci were observed on the basal side of the scutellum (Figure 21, p 57), the average number of GUS foci/explant increased applying preculture treatment from 12.51 to 16.27 on average and the percentage of survival rate from 79.29 to 90.59 %.

A highly significant increase (71.22 %) was recorded for the percentage of explants with GUS foci on average; mean value of three independent experiments, when intact scutellum of immature embryos was precultured for 3 days before inoculation in comparison to the control treatment without preculture (8.77 % on average).

Again as with half embryos the distribution of the GUS foci was only on the upper side of the scutellum of the explants without preculture, while with preculture the GUS foci

increased on the upper side as well as a high number of GUS foci was detected on the basal side of the scutellum of intact immature embryos (Figure 22, p. 58). Moreover, the average number of GUS foci/explant increased from 10.30 to 17.51, also the percentage of the survival rate increased from 83.79 to 91.40 %.

Therefore, 3-days preculture treatment was used in the subsequent experiments.

Table 17: Effect of the preculture period on transient GUS expression using pWBVec10

Explants	Preculture period (days)	No. of explants/ experiment	Percentage of explants with GUS foci	Mean of GUS foci/ explant	Survival rate (%)
Intact immature scutellum	0	50	8.00	9.66	82.00
		50	2.00	10.00	86.00
		49	16.32	11.25	83.37
		T= 149	**A = 8.77**	**A = 10.30**	**A = 83.79**
	3	61	85.25	15.23	90.16
		50	30.00	19.13	92.00
		63	98.41	18.19	92.06
		T= 174	**A = 71.22**	**A = 17.51**	**A = 91.40**
Half immature scutellum	0	172	69.18	9.88	79.07
		245	60.40	13.11	76.73
		201	57.71	14.54	82.09
		T= 618	**A = 62.43**	**A = 12.51**	**A = 79.29**
	3	296	86.48	15.76	92.91
		187	68.45	15.22	90.37
		287	74.21	17.84	88.50
		T= 770	**A = 76.38**	**A = 16.27**	**A = 90.59**

T= Total A= Average

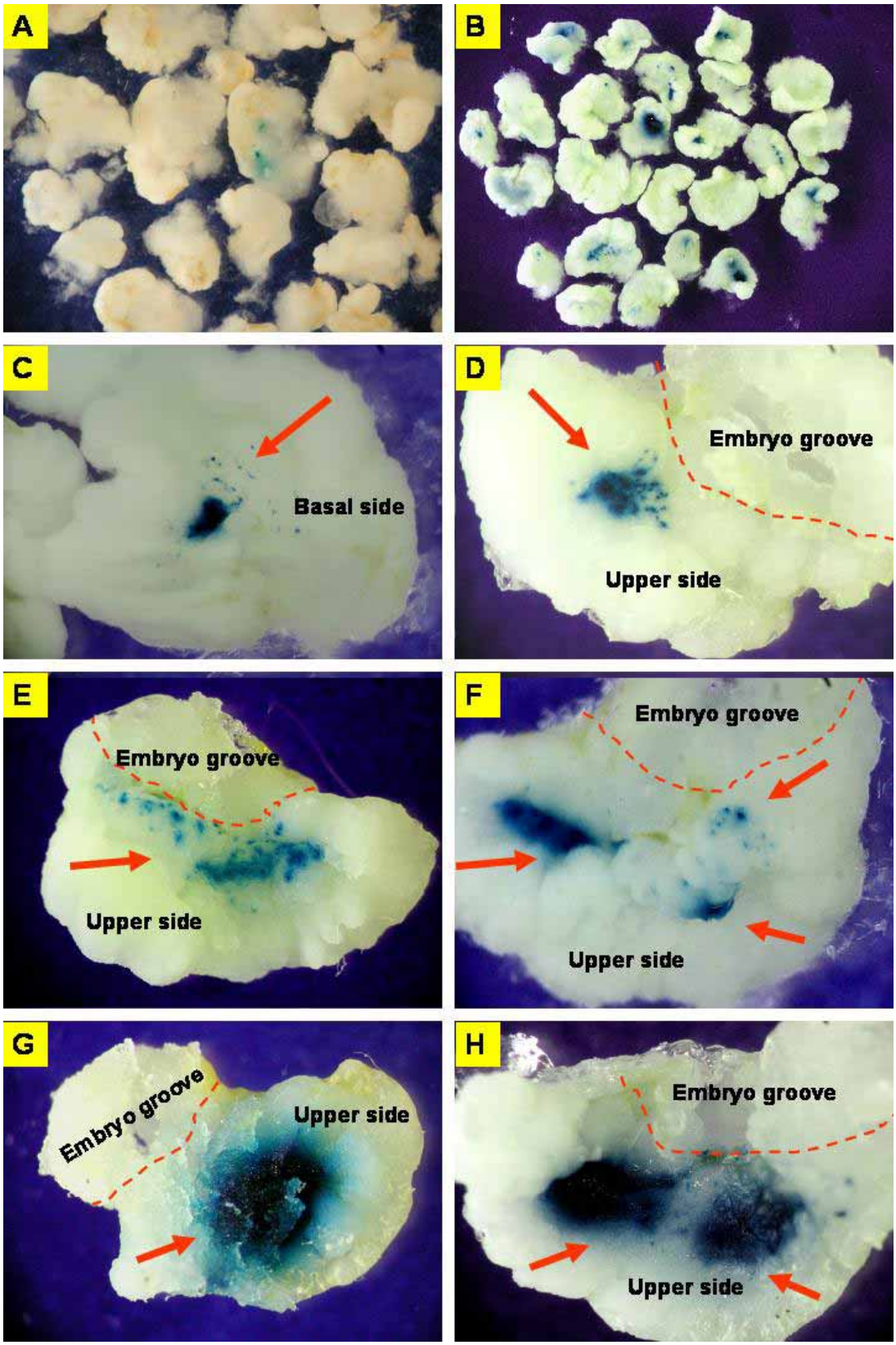

Figure 21: Transient GUS expression after 7 days of inoculation with *Agrobacterium tumefaciens* AGL1 pWBVec10 of half scutellum of immature embryos **(A)** Without preculture. **(B)** With the 3 days preculture on CIM before inoculation. **(C-H)** GUS expression in explants precultured for 3 day, **(C)** The GUS foci on the basal side of the scutellum. **(D-H)** Photos show the distribution of GUS foci on the upper side of the scutellum close to the groove area of the embryo axis, *arrows point to* the GUS foci.

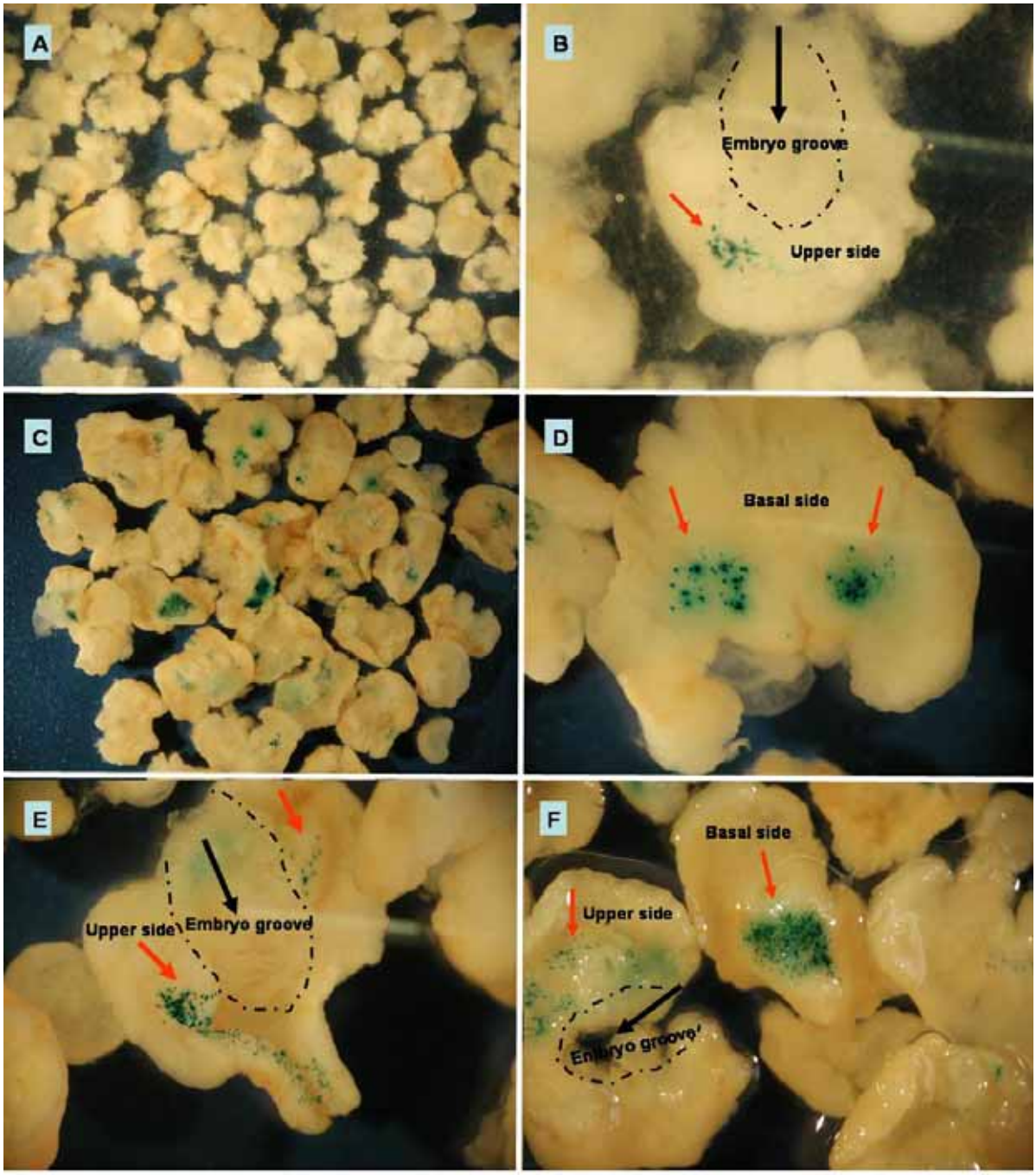

Figure 22: Transient GUS expression of intact scutellum of immature embryos after 7 days of inoculation with *Agrobacterium tumefaciens* AGL1 pWBVec10 **(A)** Without preculture, a few numbers of explants contain GUS foci on the upper side of scutellum. **(B)** Without preculture and *red arrow* points to GUS foci on the upper side of scutellum close to the groove area of the embryo axis *black arrow.* **(C-F)** With the 3-days preculture most of the explants contain GUS foci on both sides of the scutellum. **(D)** A distinguished GUS Foci on the basal side of the scutellum; *red arrows.* **(E)** Many of GUS foci on the upper side of the scutellum; *red arrows,* close to the groove area of embryo axis. **(F)** The distribution of distinguished GUS foci on the upper side and on the basal side of the scutellum; *red arrows.*

3.2.2.2. Production of stable transgenic barley plants using pWBVec10

A reproducible and efficient transformation system of barley using AGL1 pWBVec10 was established through a set of experiments (Table 18, p. 61, Figure 23, p. 60). Fourteen successful experiments were performed using the binary vector pWBVec10 which resulted in 158 lines positive for *uidA* and *hpt* genes (each line derived from one explant; half embryo). The transformation rate ranged between 4 and 23 %, with an average of 13.42 %. The number of positive plants/line ranged between 1 and 6 (on average 2.5 plants/line). This average number is higher than the average number recorded with the bialaphos system (1.53 plants/line).
The transgenic T_0 plants were tested using GUS assay and PCR analysis for *uidA* and *hpt* genes as well as for *vir* D and *vir* G of *Agrobacterium* to detect any *Agrobacterium* contamination of genomic DNA of the T_0 plants. Southern blot analysis of representative plants confirmed the presence of the *uidA* gene and integration into the genome of T_0 lines.

It was observed that callus tissues appeared in higher numbers on hygromycin selection medium (CIM + Hygromycin B 50 mg/l + Timentin 150 mg/l) Figure 23, p. 60, more than on bialaphos selection medium (Figure 14, p. 45), and a high percentage of callus lines shows remarkable somatic embryo like structures.

Moreover, successful regeneration of transgenic calli occurred frequently and it was observed that each callus line produced many plantlets, with roots (Figure 23F,G) which proofs evidently that most of these plantlets regenerated through somatic embryogenesis. This observation indicates that not only the transformation rate under hygromycin selection system is high but also the efficiency of the system. A high number of transgenic plants were generated from each callus line (Table 18, p. 61) in comparison to the bialaphos system (Table 14, p. 46 & 47).

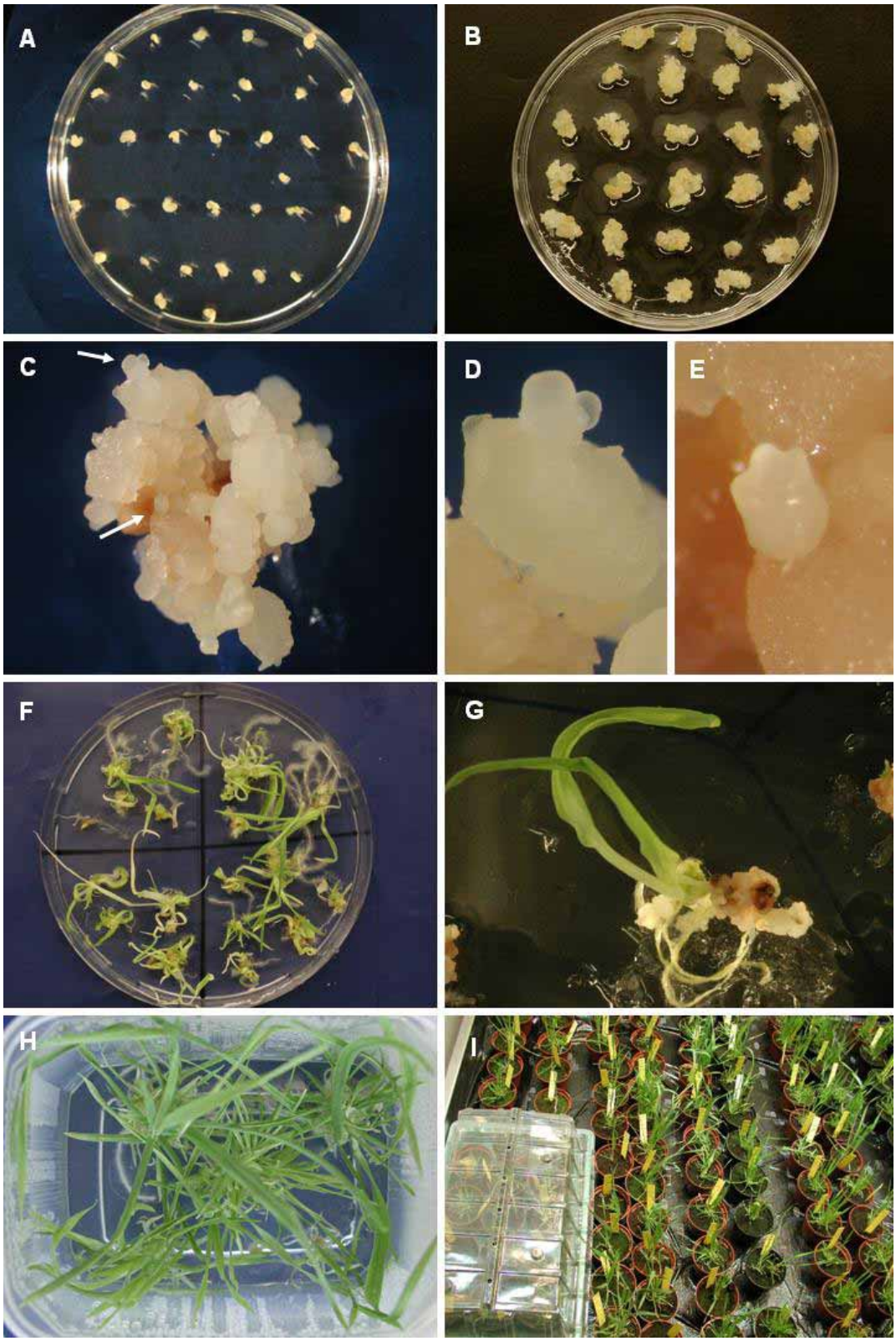

Figure 23: Production of transgenic barley plants using *Agrobacterium tumefaciens* AGL1 pWBVec10. **(A)** Callus induction after 7 days on CIM+ Hygromycin B 50 mg/l + Timentin 150 mg/l. **(B&C)** The embryogenic callus derived from the scutellum tissues after 4-week on CIM + Hygromycin B 50 mg/l + Timentin 150 mg/l, *arrows* point to somatic embryos. **(D&E)** A distinguished somatic embryos like structure. **(F)** Regeneration of putative transformed plants after 3 weeks on FHG + Hygromycin B 50 mg/l + Timentin 150 mg/l. **(G)** 3-week old individual transgenic plant on the same medium. **(H)** Transgenic plants after 3 weeks on root developing medium + Hygromycin B 50 mg/l + Timentin 150 mg/l. **(I)** Transgenic plants after 2-week in the soil.

Table 18: Transformation of barley immature embryos using AGL1 pWBVec10 (Hygromycin selection)

Experiment No.	No. of explants (half embryos)	No. of callus lines	No. of lines with green plantlets	No. of plantlets / line	Name of plantlets	Expression of GUS	PCR Test *uidA*	PCR Test *hpt*	Transformation frequencies %
H1	179	28	11	1 line x 5 1 line x 4 4 lines x 3 3 lines x 2 2 lines x 1	H1-L1 (1), (2), (3), (4), (5) L2 (1), (2), (3), (4) L3 (1-3), L4 (1-3), L5 (1-3) L6 (1-3), L7 (1-2), L8 (1-2), L9 (1-2) L10 (1), L11 (1)	L1 (1), (2), (3)+ L2 (4)+ L4 (1), (3)+ L6 (3)+ L7 (1)+ L8 (1), (2)+	L1 (1), (2), (3)+ L2 (4)+ L4 (1), (3)+ L6 (1), (2), (3)+ L7 (1)+ L8 (1), (2)+	L1 (1), (2), (3)+ L2 (3), (4)+ L4 (1),(2), (3)+ L6 (1), (2)+ L7 (1), (2)+ L8 (1), (2)+ L9 (1)+	4.47
H2	48	9	2	1 line x 3 1 line x 2	H2-L1 (1), (2), (3) L2 (1), (2)	L1 (1), (3)+ L2 (1), (2)+	L1 (1), (3)+ L2 (1), (2)+	L1 (1), (3)+ L2 (1), (2)+	4.16
H3	229	69	48	5 lines x 6 6 lines x 5 5 lines x 4 6 lines x 3 6 lines x 2 20 linesx 1	H3-L1 – L5 (1-6) L6 – L11 (1-5) L12 – L16 (1-4) L17 - L22 (1-3) L23 – L28 (1-2) L29 –L48 (1)	42 lines + 6 lines ─ (15,32,25,23, 44,47)	42 lines + 6 lines ─ (15,32,25,23, 44,47)	42 lines + 6 lines ─ (15,32,25,23, 44,47)	18.34
H4	41	12	2	1 line x 4 1 line x 2	H4-L1 (1), (2), (3), (4) H4-L2 (1), (2)	L1 (1),(2), (3) + L2 (1), (2)+	L1 (1),(2), (3) + L2 (1), (2)+	L1 (1),(2), (3) + L2 (1), (2)+	4.87
H5	35	9	5	1 line x 7 2 lines x 5 2 lines x 2	H5-L1(1),(2),(3),(4),(5),(6),(7) L2(1),(2),(3),(4),(5) L3(1),(2),(3),(4),(5) L4(1),(2) L5(1),(2)	L1(1),(2),(3),(7) L2(1), (4) L3(1),(2),(3),(4) L5(2) (+)	L1(1),(2),(3),(6),(7) L2(1), (4), (5) L3(1),(2),(3),(4) L5(2) (+)	L1(1),(2),(3),(6),(7) L2(1), (4), (5) L3(1),(2),(3),(4) L5(1),(2) (+)	11.42
H6	69	22	9	5 lines x 3 4 lines x 2	H6-L1, L2, L3, L4, L5 (1-3) H6-L6, L7, L8, L9 (1-2)	7 lines + 2 lines ─ (L2, L4)	7 lines + 2 lines (L2, L4) ─	7 lines + 2 lines (L2, L4) ─	10.14
H7	39	14	2	1 line x 4 1 line x 1	H7-L1 (1),(2),(3),(4) L2 (1)	L1(1),(2),(3),(4) L2 (1) (+)	L1 (1),(2),(3),(4) L2 (1) (+)	L1 (1),(2),(3),(4) L2 (1) (+)	5.12
H8	116	28	26	4 lines x 6 1 line x 5 3 lines x 3 6 lines x 2 12 linesx 1	H8- L1, L2, L3, L4 (1-6) L5(1),(2),(3),(4),(5) L6, L7, L8 (1-3) L9, L10, L11, L12, L13, L14 (1-2) L15, L16, L17, L18, L19, L20, L21, L22, L23, L24, L25, L26 (1)	21 lines + 5 lines ─ (L11, L18, L22, L23, L26)	21 lines + 5 lines ─ (L11, L18, L22, L23, L26)	21 lines + 5 lines ─ (L11, L18, L22, L23, L26)	18.10
H9	85	20	20	4 lines x 4 1 line x 3 4 lines x 2 11 linesx1	H9-L1, L2, L3, L4 (1-4) L5(1),(2),(3) L6, L7, L8, L9 (1-2) L10, L11, L12, L13, L14, L15, L16, L17, L18, L19, L20	16 lines + 4 lines ─ (L5, L16, L18, L19)	16 lines + 4 lines ─ (L5, L16, L18, L19)	16 lines + 4 lines ─ (L5, L16, L18, L19)	18.82
H10	148	33	32	2 lines x 5 4 lines x 4 6 lines x 3 11lines x 2 9 lines x 1	H10-L1, L2 (1-5) L3, L4, L5, L6 (1-4) L7,L8,L9, L10,L11,L12, (1-3) L13 – L23 (1-2) L24 – L32 (1)	25 lines + 7 lines ─ (L7,L12,L17, L23,L25,L30)	25 lines + 7 lines ─ (L7,L12,L17, L23,L25,L30)	25 lines + 7 lines ─ (L7,L12,L17, L23,L25,L30)	16.89
H11	54	14	12	2 lines x 3 7 lines x 2 3 lines x 1	H11-L1, L2 (1),(2),(3) L3 – L9 (1), (2) L10, L11, L12 (1)	9 lines + 3 lines ─ (L6, L7, L9)	9 lines + 3 lines ─ (L6, L7, L9)	9 lines + 3 lines ─ (L6, L7, L9)	16.66
H12	39	17	11	3 lines x 6 3 lines x 3 1 line x 2 4 lines x 1	H12-L1, L2, L3 (1-6) L4, L5, L6 (1-3) L7 (1), (2) L8, L9, L10, L11 (1)	9 lines + 2 lines ─ (L7, L8)	9 lines + 2 lines ─ (L7, L8)	9 lines + 2 lines ─ (L7, L8)	23.07
H13	70	31	14	2 lines x 4 1 line x 3 1 line x 2 10 linesx 1	H13- L1, L2 (1-4) L3 (1), (2, (3) L4 (1), (2) L5 – L14 (1)	10 lines + 4 lines ─ (L3, L9, L13, L14)	10 lines + 4 lines ─ (L3, L9, L13, L14)	10 lines + 4 lines ─ (L3, L9, L13, L14)	14.28
H14	25	8	2	1 line x 2 1 line x 1	H14-L1 (1), (2) L2 (1)	L1 (1) ─ L1 (2) + L2 (1) +	L1 (1) ─ L1 (2) + L2 (1) +	L1 (1), (2) + L2 (1) +	8.00

H = Hygromycin B, L = line, individual numbers of regenerants given in parenthesis, Transformation frequency % = percentage of lines positive for at least one gene (each line derived from one explant) in relation to the total No. of explants (half embryos) used.

Histochemical Assay of GUS Activity

To determine transformation efficiency, GUS assay was performed 2 weeks after *Agrobacterium* inoculation with about 10 % of the embryogenic callus tissues of each transformation experiment (Figure 24). After regeneration, GUS assay of leaf samples of the T_0 transgenic plants was carried out to demonstrate the positive GUS lines (Figure 25, p. 63).

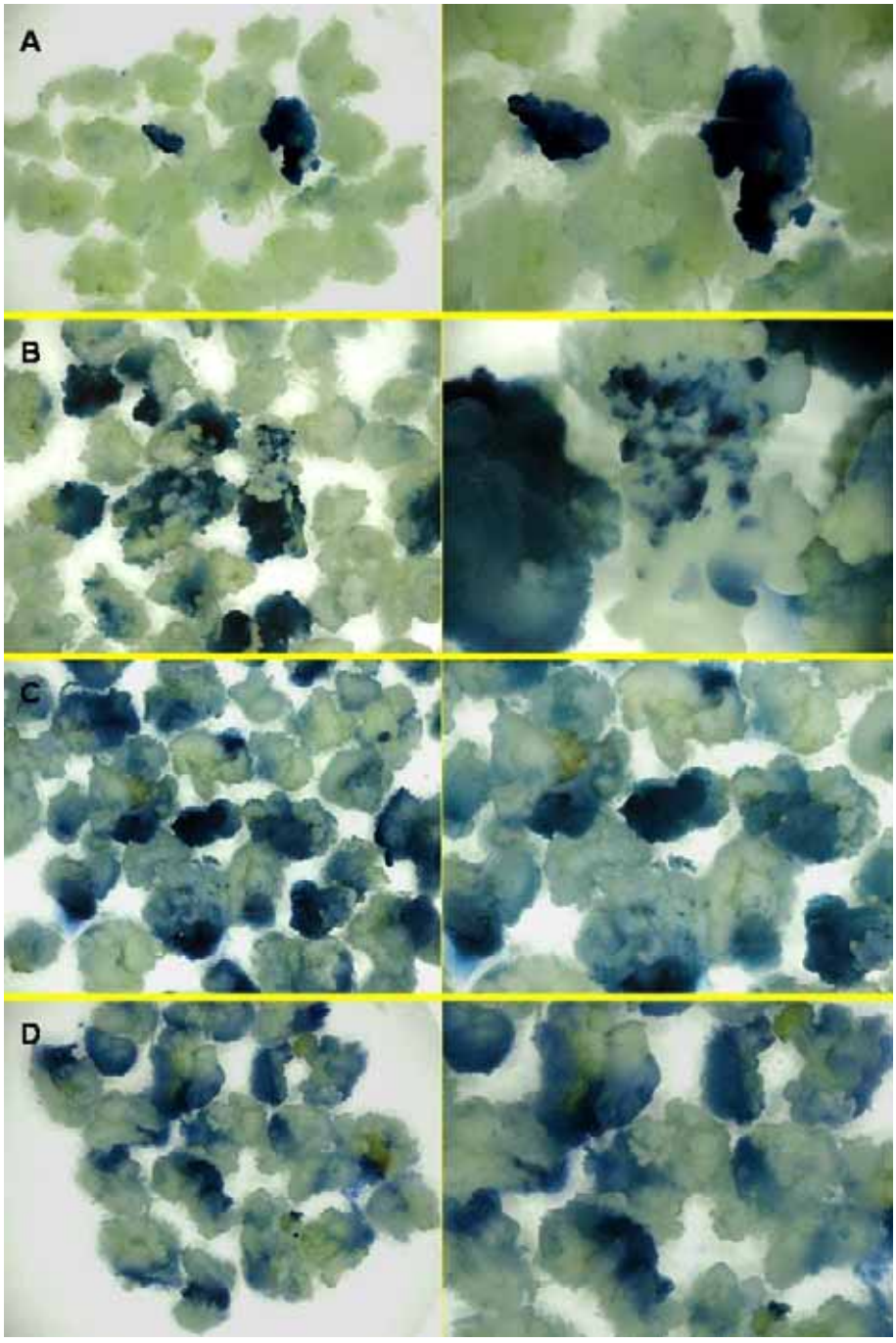

Figure 24: Stable expression of *uidA* gene after 2 weeks of inoculation with *Agrobacterium tumefaciens* pWBVec10. **(A)** Callus tissues derived from immature half embryos without preculture. **(B, C and D)** Callus tissues from three independent experiments of preculture for 3 days, *right side in focus* callus tissues expressing GUS.

Figure 25: Histochemical GUS assay of leaf sample from T_0 transgenic plant; leaf of the control non-transformed plant (*left*).

Southern blot analysis of the *uidA* gene

The binary plasmid vector pWBVec10 was digested with HindIII to generate one fragment of the intact *uidA* gene (*ubi1*+ *uidA* + *nos* = 4260 bp). Figure 26A, p. 64 shows the expected size. Hybridization with the *uidA* probe detected this fragment in the digested plasmid as well as in digested genomic DNA of four independent T_0 lines (Figure 26B). This result confirmed that the *uidA* gene was successfully transferred to all tested lines. A variation in the signal intensity between the samples was observed. This may due to a varying number of copies of the T-DNA being present in the individual transformants.

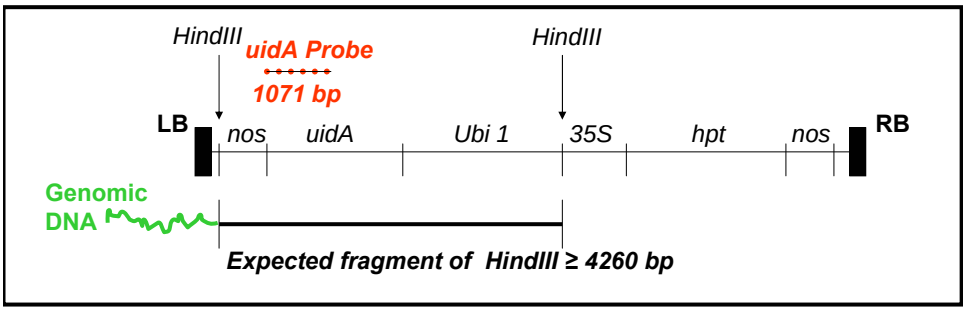

Figure 26A: T-DNA of the pWBVec10 vector and the expected fragment of the *uidA* gene when the plasmid or genomic DNA is digested with HindIII.

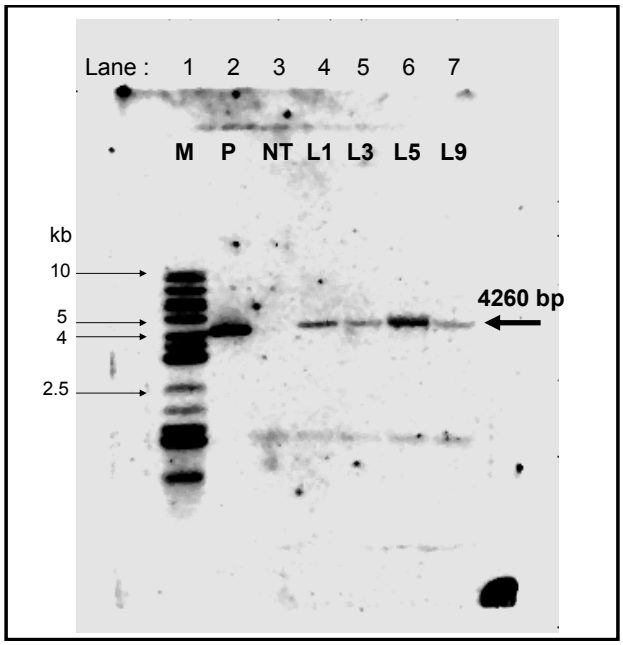

Figure 26B: Southern blot analysis of barley genomic DNA isolated from four independent T_0 transgenic lines (L1, L3, L5 and L9), digested with HindIII to cut out the intact *uidA* gene fragment 4260 bp from the T-DNA to select the transgenic lines. (P) The plasmid vector pWBVec10 digested with HindIII. (NT) Genomic DNA of non-transgenic plant digested with HindIII. (M) DNA marker size.

To determine the copy number of the *uidA* gene, line number 5 (L5: lane 6, Fig 26B) which showed an intensive fragment after HindIII digestion was chosen for this purpose. The expected fragment from digestion of the plasmid vector pWBVec10 or the genomic DNA with HindIII is 4260 bp, digestion of BamHI or XbaI is ≥ 2260 bp as shown in Figure 27A, p. 65. The result of hybridization of the membrane with the *uidA* probe confirmed the presence of the *uidA* gene in two copies in the genome of this transgenic line (Figure 27B, p. 65).

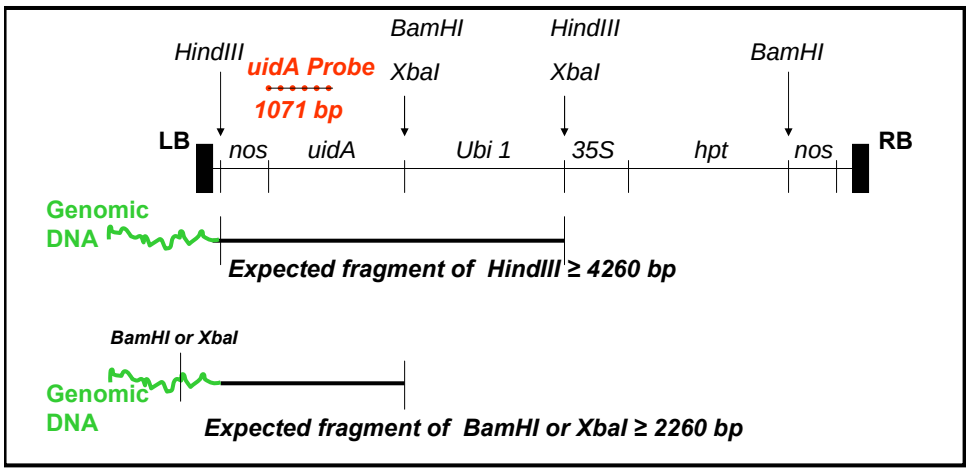

Figure 27A: T-DNA of the pWBVec10 vector and the expected fragments of the *uidA* gene when plasmid or genomic DNA is single digested with different enzymes (HindIII *or* BamHI *or* XbaI).

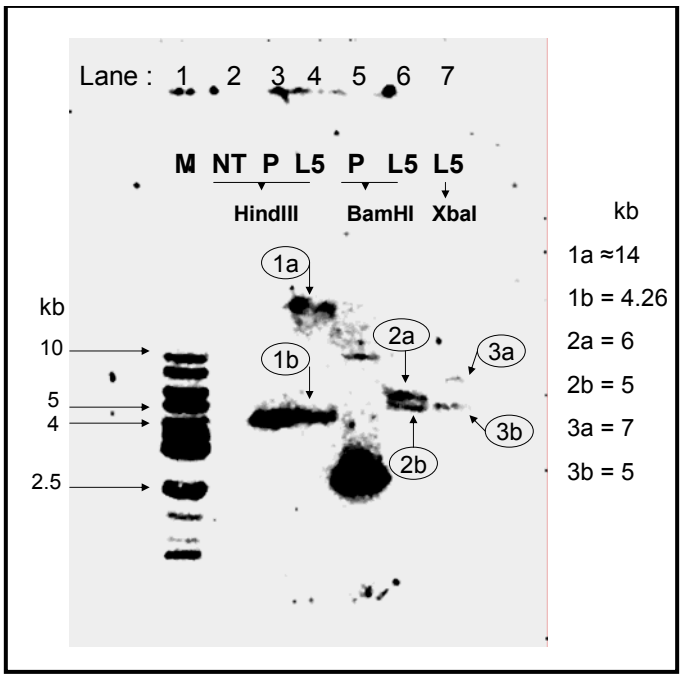

Figure 27B: The genomic DNA of L5 (20 µg) was digested with HindIII in lane number 4, with BamHI in lane number 6, and with XbaI in lane number 7 to detect the copy number of the integrated *uidA* gene in the genome of this transgenic T_0 line. (NT) Genomic DNA of non-transgenic plant digested with HindIII in lane number 2, and (P) plasmid vector pWBVec10 digested with HindIII in lane number 3 and with BamHI in lane 5. (M) DNA marker size. Two copies of the *uidA* gene were detected using different restriction enzymes. Fragments 1a and 1b, 2a and 2b, and 3a and 3b represent the two copies of the *uidA* gene.

Inheritance of the *uidA* and *hpt* genes in the T_1 generation

Histochemical GUS assay was carried out for 78 individual T_1 plants of 7 independent T_0 lines (Figure 28) to investigate the activity of the *uidA* gene in the T_1 generation. PCR reactions were performed to detect the presence of both the *uidA* gene and the selectable marker *hpt* gene in the progeny of T_0 plants (Figure 29, p. 67). Results of GUS assay and PCR reactions were used to assess the inheritance of *uidA* and *hpt* genes in the T_1 generation (Table 19, p. 67). The segregation ratio on average of both genes (*uidA* and *hpt*) exhibited the Mendelian pattern of the single gene segregation (3:1).

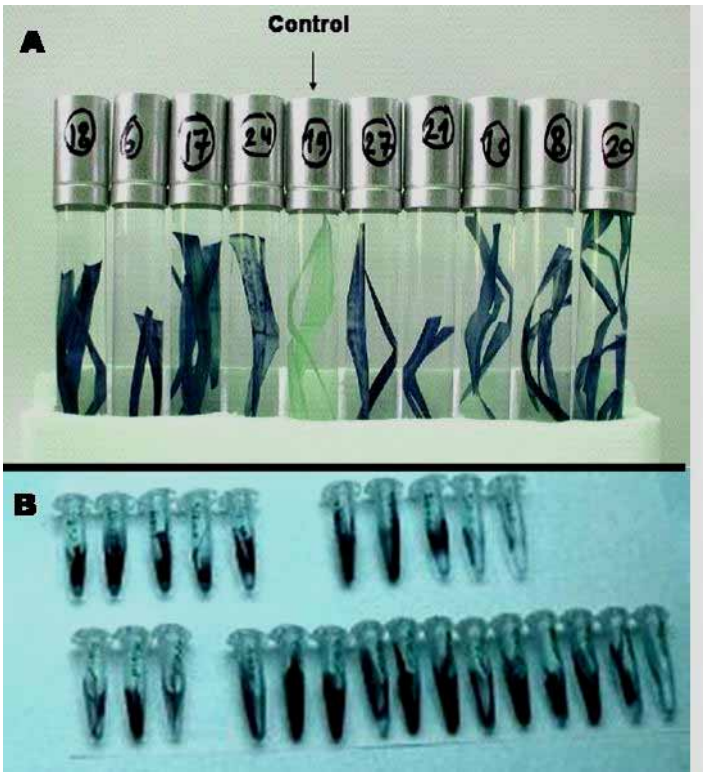

Figure 28: Histochemical GUS assay of the T_1 transgenic plants. **(A)** Leaf samples of positive T_1 plants in comparison to the control non-transformed plant. **(B)** GUS assay of the T1 plants (leaf samples in Eppendorf tubes) derived from two T_0 plants H14-L2 (1) and H10-L5 (4), all tested plants were positive.

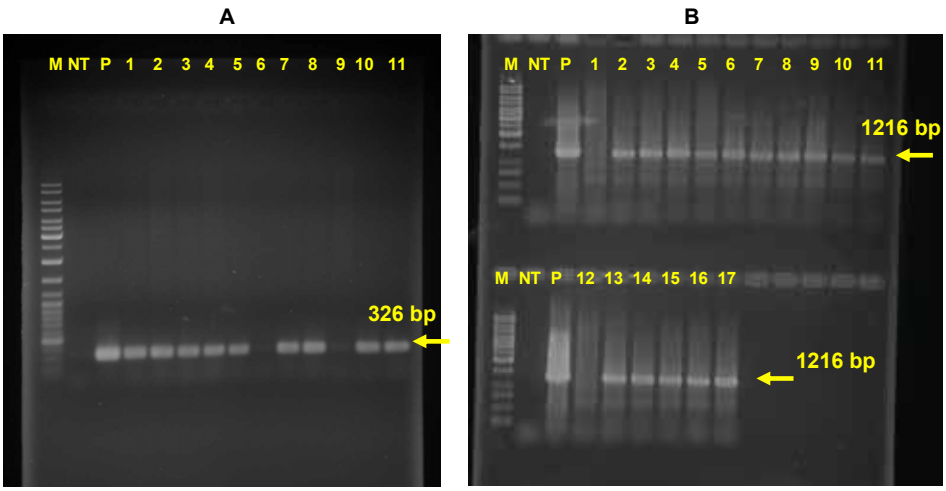

Figure 29: PCR analysis of genomic DNA from leaf tissues of control and T_1 transgenic plants.
(A) Genomic DNA from non-transgenic control (NT), (P) plasmid pWBVec10 and T1 plants of individual T_0 transformed lines were used in PCR reactions with the primer set GUS Ting 97 to amplify the 326 bp of the *uidA* fragment (*arrow*). **(B)** The primer set HPT was used to amplify the coding region 1216 bp fragment of the *hpt* gene (*arrow*).

Table 19: Segregation of the *uidA* and *hpt* genes in the T_1 generation

Name of T_0 Transgenic plant	Total No. of T1 plants tested by GUS assay and PCR	Segregation of *uidA* and *hpt* genes both (+) : both (-)
H5-L2 (1)	11	7 : 4
H7-L1 (1)	12	8 : 4
H8-L9 (2)	5	3 : 2
H10-L5 (4)	15	15 : 0
H12-L3 (1)	12	9 : 3
H13-L7 (1)	13	8 : 5
H14-L2 (1)	10	10 : 0
Total	78	60 : 18 = (3:1)

Results of the transformation experiments using the binary vector pWBVec10 in comparison to the pDM805 vector:

The obtained results of the transformation experiments using the *bar* gene as a selectable marker for achieving a high efficient barley transformation system proved non-profitable in contrast to the use of the *hpt* gene as a selectable marker. A great difference was

observed in the transformation rate between pWBVec10 and pDM805 vectors. In case of pWBVec10 the transformation rate ranged between 4 % and 23 % (average value 13.42 %), with the pDM805 vector the transformation rate ranged between 0.69 % and 3.13 % (average value 1.51 %). Only when the Post Regeneration Selection protocol with pDM805 vector was used the transformation rate increased up to 6.66 % and on average 3.95%.

Moreover, Figure 30 shows a representative impact of both selection systems on the regeneration of transgenic plantlets from transgenic calli. Transgenic callus tissue was restrained under bialaphos selection. Therefore, bialaphos seems to be a restraining factor playing an inhibitory role for regeneration. Consequently only few transgenic calli lines could regenerate and produce a small number of transgenic plantlets. On the contrary, with hygromycin most of transgenic callus lines could regenerate and produced a great number of transgenic plantlets.

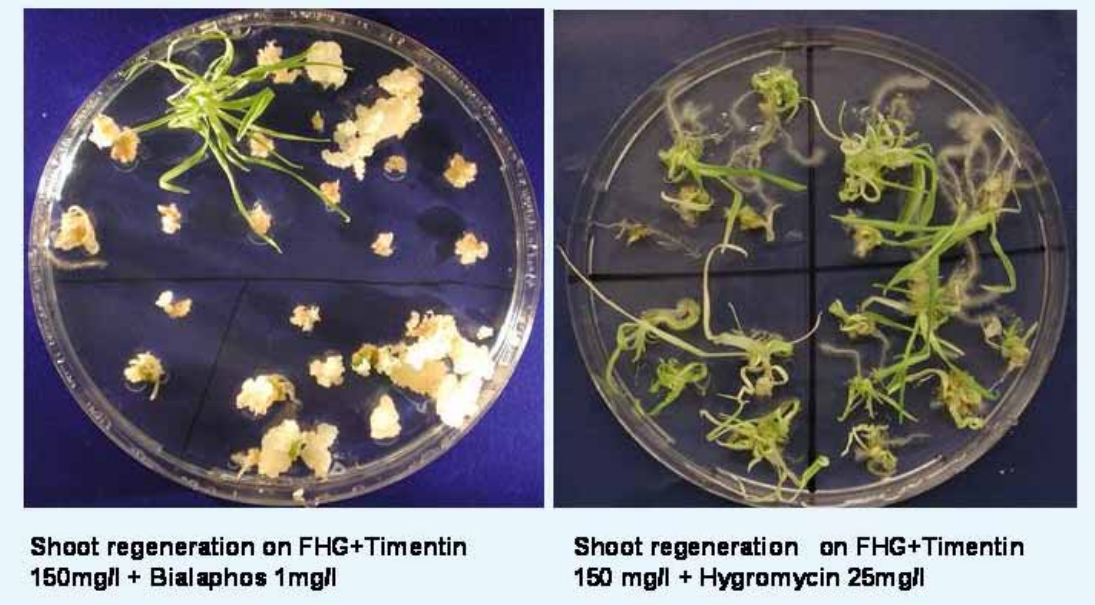

Figure 30: A representative impact of the bialaphos selection system on the regeneration of barley in comparison to the hygromycin B selection system.

Finally, it was decided to use the backbone of pWBVec10 for construction of new transformation vectors including the target genes *dapA* and *lysC*.

3.3. Transformation of barley with "high lysine" vectors

3.3.1. Construction of the amino acid transformation vectors

3.3.1.1. Transient expression of the *uidA* gene under the control of the *D-Hor* promoter

In a qualitative particle gun experiment the activity of the *D-hor3* promoter in the endosperm was tested. 25 isolated endosperms were bombarded either with the *ubiquitin* promoter *uidA* construction or with the *D-hordein* promoter *uidA* gene. Both promoters are highly active in the endosperm tissue (Figure 31).

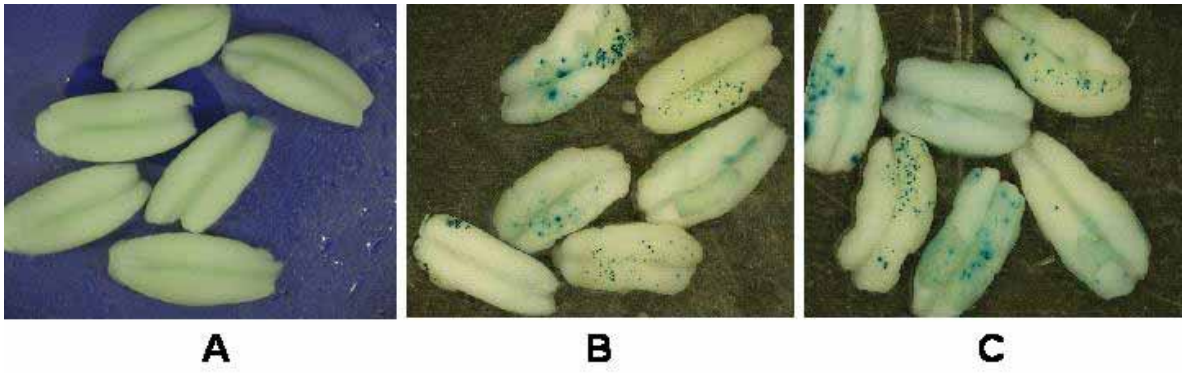

Figure 31: Transient GUS expression in the endosperm of barley. (A) Control non-transformed endosperms. (B) Expression of the *uidA* gene driven by the *ubi1* promoter. (C) Expression of the *uidA* gene driven by the endosperm-specific *D-hor* promoter.

3.3.1.2. Construction of the transformation vectors including the target genes *dapA* and *lysC* under the control of different promoters

The new *Agrobacterium* vectors pSMW12 and pSMW16 (Figure 32 & 33, p. 70) carry the *dapA* gene encoding dihydrodipicolinate synthase (DHDPS) and the *lysC* gene encoding aspartate kinase (AK), respectively. Both genes are driven by the endosperm-specific *D-hordein* promoter of the barley *D-Hor3* gene for production of high free lysine and threonine during development of the endosperm.

Five different T-DNA vectors of each gene were constructed for introduction of the target genes into the barley genome (Figure 34, p. 71). The *dapA* and *lysC* genes are driven by the *D-hor*, *ubi1* and 35S promoters. The selection gene *hpt* is located downstream of the

35S promoter. Vectors pSMW 11, 13, 15, 17 without a plant selection marker are used for marker free transformation or co-transformation experiments.

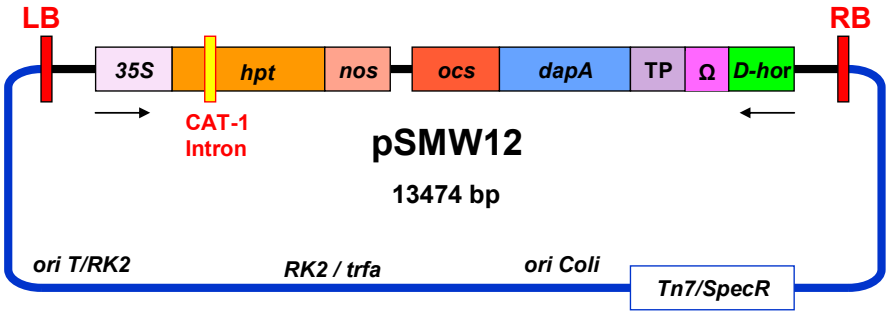

Figure 32: Structure of the *Agrobacterium* transformation vector pSMW12
Abbreviations : (*D-hor*), sequences of the promoter of the *D-Hor3* gene of barley; (Ω); omega leader sequence from the tobacco mosaic virus mRNA; (*TP*) the sequence coding for the chloroplast targeting sequence of the pea small subunit of ribulose 1,5-bisphosphate carboxylase(*rbcS*); (*dapA*), the *E coli* gene encoding DHDPS; (*ocs*), the polyadenylation signal of the octopine synthase gene of *Agrobacterium tumefaciens*, (*35S*), CaMV 35S promoter; (*hpt*) coding region of the *Streptomyces hygroscopicus* hygromycin B phosphotransferase gene; (*CAT-1*), intron of the castor bean catalase gene; (*nos*), 3` transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene LB, T-DNA left border sequence; RB, T-DNA right border sequence; *Tn7/SpecR*, spectinomycin resistance gene .

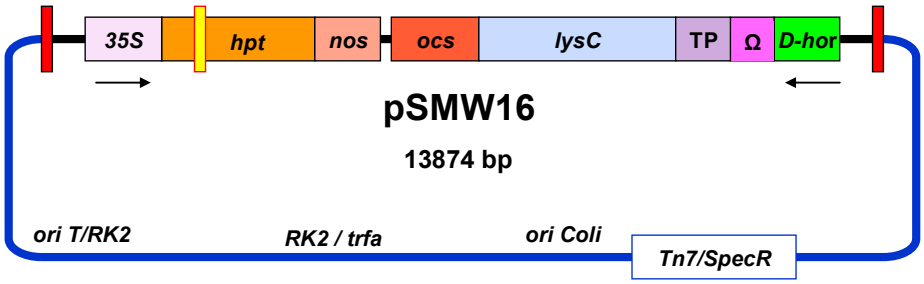

Figure 33: Structure of the *Agrobacterium* transformation vector pSMW16
Abbreviations : (*D-hor*), sequences of the promoter of the *D-Hor3* gene of barley; (Ω); omega leader sequence from the tobacco mosaic virus mRNA; (*TP*) the sequence coding for the chloroplast targeting sequence of the pea small subunit of ribulose 1,5-bisphosphate carboxylase(*rbcS*); (*lysC*), the gene of *E coli* encoding a desensitized AK-III; (*ocs*), the polyadenylation signal of the octopine synthase gene of *Agrobacterium tumefaciens*, (*35S*), CaMV 35S promoter; (*hpt*) coding region of the *Streptomyces hygroscopicus* hygromycin B phosphotransferase gene; (*CAT-1*), intron of the castor bean catalase gene; (*nos*), 3` transcript termination region of the *Agrobacterium tumefaciens* nopaline synthase gene LB, T-DNA left border sequence; RB, T-DNA right border sequence; *Tn7/SpecR*, spectinomycin resistance gene .

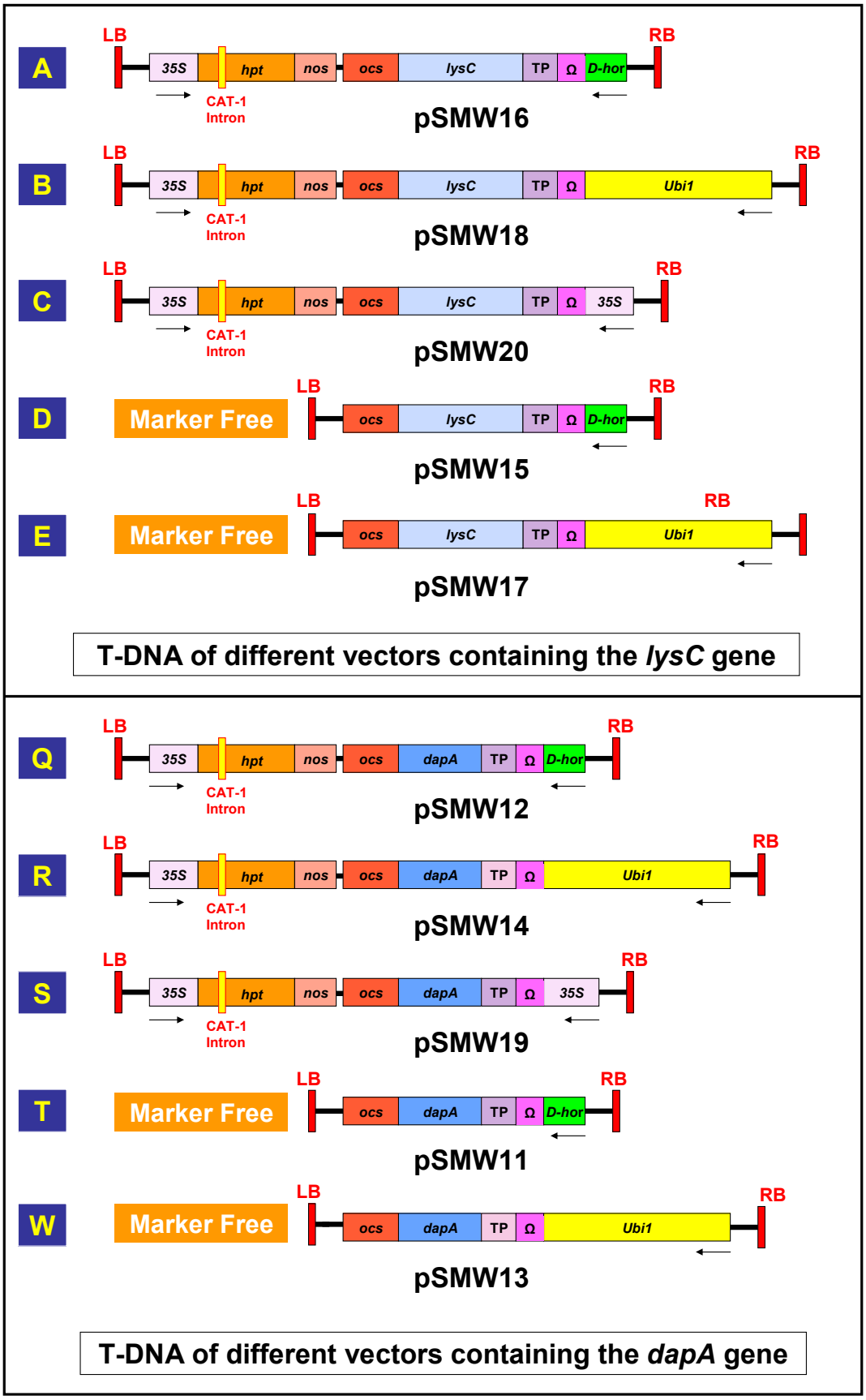

Figure 34: The construction of the expression cassettes of the *Agrobacterium* transformation vectors containing the gene of *lysC* and *dapA*.
A, B, C,… ..W = Name of the vectors used in different transformation experiments.

3.3.2. Production of barley plants expressing *dapA* and *lysC* genes

33 transformation experiments were performed using the new constructed vectors to produce T_0 transgenic barley plants expressing either the *dapA* or *lysC* gene (Figure 35, p. 74). 103 transgenic-positive lines were detected in a total number of 226 individual plants in 21 successful transformation experiments using constructions of *lysC* and *dapA* genes as shown in Tables 20 and 21. Using the *lysC* constructions in 8 successful experiments resulted in 49 T_0 independent positive lines with 127 plants. Also a total of 54 positive lines with 99 plants were detected in 13 successful experiments using the *dapA* gene constructions.

Table 20: Transformation experiments with different constructs containing the *lysC* (AK) gene

Experi-ment	Plasmid name	No. of explants (half embryos)	No. of callus lines	No. of lines with green plantlets	No. of plantlets / line	Name of plantlets	PCR test *lysC* (+)	PCR test *hpt* (+)	Transformation frequencies %
A1	pSMW16	61	14	3	1 line x 20 1 lines x 7 1 line x 12	A1-L1 (1-20) A1-L2 (1-7) A1-L3 (1-12)	L1 (1-20) L2 (1-7) L3 (1-12)	L1 (1-20) L2 (1-7) L3 (1-12)	4.91
A2	pSMW16	95	18	2	2 lines x1	A2-L1 (1) A2-L2 (1)	L1 (1) L2 (1)	L1 (1) L2 (1)	2.10
A3	pSMW16	70	23	*Stopped (low effi)	-	-	-	-	-
B1	pSMW18	230	12	*Stopped (low effi)	-	-	-	-	-
B2	pSMW18	108	8	*Stopped (low effi)	-	-	-	-	-
B3	pSMW18	65	26	14	3 lines x 3 4 lines x 1	B3-L1, L3, L4 (1- 3) B3-L2, L5, L6, L7 (1)	L1, L3, L4 (1- 3) L2, L5, L6, L7 (1)	L1, L3, L4 (1- 3) L2, L5, L6, L7 (1)	10.77
B4	pSMW18	55	6	*Stopped (low effi)	-	-	-	-	-
B5	pSMW18	42	12	*Stopped (low effi)	-	-	-	-	-
B6	pSMW18	84	11	2	2 lines x 2	B6-L1 (1), (2) B6-L2 (1), (2)	L1 (1), (2) L2 (1), (2)	L1 (1), (2) L2 (1), (2)	2.38
B7	pSMW18	95	16	1	1line x 1	B7-L1 (1)	L1 (1)	L1 (1)	1.05
B8	pSMW18	115	9	8	1line x 5 1line x 6 1line x 7 1line x 4 2 lines x 3 2 lines x 2	B8-L1 (1 - 5) B8-L2 (1 - 6) B8-L5 (1 - 7) B8-L4 (1 - 4) B8-L3, L7 (1 - 3) B8-L6, L8 (1), (2)	L1 (1 - 5) L2 (1 - 6) L5 (1 - 7) L4 (1 - 4) L3, L7 (1 - 3) L6, L8 (1), (2)	L1 (1-5) L2 (1 - 6) L5 (1 - 7) L4 (1 - 4) L3, L7 (1 - 3) L6, L8 (1), (2)	6.95
C1	pSMW20	42	3	*Stopped (conta.)	-	-	-	-	-
C2	pSMW20	342	37	21	1 line x 5 1 line x 4 1line x 3 2 linesx 2 16 lines x 1	C2-L15 (1- 5) C2-L16 (1- 4) C2-L1 (1- 3) C2-L 5, L17 (1), (2) C2-L2, L3, L4, L6-L14, L18-L21 (1)	L15 (1), (2), (3), (5) L16 (1), (2) , (3), (4) L1 (2) , (3) L 5, L17 (1), (2) L2, L3, L4, L6-L14, L18-L21 (1)	L15 (1), (2), (3), (5) L16 (1), (2) , (3), (4) L1 (2) , (3) L 5, L17 (1), (2) L2, L3, L4, L6-L11, L14, L18-L21 (1)	5.56
C3	pSMW20	68	25	8	3 lines x 2 5 lines x 1	C3-L4, L7, L8 (1), (2) C3-L1, L2, L3, L5, L6 (1)	L4 (1), L8 (1), (2) L7 (2) L1, L2, L3, L5, (1)	L4 (1), L8 (1), (2) L7 (2) L1, L2, L3, L5, (1)	10.29

L = line, individual numbers of regenerants given in parenthesis, Transformation frequency % = percentage of lines positive for at least one gene (each line derived from one explant) in relation to the total No. of explants (half embryos) used. *****Experiments were stopped due to the contamination (conta.) or low transformation efficiency (low effi) which determined by the transient GUS expression in a parallel transformation experiment using pWBVec10 vector.

Table 21: Transformation experiments with different constructs containing the *dapA* (DHDPS) gene

Experi-ment	Plasmid name	No. of explants (half embryos)	No. of callus lines	No. of lines with green plantlets	No. of plantlets / line	Name of plantlets	PCR test *dapA* (+)	PCR test *hpt* (+)	Transformation frequencies %
Q1	pSMW12	142	-	*Stopped (conta.)	-	-	-	-	-
Q2	pSMW12	222	-	*Stopped (conta.)	-	-	-	-	-
Q3	pSMW12	280	6	2	1 line x 1 1 line x 2	Q3-L1 (1) Q3-L2 (1), (2)	L1 (1) L2 (1) , (2)	L1 (1) L2 (1) , (2)	0.71
Q4	pSMW12	233	22	7	2 lines x 4 3 lines x 3 2 line x 1	Q4-L5, L6 (1-4) Q4-L1, L2, L4 (1-3) Q4-L3, L4 (1)	L5, L6 (1-4) L1, L2, L4 (1-3) L3, L4 (1)	L5, L6 (1-4) L1, L2, L4 (1-3) L3, L4 (1)	3.00
Q5	pSMW12	193	-	*Stopped (conta.)	-	-	-	-	-
Q6	pSMW12	162	4	*Stopped (low effi)	-	-	-	-	-
Q7	pSMW12	119	20	*Stopped (low effi)	-	-	-	-	-
Q8	pSMW12	54	-	*Stopped (conta.)	-	-	-	-	-
Q9	pSMW12	100	24	2	2 lines x 1	Q9-L1 (1) Q9-L2 (1)	L1 (1) L2 (1)	L1 (1) L2 (1)	2.00
Q10	pSMW12	98	11	1	1 line x 3	Q10-L1 (1), (2), (3)	L1 (1), (2), (3)	L1 (1), (2), (3)	1.02
Q11	pSMW12	103	28	1	1 line x 2	Q11-L1 (1), (2)	L1 (1), (2)	L1 (1), (2)	0.97
Q12	pSMW12	88	16	1	1 line x 4	Q12-L1 (1), (2), (3), (4)	L1 (1), (2), (3), (4)	L1 (1), (2), (3), (4)	1.13
R1	pSMW14	290	13	10	10 lines x 1	R1-L1 – L10 (1)	L1 – L10 (1)	L1 – L10 (1)	3.44
R2	pSMW14	103	25	2	1 line x 2 1 line x1	R2-L1 (1), (2) R2-L2 (1)	L1 (1), (2) L2 (1)	L1 (1), (2) L2 (1)	1.94
R3	pSMW14	70	17	6	1 line x 3 5 lines x 1	R3-L5 (1), (2), (3) R3-L1, L2, L3, L4, L6 (1)	L5 (1), (2), (3) L1, L3, L4, L6 (1)	L5 (1), (2), (3) L1, L3, L4, L6 (1)	7.14
S1	pSMW19	44	6	2	2 lines x 3	S1-L1 (1), (2), (3) S1-L2 (1), (2), (3)	L1 (1), (2), (3) L2 (1), (2), (3)	L1 (1), (2), (3) L2 (1), (2), (3)	4.55
S2	pSMW19	40	3	1	1 line x 3	S2-L1 (1), (2), (3)	L1 (1), (2), (3)	L1 (1), (2), (3)	2.50
S3	pSMW19	300	42	7	1 line x 6 1 line x 4 2 lines x 3 3 lines x 1	S3-L1 (1), (2), (3), (4), (5), (6) S3-L4 (1), (2), (3), (4) S3-L2, L3 (1), (2), (3) S3-L5, L6, L7 (1)	L1 (1), (2), (3), (4), (5), L4 (1), (2), (3), (4) L2, L3 (1), (2), (3) L5, L6, L7 (1)	L1 (1), (2), (3), (4), (5), L4 (1), (2), (3), (4) L2, L3 (1), (2), (3) L5, L6, L7 (1)	2.33
S4	pSMW19	68	28	13	1 line x 5 1 line x 4 1 line x 3 5 line x 2 5 line x 1	S4-L7 (1), (2), (3), (4), (5) S4-L1(1), (2), (3), (4) S4-L10 (1), (2), (3) S4-L2, L3, L13, L11, L6 (1), (2) S4-L4, L5, L8, L9, L12 (1)	L7 (1), (2), (3), (5) L1(1), (2), (3), (4) L10 (1), (2), (3) L3, L13, L11, L6 (1), (2) L4, L5, L8, L9, L12 (1) L2 (2)	L7 (1), (2), (3), (5) L1(1), (2), (3), (4) L10 (1), (2), (3) L3, L13, L11, L6 (1), (2) L4, L5, L8, L9, L12 (1) L2 (2)	19.11

L = line, individual numbers of regenerants given in parenthesis, Transformation frequency % = percentage of lines positive for at least one gene (each line derived from one explant) in relation to the total No. of explants (half embryos) used. *****Experiments were stopped due to the contamination (conta.) or low transformation efficiency (low effi) which determined by the transient GUS expression in a parallel transformation experiment using pWBVec10 vector.

Figure 35: Transgenic T_0 plants transformed with different constructions of *lysC* (AK) and *dapA* (DHDPS) genes. (A) Plants of different constructions in small pots during adaptation in green house. (B) Plants 3 months-old during anthesis.

Co-transformation of the *dapA* and *lysC* genes

Seven co-transformation experiments were performed to introduce both genes into the barley genome as shown in Table 22. Only two transgenic lines using the constructions (B + Q) with five plants were detected. However, all of these plants were positive for the B (*ubi1-lysC*) and negative for the Q (*D-hor- dapA*) construction.

Table 22: Co-transformation experiments with two plasmids

Experiment	Plasmid Name	Construction	No. of experiments	Total No. of positive lines tested by PCR
B T	pSMW18 & pSMW11 Marker free vector	*Ubi1-lysC +hpt* /pSMW18 & *D-hor- dapA*/pSMW11	3	-
E T	pSMW17 & pSMW11 Both are Marker free vectors	*Ubi1-lysC* /pSMW17 & *D-hor- dapA*/pSMW11	3	-
B Q	pSMW18 & pSMW12	*Ubi1-lysC +hpt* /pSMW18 & *D-hor- dapA +hpt* /pSMW12	1	2 lines (5 plants) All were positive for the (*lysC*) B construction

All transgenic (T_0) plants were tested by PCR (for example, Figures 36, 37), and a representative lines of each construction were used for southern blot analysis to confirm the integration of the transgenes into the barley genome.

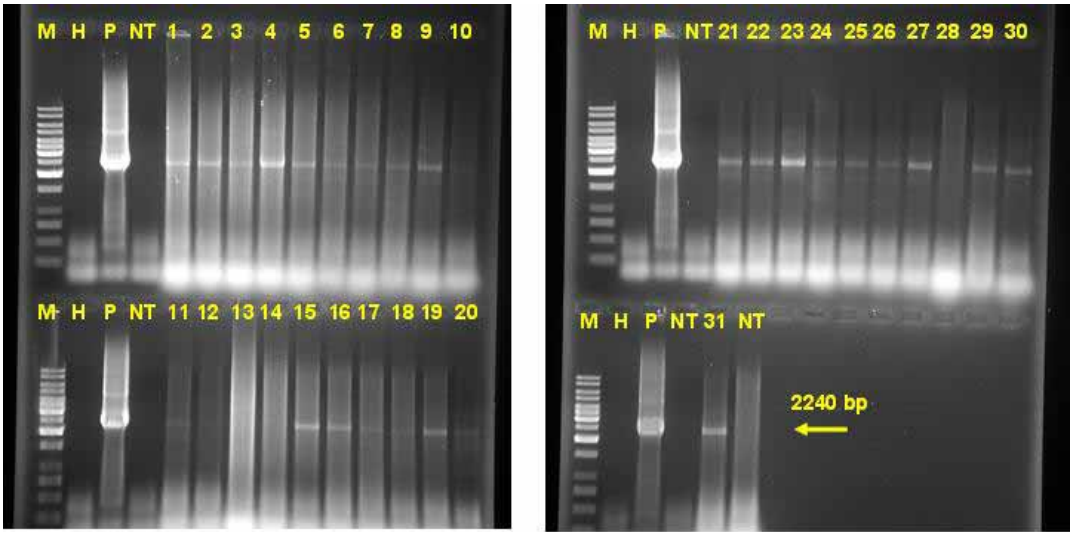

Figure 36: PCR analysis of genomic DNA from leaf tissues of a control non-transgenic plant (NT) and 31 T_0 plants derived from 26 independent cell lines transformed with the pSMW20 vector (*lysC* driven by the 35S promoter). Genomic DNA and Plasmid pSMW20 (P) were used in PCR reactions with the specific primers Omega BamHI Fwd and OCS SacI Rev to amplify a 2240 bp fragment (*arrow*)**.**

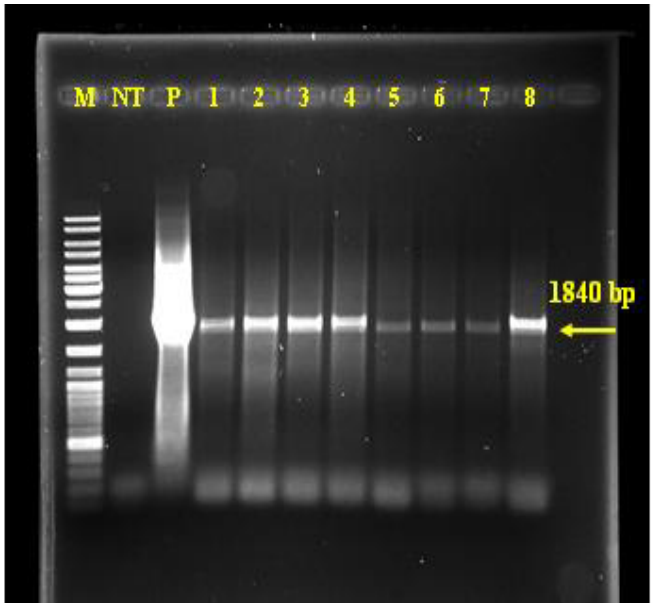

Figure 37: PCR analysis of genomic DNA from leaf tissues of a control non-transgenic plant (NT) and 8 T_0 plants derived from 8 independent cell lines transformed with the pSMW19 vector (*dapA* driven by 35S promoter). Genomic DNA and Plasmid pSMW19 (P) were used in PCR reactions with the specific primers Omega BamHI Fwd and OCS SacI Rev to amplify a 1840 bp fragment (*arrow*).

Southern blot analysis of T_0 transgenic plants derived from different transformation experiments

Southern blot of T_0 plants derived from cell lines transformed with the *dapA* and *lysC* genes confirmed the presence and integration of the transgenes into the genome of transformed plants, Figures 38, 39, 40, and 41.

An unexpected single copy integration pattern of the *lysC* gene was detected when genomic DNA of 4 individual plants derived from 4 independent transformed lines was digested by NotI, which cuts only one time inside the T-DNA between the *lysC* and *hpt* genes, Figure 38. To further investigate this result, a single digestion was performed using BglII (non-cutter of the T-DNA) and genomic DNA of 4 plants: 2 new plants C3-L4 (2), C2-L16 (4), and the previous tested plants C2-L2 (1) and C2-L4 (1). A single copy was detected in all tested plants of 4 independent lines in a big fragment (size ranged between 16 and 18 k bp.) as shown in Figure 38. This result confirmed the integration of a single copy of the *lysC* gene into the genome of barley.

Generally, southern blot analysis of the copy number of *lysC*, *dapA* and *hpt* genes showed that most of the tested plants carried one or two copies of the transgene and the highest number detected was 4 copies of *lysC* gene in the transgenic line B6-L2 (2) (see lane 8, Figure 41B, p 80).

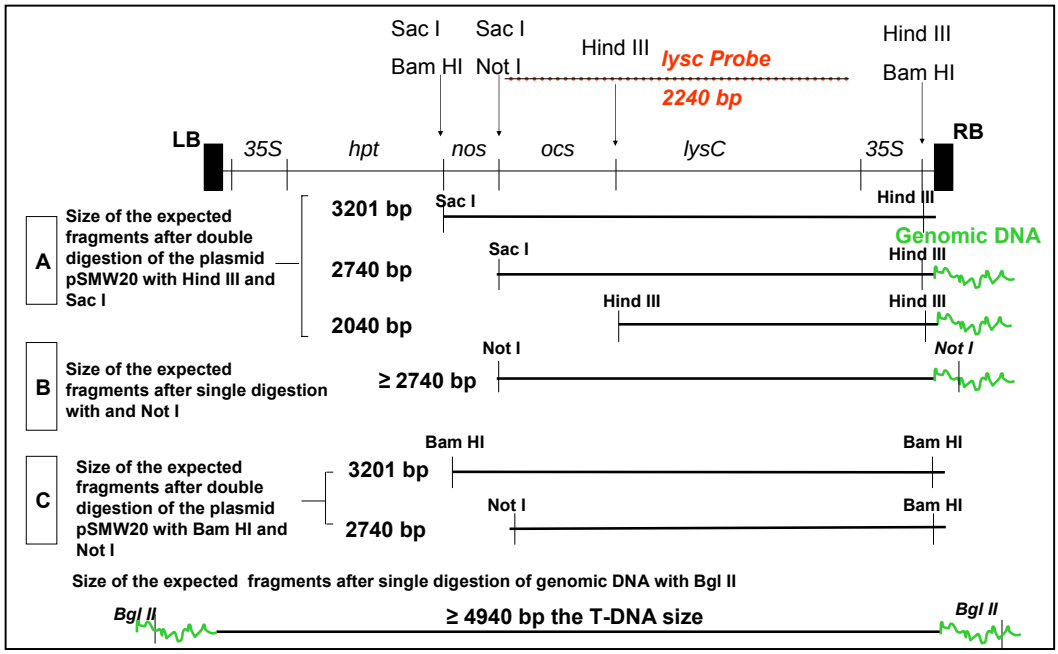

Figure 38A: Digestion of the transformation vector pSMW20 using different restriction enzymes to identify the transgenic T_0 lines and to detect the copy number of the *lysC* gene in transgenic plants.

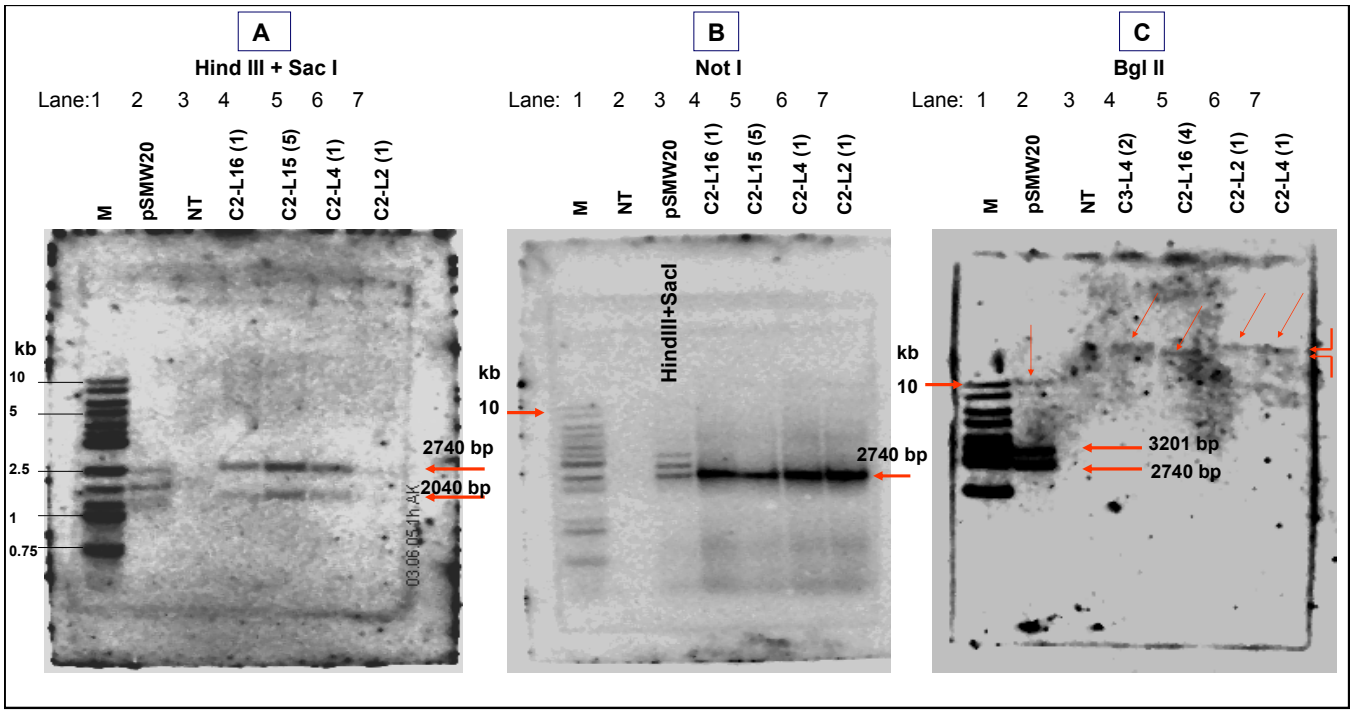

Figure 38B: Southern blot analysis of T_0 plants transformed with the *lysC* gene under the control of the 35S promoter. (A) Double digestion of genomic DNA was performed to identify the transgenic lines using HindIII and Sac I to cut the *lysC* gene from the T-DNA (two expected fragments; 2740bp fragment of the intact gene, and 2040 bp of the gene without the terminator *ocs*. (B) Single digestion of the genomic DNA of the transgenic lines was performed using NotI to detect the copy number of the *lysC* gene. (C) Lane 2 mixture of pSMW20 fragments (2 fragments derived from HindIII+SacI digestion and super coiled intact plasmid after digestion with BglII).The restriction enzyme BglII (non-cutter of the vector backbone or the T-DNA) was used to detect the copy number. A single copy was detected in all tested plants of 4 independent lines: 3 fragments in size of 18 k bp and 1 fragment in size of 16 kb in comparison to digested plasmid with BglII (fragment in super coiled form corresponding to the 10 kb of marker fragment).

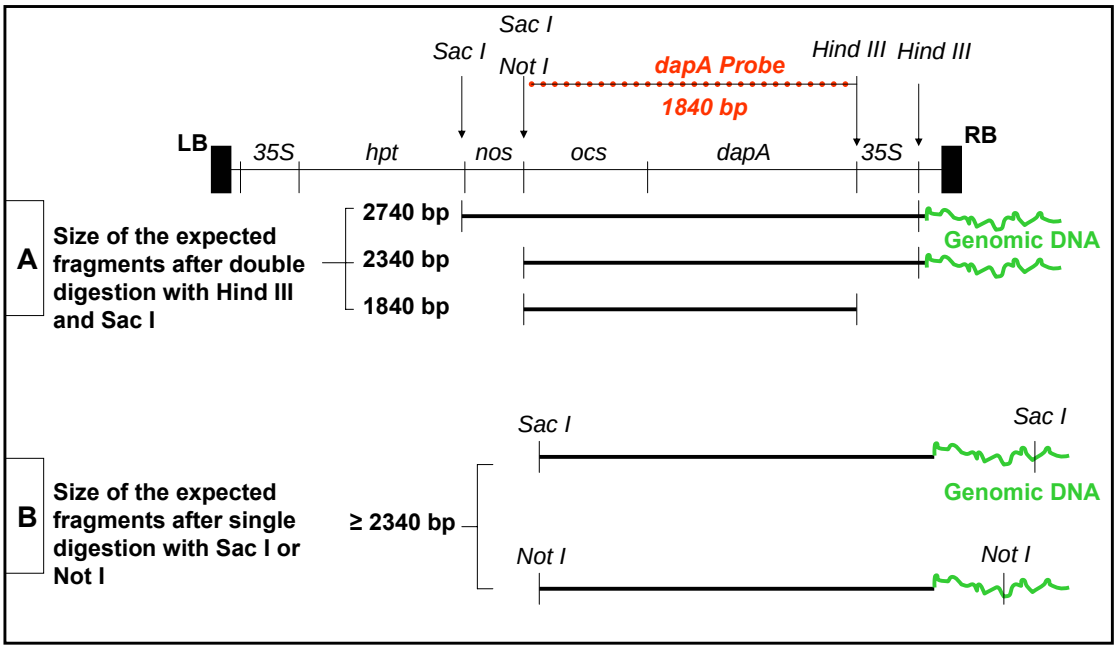

Figure 39A: Digestion of the transformation vector pSMW19 using different restriction enzymes to identify the transgenic T_0 lines and to detect the copy number of the *dapA* gene in transgenic plants.

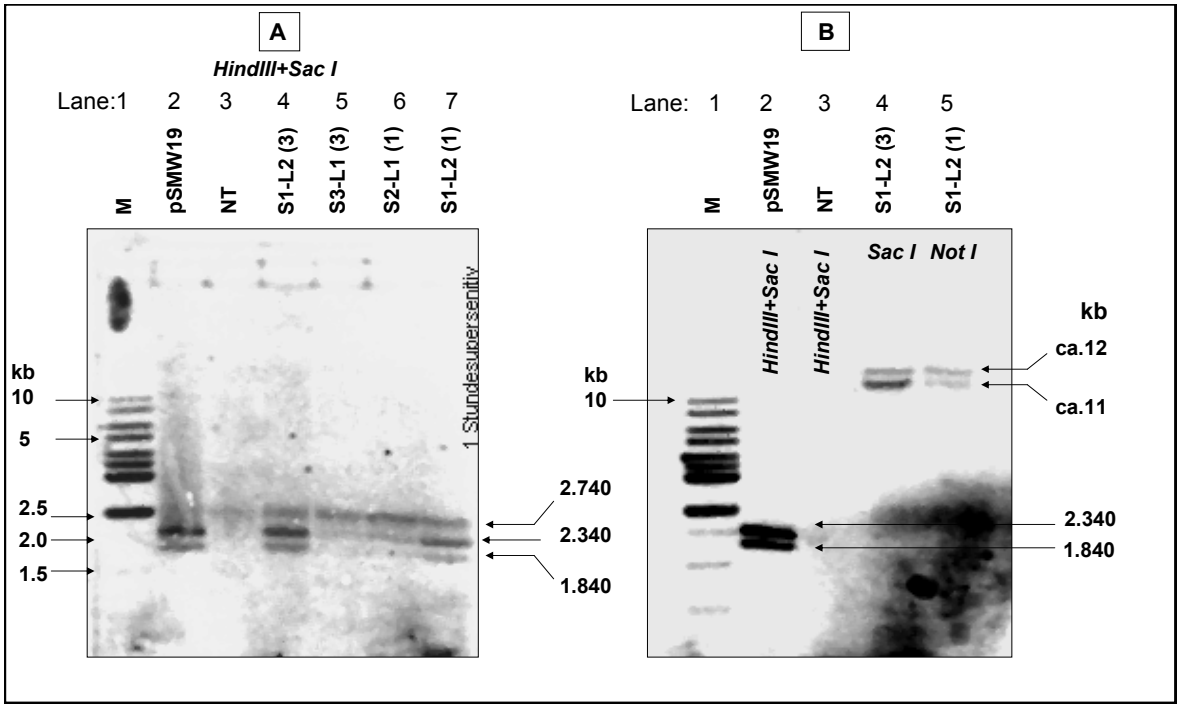

Figure 39B: Southern analysis of T_0 plants transformed with the *dapA* gene under control of the 35S promoter. (A) Double digestion of plasmid pSMW19 and genomic DNA was performed using HindIII and SacI to cut the *dapA* gene from the T-DNA three expected fragments different in size: *arrows* to identify the transgenic lines. (B) Two copies of *dapA* were detected in the transgenic line (S1-L2) in two individual plants (3) and (1) when single digestion of the genomic DNA was performed by SacI and NotI, respectively.

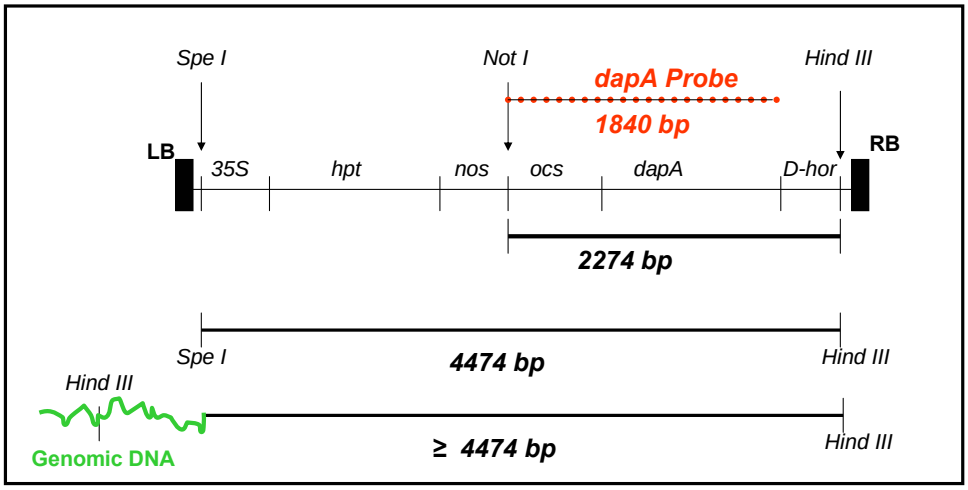

Figure 40A: Digestion of the transformation vector pSMW12 using different restriction enzymes to identify the transgenic T_0 lines and to detect the copy number of the *dapA* gene in transgenic plants.

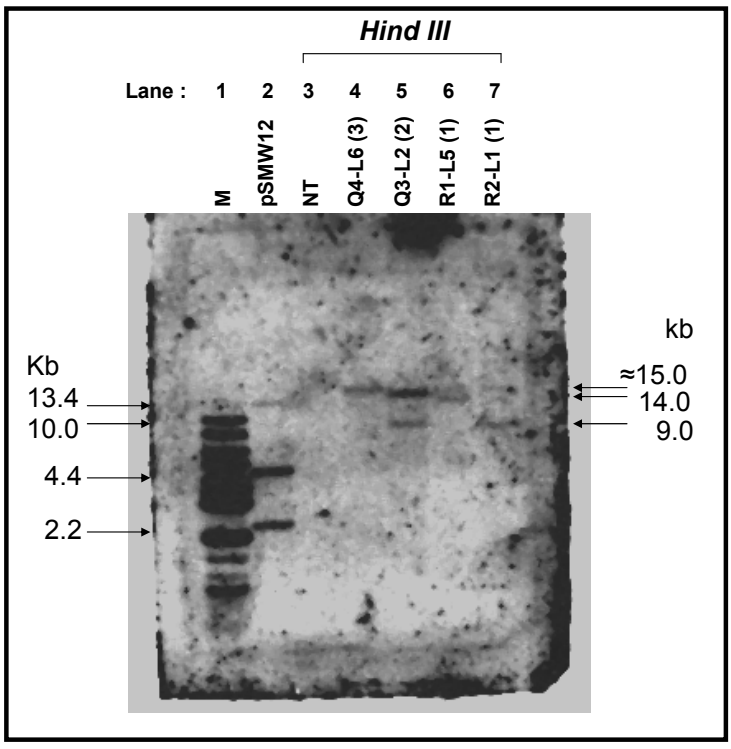

Figure 40B: Southern blot analysis of 2 T_0 plants transformed with the *dapA* gene under the control of the *D-hor* promoter (lane 4 and 5) and 2 plants transformed by *dapA* under the control of the *ubi1* promoter (lane 6 and 7). Single digestion of genomic DNA was performed using HindIII to identify the transgenic lines as well as the copy number of the *dapA* gene. Two copies of the *dapA* were detected in the transgenic lines Q3-L2 (2) and R2-L1 (1) lane 5 and 7, respectively. While the other two lines showed a single copy of the *dapA* gene in the genome of barley; lane 4 and 6. Plasmid vector pSMW12 was digested by HindIII (13.474 kb = opened vector), by HindIII + SpeI (4.474 kb = intact T-DNA), and by HindIII + NotI (2.274 kb = *dapA* gene).

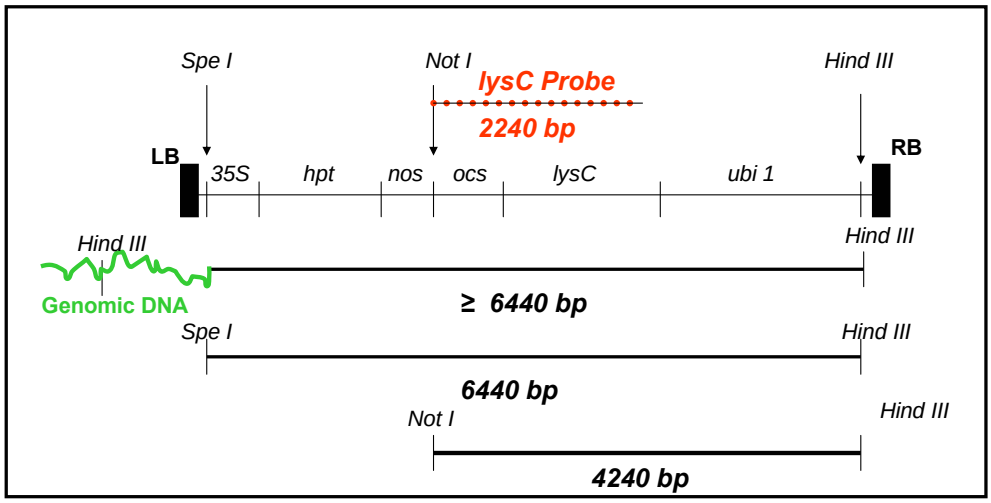

Figure 41A: Digestion of the transformation vector pSMW18 using different restriction enzymes to identify the transgenic T_0 lines and to detect the copy number of the *lysC* gene in transgenic plants.

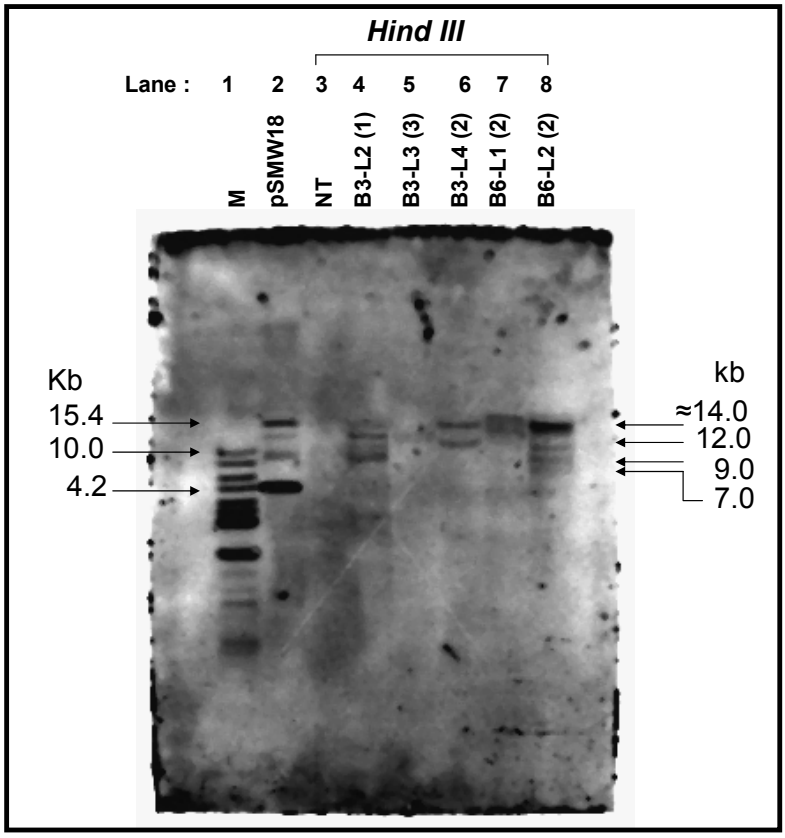

Figure 41B: Southern blot analysis of 5 T_0 plants transformed with the *lysC* gene under the control of the *ubi1* promoter. The plasmid vector pSMW18 in lane 2 digested by HindIII (15.440 kb = opened vector), by HindIII + SpeI (6.440 kb = intact T-DNA) not detected, and by HindIII + NotI (4.240 kb = *lysC* gene). Single digestion of genomic DNA was performed using HindIII to identify the transgenic lines as well as the copy number of *dapA* gene. 4 copies of the *lysC* were detected in the transgenic line B6-L2 (2). 2 copies were detected in 3 lines (lane 4, 6 and 7). Single copy was detected in the line B3-L3 (3) lane 5.

Southern blot for *hpt* gene

The presence and integration of the *hpt* gene in the genome of T_0 transgenic plants was confirmed by Southern blot analysis of some representative plants derived from cell lines transformed either by *dapA* and *lysC* constructions as shown in Figure 42.

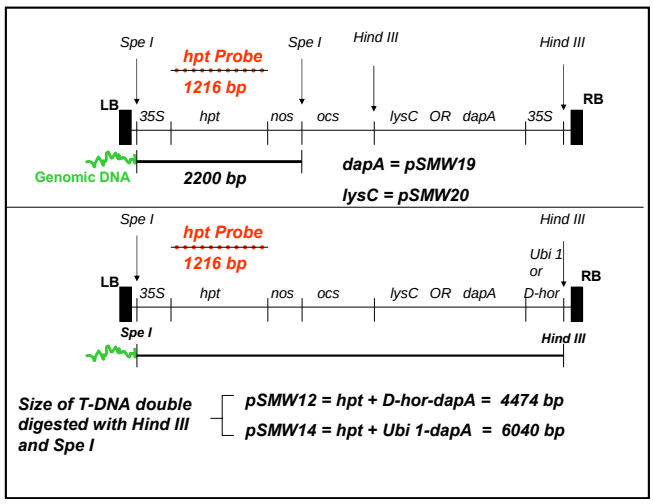

Figure 42A: Digestion of the transformation vector pSMW19 and pSMW20 with SpeI (above 2.2kb). Double digestion with HindIII and SpeI of different transformation vectors pSMW12 and pSMW14 to detect the *hpt* gene in transgenic plants.

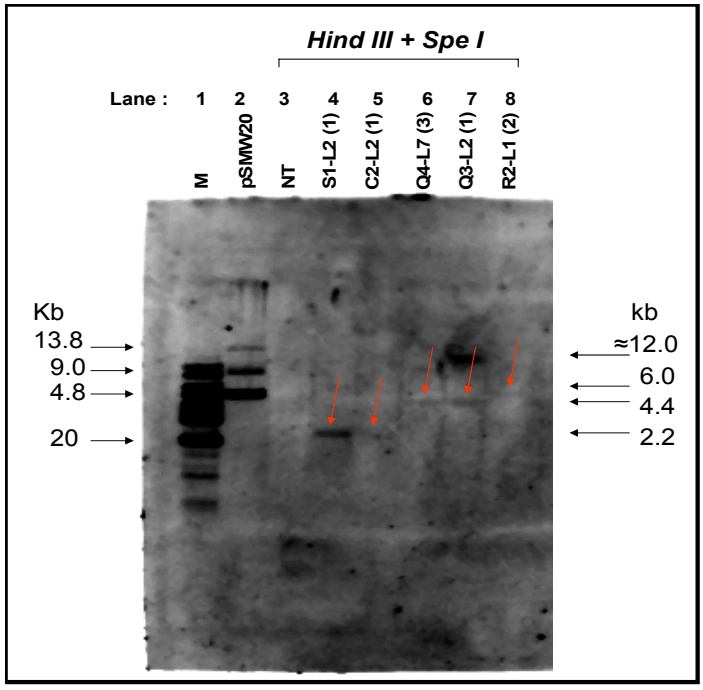

Figure 42B: Southern blot analysis of 5 T_0 transgenic plants transformed by different vectors including the *hpt* gene. Double digestion of genomic DNA was performed using HindIII + SpeI to identify the transgenic lines containing the *hpt* gene. 2 copies of the *hpt* were detected in the transgenic line Q3-L2 (1) lane 7. Single copies were detected in 4 lines. The plasmid vector pSMW16 in lane 2: a mixture of different fragments derived from different digestion reactions using HindIII: 13.874 kb = opened vector, HindIII + SpeI: 4.874 kb = intact T-DNA. In lanes 4 and 5 the intact *hpt* gene was isolated in 2.200 kb fragment (SpeI –SpeI) from transgenic plants transformed with the vectors pSMW19 and pSMW20. Intact T-DNA was isolated from plants transformed with the vectors pSMW12 (lane 6 and 7 = 4.474 kb) and pSMW14 (lane 8 = 6.040 kb).

Inheritance of the *dapA* and *lysC* genes in the T_1 generation

So far, inheritance of the introduced genes *dapA* and *lysC* has been analyzed using PCR reactions with the progeny of the T_0 transgenic plants transformed with the vectors pSMW12, pSMW14, pSMW19 and pSMW20 Figure 43, p. 83. The *lysC* gene was screened in 60 plants of the T_1 generation of 6 transgenic plants representing 5 (T_0) individual lines as shown in Table 23 and Figure 44A, p. 83. For the *dapA* gene 221 plants of the T_1 generation of 21 transgenic plants representing 13 (T_0) individual lines were screened as shown in Table 23 and Figure 44B, C. Data in Table 23 showed that although the segregation ratio for the progeny of most of the T_0 transgenic plants is different than 3:1, the ratio between the total number of positive plants and the negative plants is about 3:1. This may point to the fact that many of the T_0 transgenic plants carry a single copy of the transgene.

Table 23: Segregation of the *lysC and dapA* genes in T_1 generation

Name of transgenic T_0 plant	Construction name	No. of T_1 plants tested by PCR	Ratio positive : negative
C2-L2 (1)	pSMW20	15	15 : 0
C2-L4 (1)		8	8 : 0
C2-L8 (1)		9	5 : 4
C2-L15 (5)		6	6 : 0
C2-L16 (1)		10	7 : 3
C2-L16 (3)		12	11 : 1
S1-L2 (3)	pSMW19	7	7 : 0
S2-L1 (1)		5	4 : 1
S3-L1 (3)		9	4 : 5
S3-L3 (1)		2	1 : 1
S4-L7 (1)		3	1 : 2
S4-L12 (1)		11	8 : 3
Q3-L2 (1)	pSMW12	16	16 : 0
Q3-L2 (2)		28	12 : 16
Q4-L2 (2)		13	8 : 5
Q4-L3 (1)		15	12 : 3
Q4-L4 (1)		30	22 : 8
Q4-L5 (1)		23	18 : 5
Q4-L5 (2)		5	5 : 0
Q4-L5 (3)		9	8 : 1
Q4-L6 (1)		8	4 : 4
Q4-L6 (2)		2	2 : 0
Q4-L6 (3)		8	8 : 0
Q4-L6 (4)		13	10 : 3
Q4-L7 (1)		8	4 : 4
R2-L1 (1)	pSMW14	4	4 : 0
R2-L1 (2)		2	2 : 0
Total		281	212 : 69 = (3 : 1)

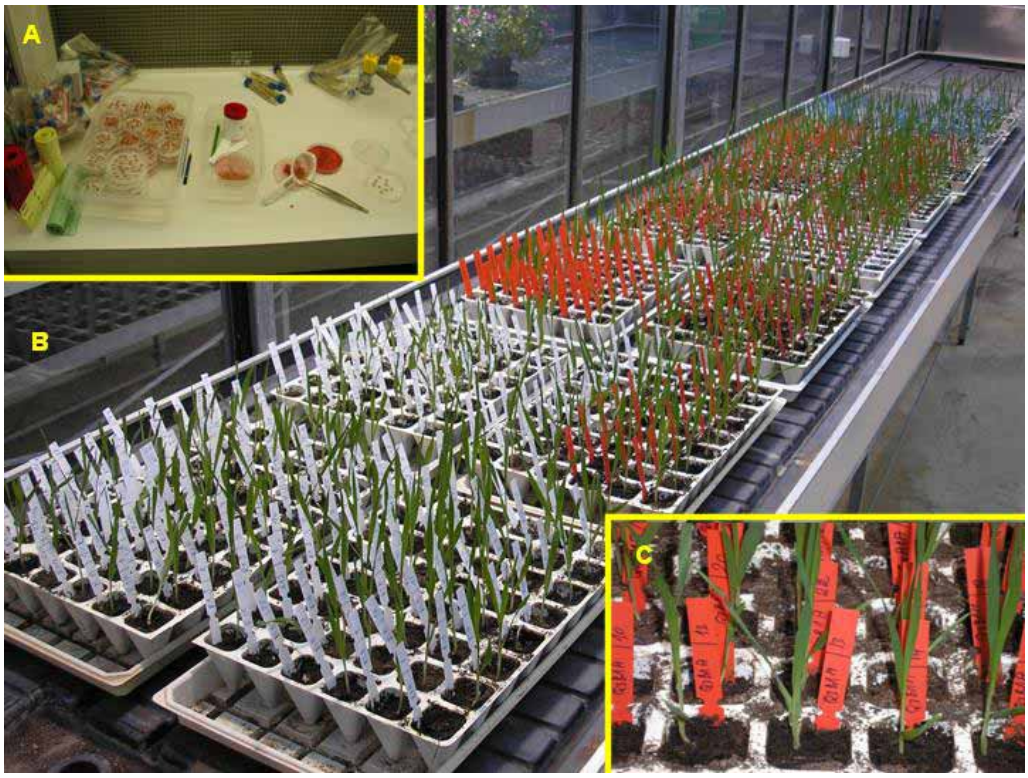

Figure 43: Seedlings of the T_1 generation of different transgenic T_0 lines. (A) Preparation of grains derived from each transgenic T_0 Plant for germination. (B) Seedlings 2 week-old in green house. (C) Seedlings of the T_0 transgenic plant Q3-L1 (1) transformed with *dapA* under the control of *D-hor* promoter.

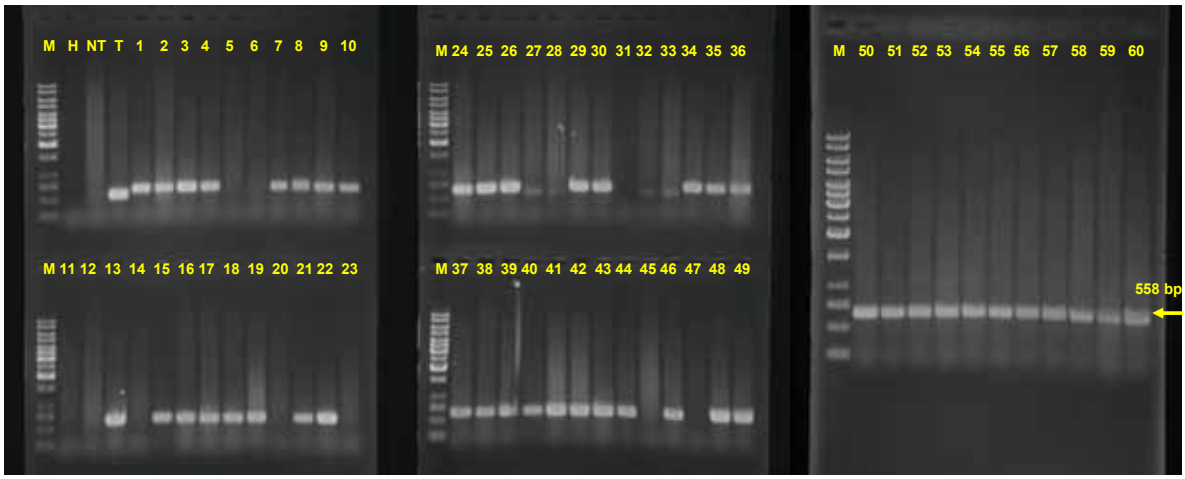

Figure 44A: PCR analysis of genomic DNA from leaf tissues of (NT) non-transgenic control plant, (T) transgenic T_0 plant B3-L3 (1), (H) H_2O and 60 T_1 plants derived from 6 individual T_0 plants of 5 independent cell lines transformed with pSMW20 vector (*lysC* driven by 35S promoter). PCR reactions were performed using the specific primers AK Fwd 997 and OCS Rev 190 to amplify the 558 bp *lysC* fragment (*arrow*). (Note): The positive control used is the transgenic T_0 plant B3-L3 (1) and the size of the amplified fragment is 508 bp; about 50 bp less than the tested plants which have a polylinker between the coding region of *lysC* and the terminator *ocs*, while this polylinker was omitted from the construction of the vector (pSMW18).

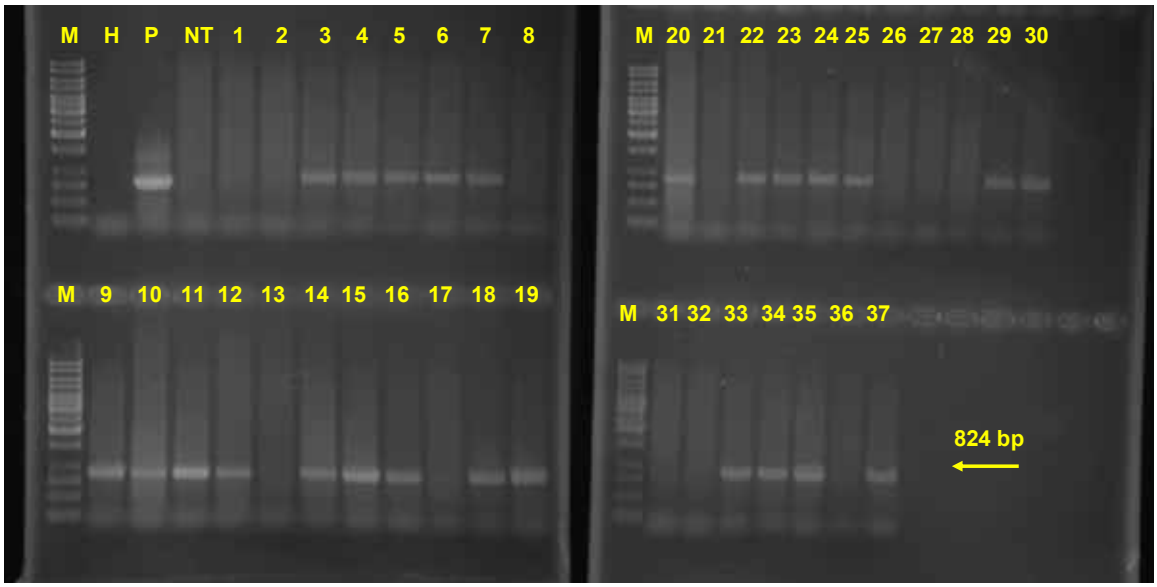

Figure 44B: PCR analysis of genomic DNA from leaf tissues of (NT) control non-transgenic, (P) plasmid pSMW19, (H) H_2O and 37 T_1 plants derived from 6 individual T_0 plants of 6 independent cell lines transformed by pSMW19 vector (*dapA* driven by 35S promoter). PCR reactions were performed using the specific primers DHPS Fwd 331 and OCS Rev 190 to amplify the 824 bp fragment (*arrow*).

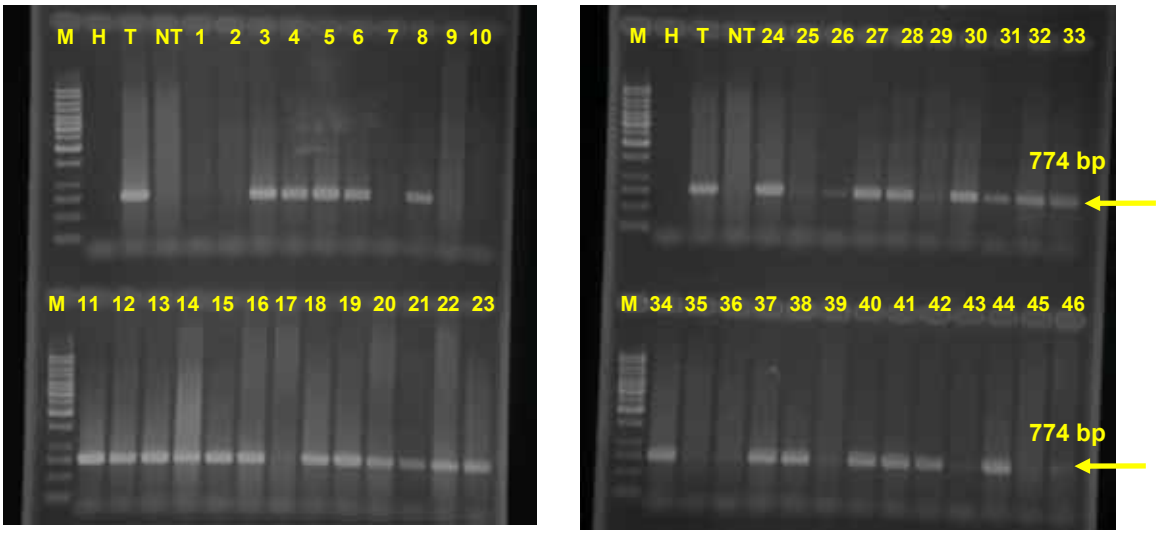

Figure 44C: PCR analysis of genomic DNA from leaf tissues of (NT) control non-transgenic plant, (T) transgenic T_0 plant Q10-L1 (1), (H) H_2O and 46 T_1 plants derived from 4 individual transgenic T_0 plants of 4 independent cell lines transformed by pSMW12 vector (*dapA* driven by *D-hor* promoter). PCR reactions were performed using the specific primers DHPS Fwd 331 and OCS Rev 190 to amplify the 774 bp *dapA* fragment (*arrow*).

3.4. Amino acid composition in T_0 plants

The percentage of 13 free amino acids was determined in the leaves of 50 transgenic T_0 plants transformed either with the *dapA* (DHDPS) or *lysC* (AK) gene (Table 24). For DHDPS leaves of 23 T_0 plants were analyzed for their free amino acid composition, 5 plants transformed with the *dapA* driven by the 35S promoter and 18 plants with *dapA* driven by the *ubi1* promoter. For AK leaves of 27 T_0 transgenic plants transformed with the *lysC* gene (10 plants with 35S-*lysC* and 17 plants with *ubi1-lysC*) were analysed for their free amino acid composition in comparison to the control non-transgenic plants (NT) at the same developmental stage (2 months old plants).

The results for free lysine in leaves of transgenic plants transformed with *dapA* under the control of the *ubi1* promoter showed a significant increase in free lysine ranging between 2 and 5-fold compared to the non-transgenic control plants (Table 24, Figure 45). In transgenic plants transformed with the *dapA* gene driven by the 35S promoter the level of lysine was increased in one plant (S3-L1-3) up to 2.5-fold. Moreover, the percentage of free lysine in transgenic plants transformed with the *lysC* gene was increased up to 2.2-fold in plant (C2-L8-1) for the *35S* construction and about 1.9-fold in plant (B3-L5-1) for the *ubi 1* construction (Table 24, Figure 45).

The percentage of free threonine was reduced in transgenic plants transformed with the *dapA* gene driven by the *ubi1* promoter which exhibited a high level of free lysine (Figure 45), while in transgenic plants transformed with *dapA* driven by the *35S* promoter a slight increase was detected up to 2-fold in plant S3-L1(3). On the other hand, the level of threonine was increased slightly up to 1.6-fold in transgenic plants transformed with the *lysC* gene driven by the *ubi1* promoter (Figure 45), while no changes were detected in transgenic plants transformed with *lysC* driven by the 35S promoter compared to the control plants.

The level of free serine was increased in 5 transgenic plants out of 18 plants transformed with the *dapA* gene driven by the *ubi1* promoter up to 5.8-fold in plant R3-L4 (1) (Figure 46), while a slight decrease was detected in transgenic plants transformed with the *dapA* driven by the 35S promoter. In addition, transgenic plants transformed with *lysC* driven by *ubi1* showed an increase in the percentage of free serine up to 4-fold, while a slight decease was detected in transgenic plants transformed with *lysC* driven by the 35S

promoter in comparison to the control plants. An increase in free alanine was detected in transgenic plants transformed with the *dapA* driven by the *ubi1* promoter up to 3-fold in plant R3-L4 (1), (Figure 46), while about 50% reduction was detected in transgenic plants transformed with *dapA* driven by the 35S promoter in comparison to the control plants. Moreover, the level of free alanine was decreased in transgenic plants transformed by the *lysC* gene either driven by *ubi1* or 35S in comparison to the control plants.

In transgenic plants transformed either with *dapA* or *lysC* both driven by the *ubi1* promoter the percentage of free glutamine was decreased, (Figure 47), while in transgenic plants transformed with the *dapA* or *lysC* gene under the control of the *35S* promoter a slight increase was detected in free glutamine compared to the control plants. The percentage of free asparagine was increased up to 2-fold in transgenic plants transformed either with the *dapA* or *lysC* gene in comparison to the control plants, (Figure 47). The level of free isoleucine was increased in some transgenic plants transformed either with the *dapA* or *lysC* gene up to 2-fold in comparison to the control plants, (Figure 48).

The percentage of free leucine was increased in transgenic plants transformed with the *dapA* gene up to 2.5-fold in comparison to the control plants, (Figure 48). While it was decreased in most of the transgenic plants transformed with the *lysC* gene, except plant C2-L8 (1) with an increase up to 2-fold. The level of free phenylalanine was increased in some transgenic plants transformed with the *dapA* gene up to 2.4-fold compared to the control plants, (Figure 49). In transgenic plants transformed with the *lysC* gene an increase was detected in some plants up to 2.6-fold.

The level of free valine was increased in some transgenic plants transformed with the *dapA* gene up to 2.6-fold compared to the control plants, Figure 49. Moreover, in transgenic plants transformed with the *lysC* gene an increase was detected in some plants up to 1.8-fold. An increase was detected in free histidine in transgenic plants transformed either with the *dapA* or *lysC* gene under the control of *ubi1* promoter up to 4.7-fold in comparison to the control plants. In some transgenic plants transformed with the *dapA* gene the percentage of free arginine was increased up to 1.7-fold in comparison to the control plants, (Figure 50). A slight decrease was detected in transgenic plants transformed with the *lysC* gene. The percentage of free proline was increased in some transgenic plants transformed either with the *dapA* or *lysC* gene up to 5-fold in comparison to the control plants (Figure 51).

Table 24: Percentage of free amino acids in control plants and T_0 transgenic barley plants transformed with the *dapA* (DHDPS) gene or *lysC* (AK) gene

Samlpe No.	Gene & Promoter	Sample Name	Free Amino Acid %												
			ASP	THR	SER	GLU	PRO	ALA	VAL	ILE	LEU	PHE	HIS	LYS	ARG
1	***dapA***	S3-L1 (3)	0.226	0.127	0.196	0.821	0.150	0.064	0.059	0.024	0.035	0.018	n.d	0.036	n.d
2	**(DHDPS)**	S3-L2 (3)	0.233	0.084	0.158	0.667	n.d	0.048	0.040	n.d	n.d	n.d	n.d	0.016	n.d
3	**driven by**	S2-L1 (1)	0.209	0.083	0.157	0.636	n.d	0.055	0.029	0.021	0.029	0.016	n.d	0.015	n.d
4	***35S***	S1-L2 (1)	0.265	0.100	0.136	0.600	n.d	0.061	0.039	n.d	n.d	n.d	n.d	0.009	n.d
5		S1-L2 (3)	0.227	0.072	0.137	0.617	n.d	0.053	0.033	n.d	n.d	n.d	n.d	0.016	n.d
6		R1-L1 (1)	0.283	0.097	0.238	0.501	0.054	0.209	0.022	n.d	n.d	n.d	n.d	n.d	n.d
7		R1-L2 (1)	0.143	0.057	0.090	0.413	0.504	0.164	0.036	0.020	0.025	0.022	0.018	0.023	n.d
8		R1-L3 (1)	0.275	0.081	0.200	0.685	n.d	0.161	0.026	0.010	0.016	0.015	n.d	0.012	n.d
9		R1-L4 (1)	0.185	0.075	0.199	0.496	0.037	0.191	0.025	0.010	0.016	n.d	0.080	0.012	n.d
10		R1-L5 (1)	0.139	0.043	0.054	0.383	0.579	0.142	0.025	n.d	n.d	n.d	n.d	n.d	n.d
11	***dapA***	R1-L6 (1)	0.134	0.047	0.049	0.418	0.672	0.128	0.025	n.d	n.d	0.017	0.012	n.d	n.d
12	**(DHDPS)**	R1-L7 (1)	0.116	0.067	0.107	0.389	0.168	0.173	0.020	n.d	n.d	n.d	n.d	n.d	n.d
13	**driven by**	R2-L1 (1)	0.121	0.062	0.271	0.362	0.294	0.181	0.035	0.016	0.019	0.015	0.030	0.053	0.054
14	***ubi 1***	R2L1 (2)	0.111	0.055	0.169	0.325	0.278	0.168	0.037	0.020	0.030	0.024	0.026	0.049	0.042
15		R2-L2 (1)	0.147	0.069	0.147	0.480	0.052	0.183	0.028	0.018	0.021	n.d	n.d	n.d	n.d
16		R3-L1 (1)	0.169	0.058	0.193	0.509	n.d	0.088	0.021	n.d	0.012	0.011	0.013	0.010	n.d
17		R3-L2 (1)	0.234	0.075	0.592	0.498	0.088	0.115	0.024	0.012	0.013	n.d	0.044	0.024	0.065
18		R3-L3 (1)	0.194	0.035	0.432	0.415	0.216	0.114	0.024	n.d	n.d	n.d	0.028	0.021	0.023
19		R3-L4 (1)	0.212	0.050	1.236	0.485	0.030	0.368	0.025	n.d	0.016	0.013	0.048	0.052	0.072
20		R3-L5 (1)	0.173	0.038	0.249	0.541	0.048	0.219	0.015	n.d	n.d	n.d	0.011	0.016	n.d
21		R3-L5 (2)	0.203	0.052	0.216	0.584	0.021	0.214	0.018	n.d	0.011	0.013	0.026	0.029	n.d
22		R3-L5 (3)	0.258	0.047	0.555	0.559	0.016	0.078	0.022	0.010	0.012	n.d	0.059	0.070	0.056
23		R3-L6 (1)	0.264	0.045	0.665	0.505	0.876	0.122	0.051	0.021	0.011	0.029	0.052	0.037	0.039
24		C2-L2 (1)	0.238	0.058	0.190	0.691	n.d	0.080	0.015	n.d	0.011	n.d	0.011	0.011	n.d
25		C2-L4 (1)	0.266	0.056	0.191	0.664	n.d	0.070	0.015	n.d	n.d	n.d	n.d	0.010	n.d
26		C2-L5 (1)	0.261	0.063	0.190	0.710	n.d	0.083	0.015	n.d	0.012	n.d	n.d	n.d	n.d
27	***lysC***	C2-L5 (2)	0.208	0.060	0.197	0.667	n.d	0.050	0.016	n.d	n.d	n.d	n.d	0.013	n.d
28	**(AK)**	C2-L8 (1)	0.168	0.067	0.136	0.585	0.680	0.088	0.040	0.022	0.030	0.018	0.012	0.032	n.d
29	**driven by**	C2-L15 (1)	0.242	0.058	0.213	0.684	n.d	0.070	0.021	n.d	0.013	0.015	n.d	0.013	n.d
30	***35S***	C2-L16 (1)	0.217	0.051	0.186	0.669	n.d	0.039	0.015	n.d	n.d	n.d	n.d	n.d	n.d
31		C2-L16 (3)	0.211	0.066	0.182	0.765	n.d	0.074	0.021	0.010	0.015	n.d	n.d	0.018	n.d
32		C2-L16 (4)	0.248	0.066	0.159	0.614	n.d	0.114	0.015	n.d	n.d	n.d	n.d	n.d	n.d
33		C3-L4 (2)	0.238	0.067	0.169	0.649	n.d	0.082	0.014	n.d	n.d	n.d	n.d	n.d	n.d
34		B3-L1 (1)	0.242	0.092	0.614	0.477	n.d	0.128	0.030	0.012	n.d	0.016	0.031	0.019	0.016
35		B3-L1 (2)	0.229	0.075	0.648	0.460	0.032	0.111	0.030	0.011	0.011	0.019	0.056	0.017	0.039
36		B3-L1 (3)	0.215	0.066	0.764	0.396	n.d	0.137	0.041	0.018	0.011	0.020	0.062	0.024	0.039
37		B3-L2 (1)	0.245	0.101	0.277	0.615	n.d	0.092	0.021	n.d	n.d	n.d	0.018	0.014	0.019
38		B3-L3 (1)	0.285	0.076	0.040	0.500	0.013	0.085	0.029	0.012	0.016	n.d	0.037	0.016	0.025
39	***lysC***	B3-L3 (2)	0.235	0.087	0.280	0.474	0.019	0.079	0.023	n.d	n.d	0.012	0.026	0.015	n.d
40	**(AK)**	B3-L3 (3)	0.264	0.100	0.567	0.502	0.077	0.094	0.026	0.012	0.011	0.013	0.039	0.018	0.030
41	**driven by**	B3-L4 (1)	0.293	0.074	0.280	0.615	0.129	0.105	0.027	0.011	0.012	0.013	0.019	0.015	n.d
42	***ubi 1***	B3-L4 (2)	0.263	0.080	0.340	0.543	n.d	0.119	0.023	n.d	n.d	0.013	0.022	0.015	0.019
43		B3-L4 (3)	0.230	0.066	0.239	0.446	0.015	0.101	0.022	n.d	0.011	0.013	0.024	0.015	n.d
44		B3-L5 (1)	0.236	0.104	0.804	0.437	0.445	0.130	0.040	0.022	0.013	0.020	0.056	0.027	0.028
45		B3-L6 (1)	0.193	0.064	0.574	0.363	0.497	0.127	0.041	0.019	0.012	0.024	0.057	0.019	0.029
46		B3-L7 (1)	0.263	0.082	0.911	0.461	n.d	0.111	0.040	0.021	0.016	0.032	0.070	0.026	0.033
47		B6-L1 (1)	0.240	0.074	0.578	0.524	0.013	0.103	0.028	0.011	0.012	0.016	0.024	0.017	0.026
48		B6-L1 (2)	0.221	0.101	0.592	0.523	0.252	0.102	0.026	n.d	n.d	0.017	0.024	0.015	0.022
49		B6-L2 (1)	0.245	0.086	0.500	0.557	0.520	0.110	0.030	0.013	n.d	0.015	0.023	0.016	n.d
50		B6-L2 (2)	0.182	0.093	0.341	0.431	0.119	0.094	0.024	0.012	n.d	0.013	0.027	0.019	n.d
Control (Non-Transgenic)															
Mean of 14 plants =			0.132	0.063	0.211	0.529	0.172	0.118	0.022	0.011	0.014	0.012	0.017	0.014	0.041
Standard deviation (SD ±) =			0.015	0.007	0.063	0.028	0.132	0.021	0.005	0.001	0.002	0.002	0.008	0.004	0.002

Values are the means of double measures of two independent extractions (the two values lie within 10% deviation).n.d = not detected.

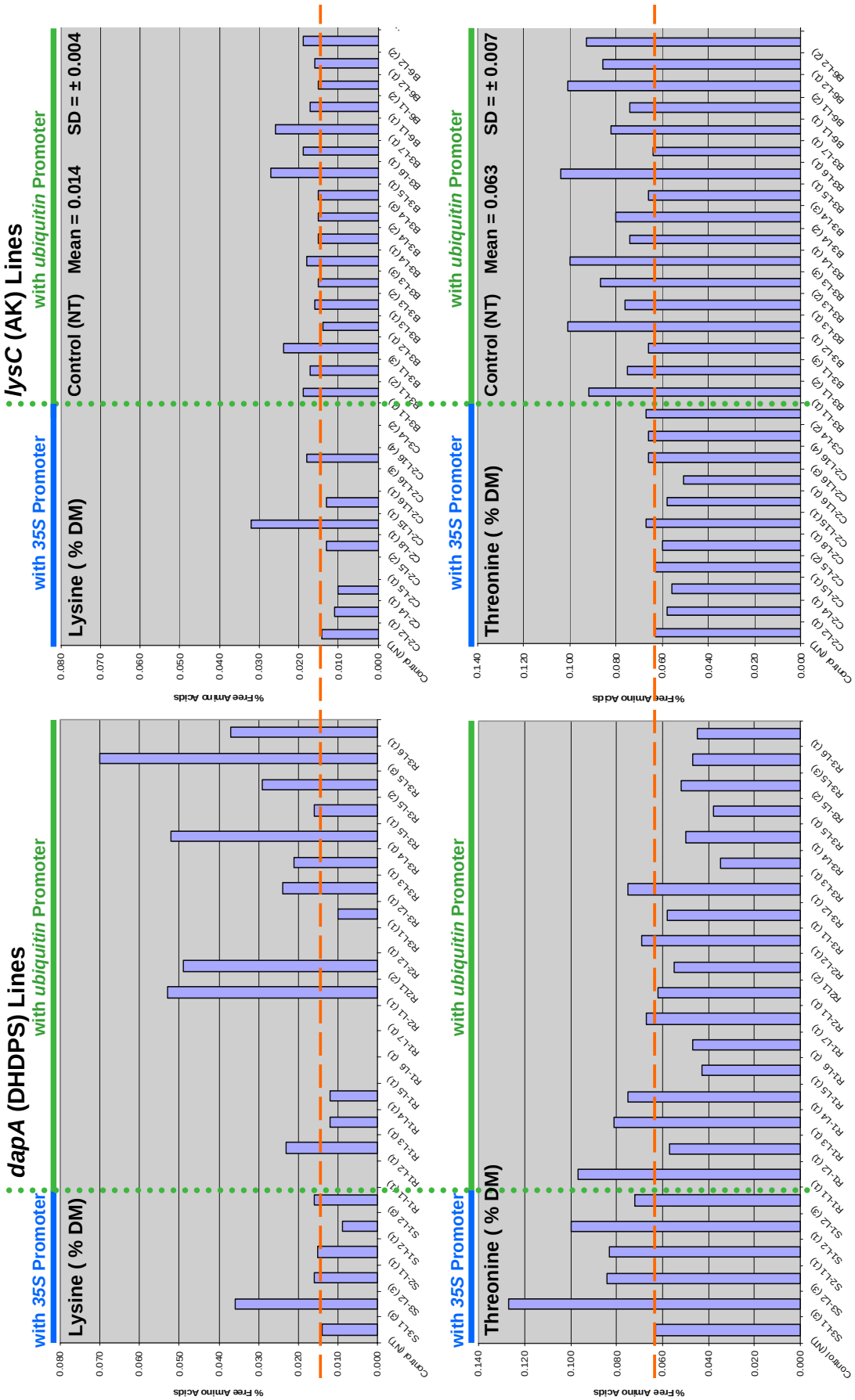

Figure 45: Percentage of free lysine and threonine in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

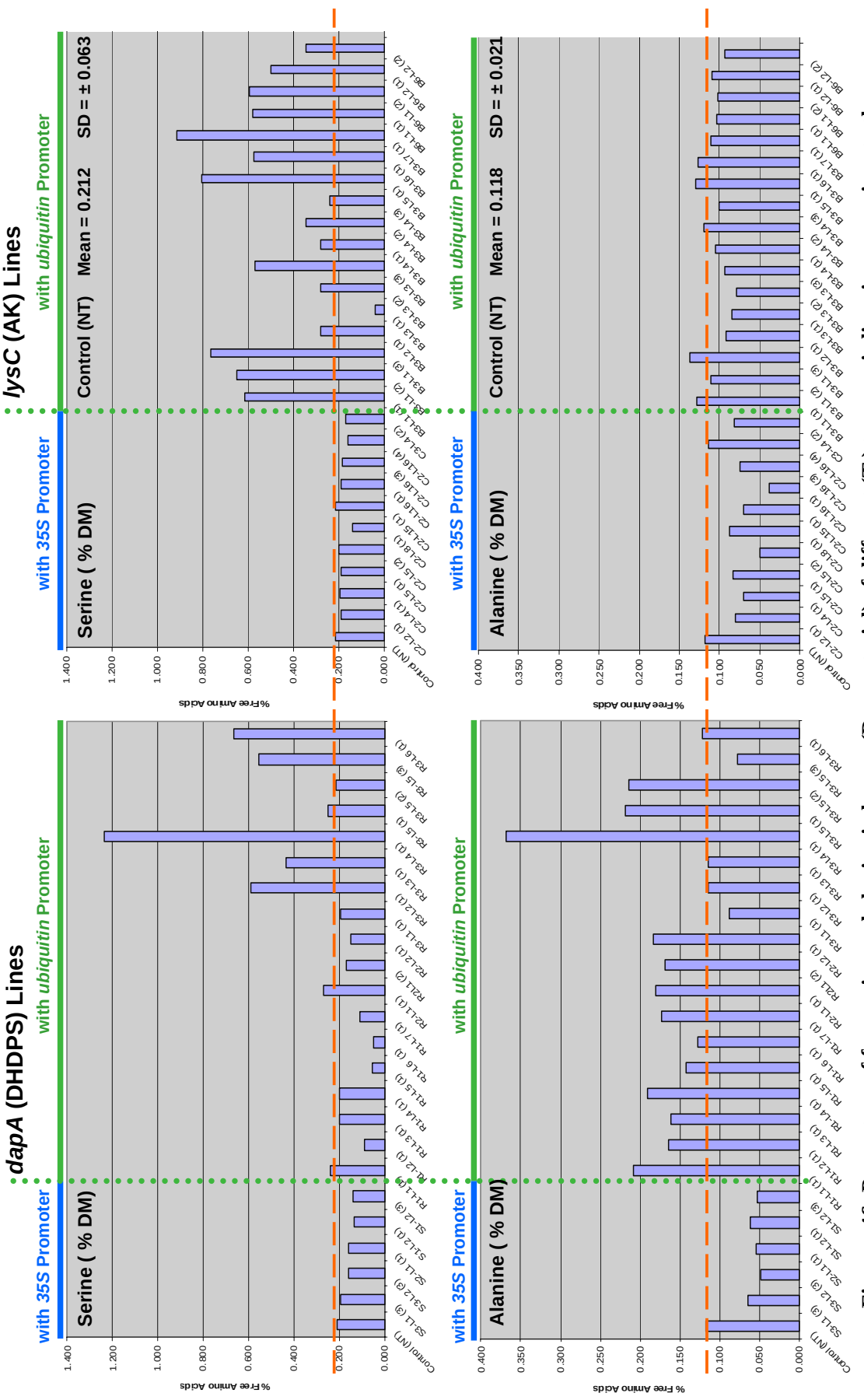

Figure 46: Percentage of free serine and alanine in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

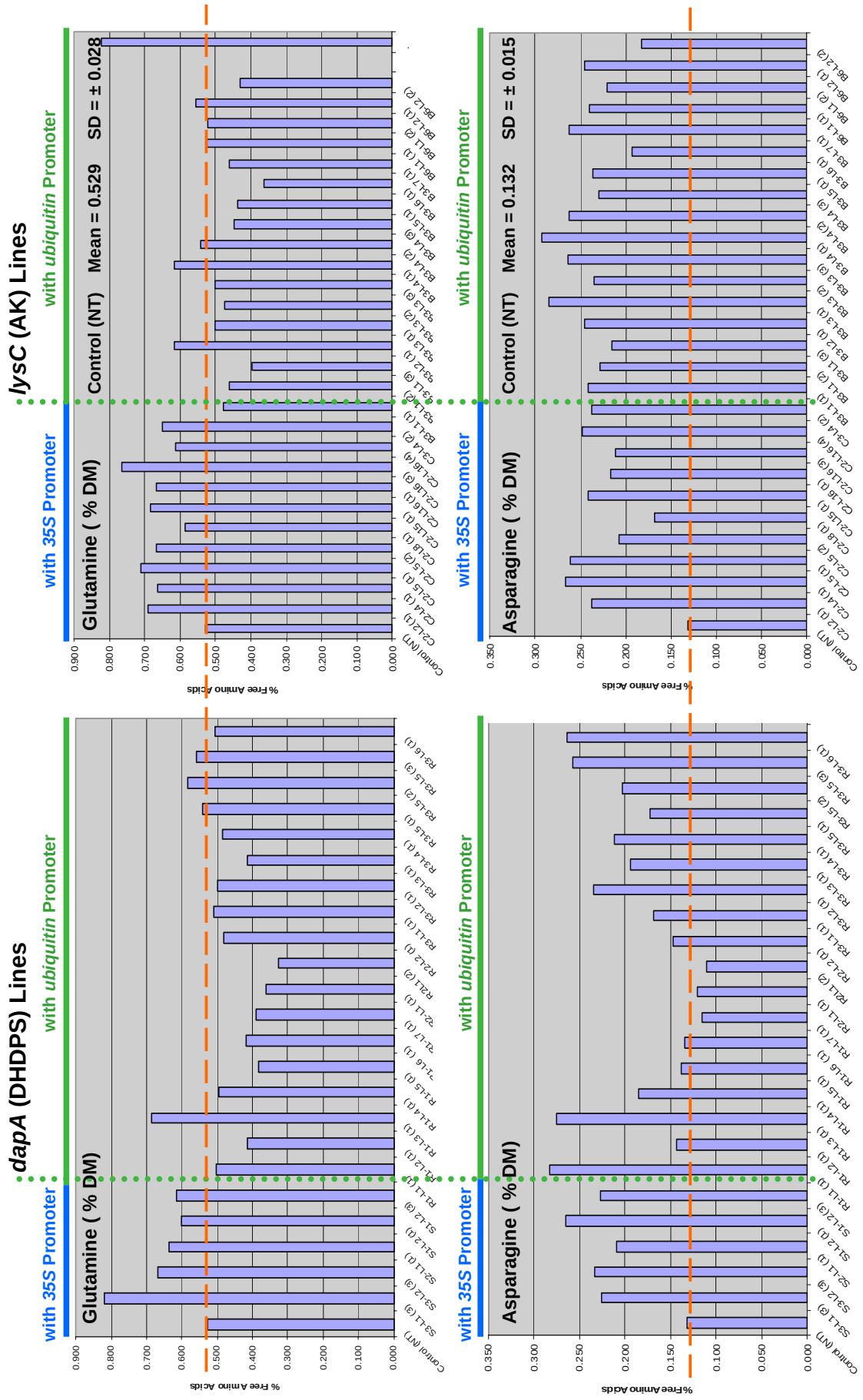

Figure 47: Percentage of free glutamine and asparagine in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

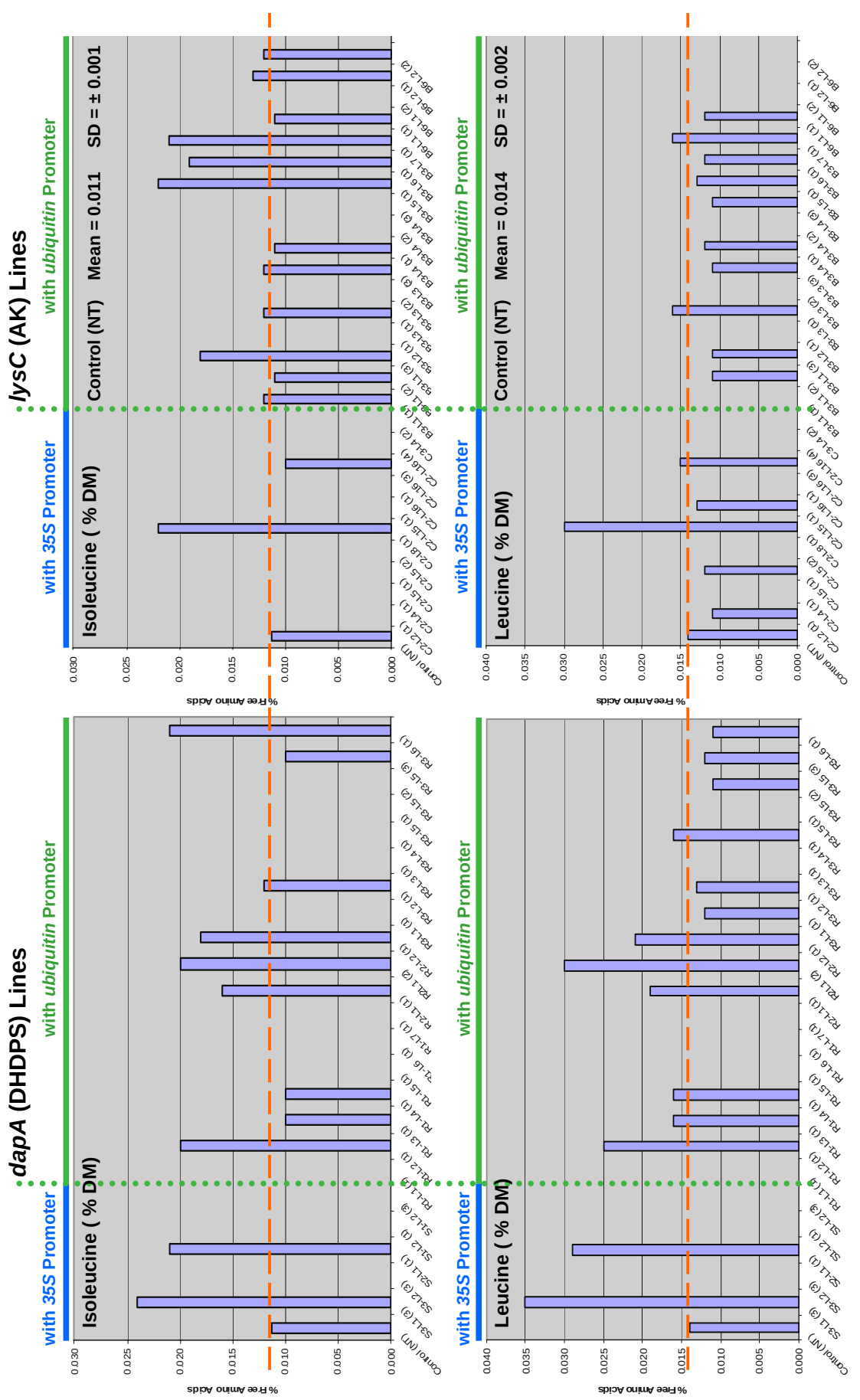

Figure 48: Percentage of free isoleucine and leucine in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

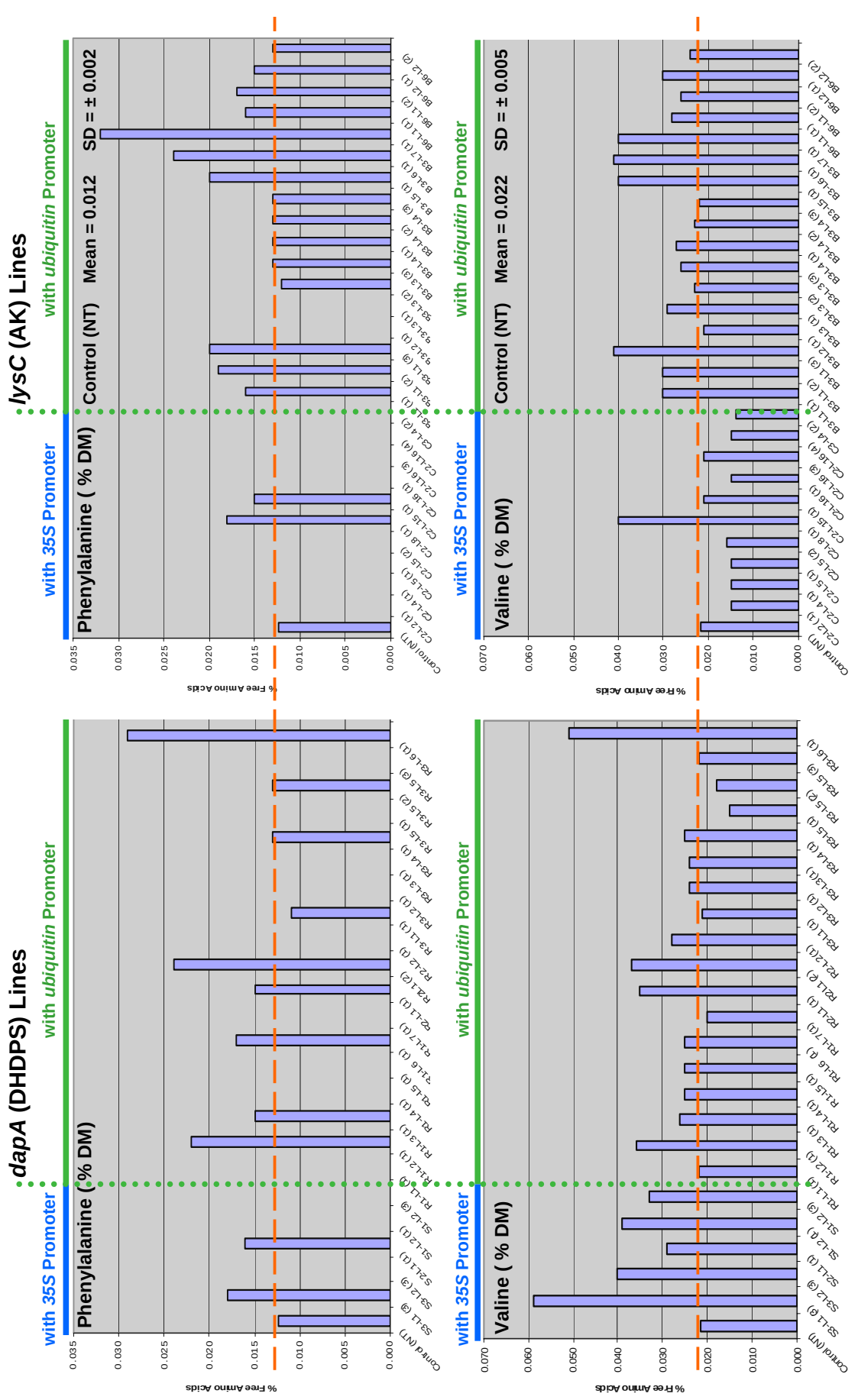

Figure 49: Percentage of free phenylalanine and valine in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

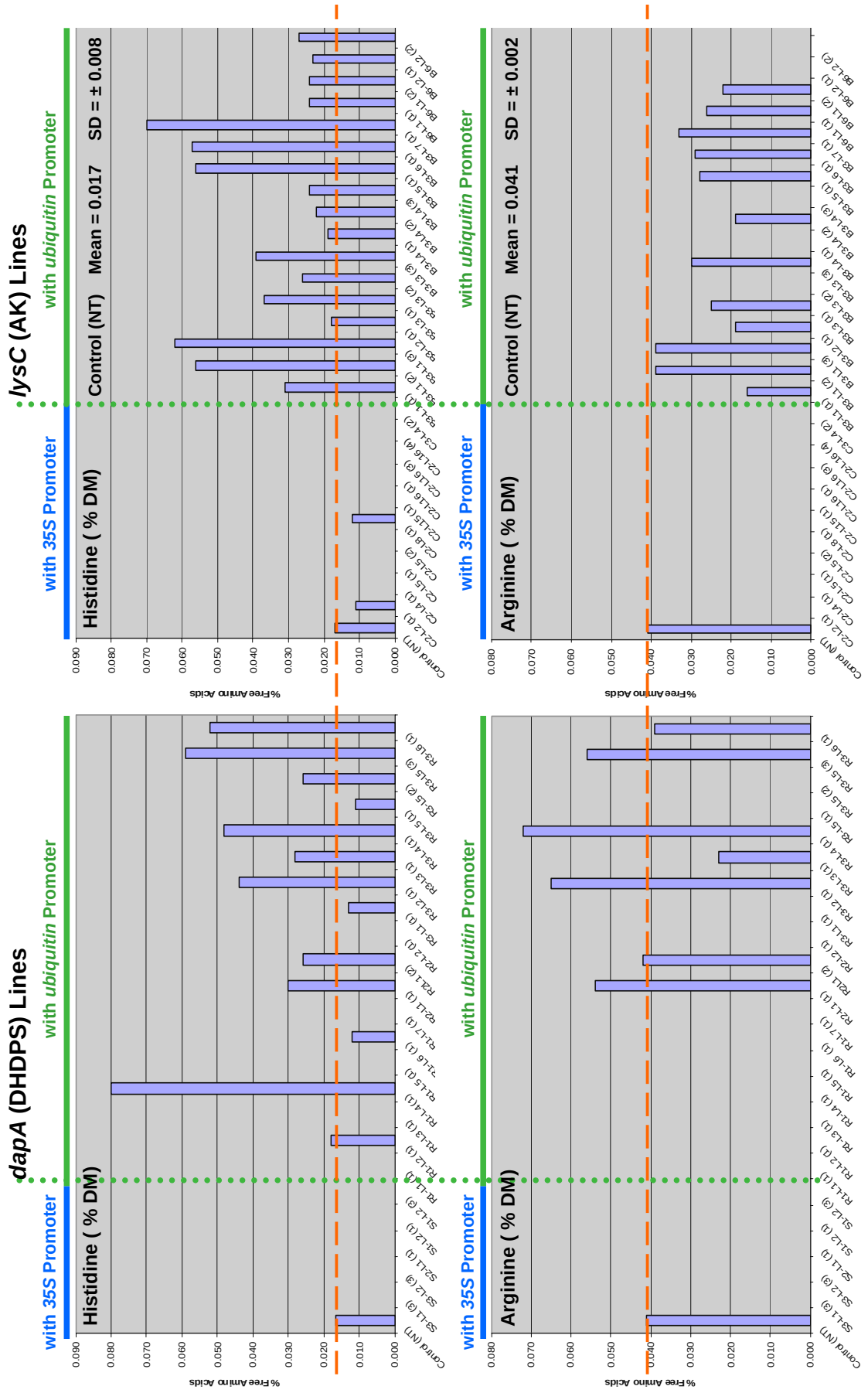

Figure 50: Percentage of free histidine and arginine in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

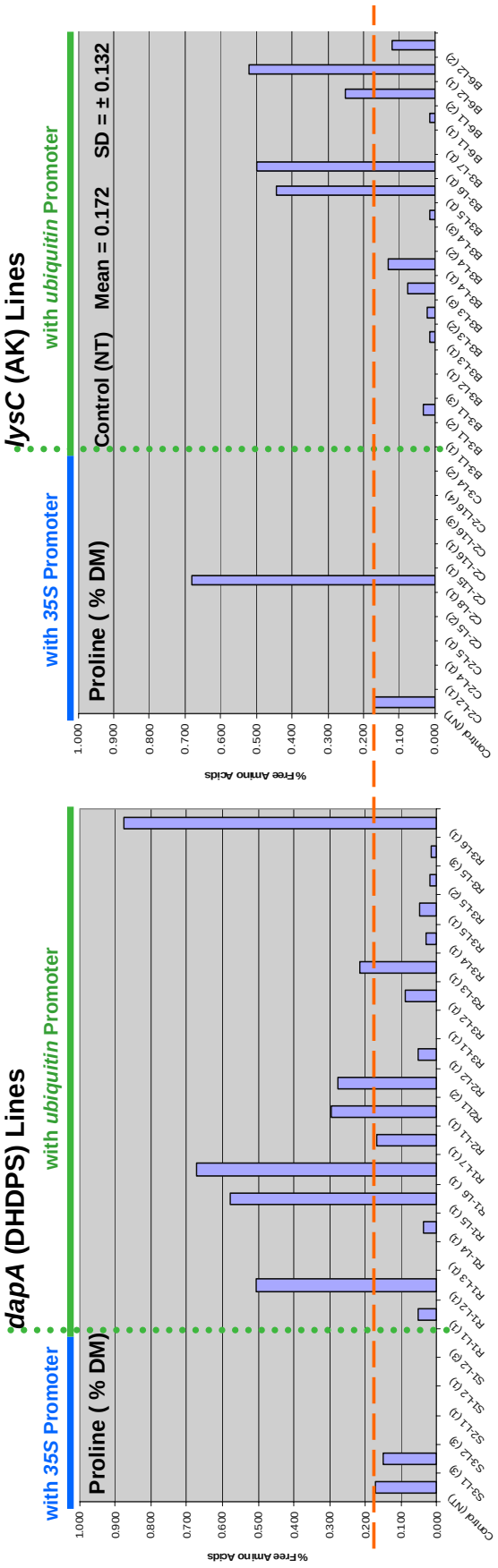

Figure 51: Percentage of free proline in leaves (Dry material) of different (T_0) transgenic lines in comparison to the control (non-transgenic plants), mean value of the control derived from 14 individual plants, SD= Standard deviation.

Relative changes in free amino acids (glutamine and 8 essential amino acids) in 6 transgenic plants transformed with the *dapA* gene were compared to the control plants. Figure 52 reveals that not only an increase in lysine up to 5-fold is the result of genetic engineering but also other amino acids are affected. Free histidine, phenylalanine and isoleucine increased up to 3.5-fold, 2.4-fold and 2-fold respectively. In addition, there was a slight increase also in other amino acids. In transgenic plants transformed with the *lysC* gene apart from elevated levels in free lysine up to 2.2-fold an increase is also shown for histidine up to 4-fold, phenylalanine up to 2.6-fold, isoleucine up to 2-fold (Figure 53).

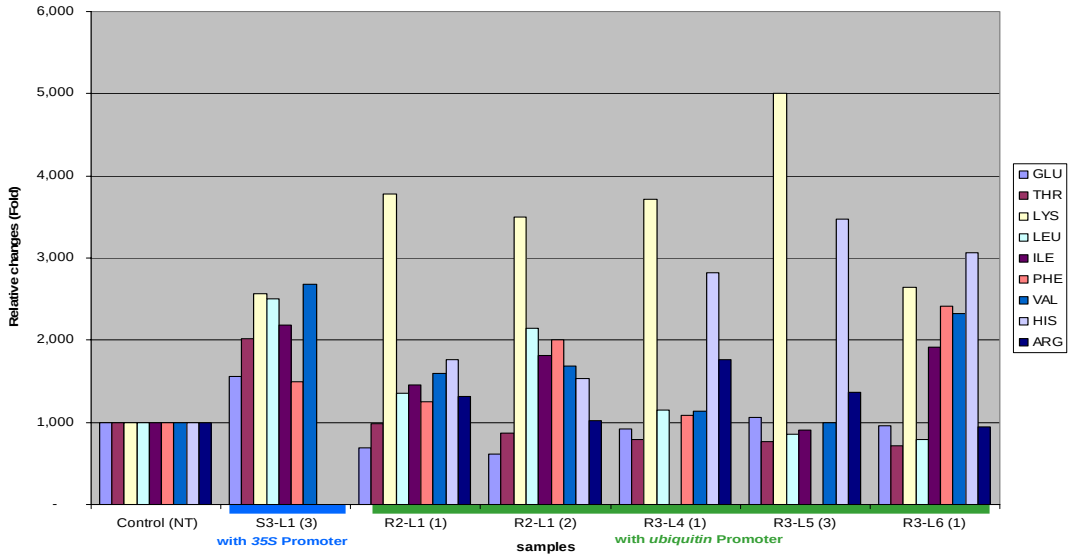

Figure 52: Relative changes in free amino acids in leaves of 6 individual T_0 transgenic barley plants derived from 5 independent cell lines transformed with the *dapA* (DHDPS) gene compared to control (NT) non-transgenic plants. The amino acid content was normalized to that found in the control plants which were given the value 1.

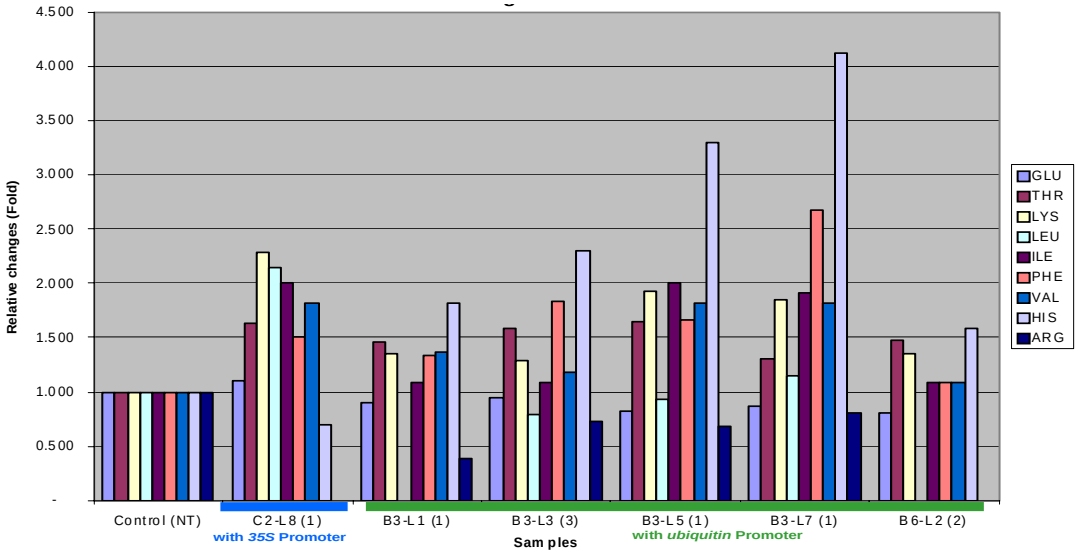

Figure 53: Relative changes in free amino acids in leaves of 6 individual T_0 transgenic barley plants derived from 6 independent cell lines transformed with the *lysC* (AK) gene compared to control (NT) non-transgenic plants. The amino acid content was normalized to that found in the control plants which were given the value 1.

The average of 5 individual transgenic plants derived from 4 independent cell lines transformed with the *dapA* gene driven by the *ubi1* promoter was compared to the non-transgenic control plants (Table 25, Figure 54). A significant increase 3.7-fold in free lysine is calculated, for histidine 2.5-fold, for phenylalanine 1.6-fold, and 1.5-fold for isoleucine and valine.

Table 25: Relative changes in free amino acids in leaves of transgenic T_0 barley plants transformed with *dapA* (DHDPS) gene in comparison to the control non-transgenic plants (NT)

Amino acid	Control (NT)	SD	Transgenic (T)	SD	Relative changes(Fold)
GLU	0.529	± 0.028	0.447	± 0.088	0.844
THR	0.063	± 0.007	0.051	± 0.006	0.809
LYS	0.014	± 0.004	0.052	± 0.010	3.714
LEU	0.014	± 0.002	0.017	± 0.006	1.214
ILE	0.011	± 0.001	0.017	± 0.004	1.545
PHE	0.012	± 0.002	0.020	± 0.006	1.666
VAL	0.022	± 0.005	0.034	± 0.010	1.545
HIS	0.017	± 0.008	0.043	± 0.012	2.529
ARG	0.041	± 0.002	0.052	± 0.012	1.268

Control (NT) = mean value of 14 plants, SD = standard deviation
Transgenic (T) = Mean value of 5 individual plants derived from 4 independent cell lines:
[R2-L1 (1), R2-L1 (2), R3-L4 (1), R3-L5 (3), R3-L6 (1)].
Relative changes (Fold) = the amino acid content was normalized to that found in the control plants which were given the value 1.

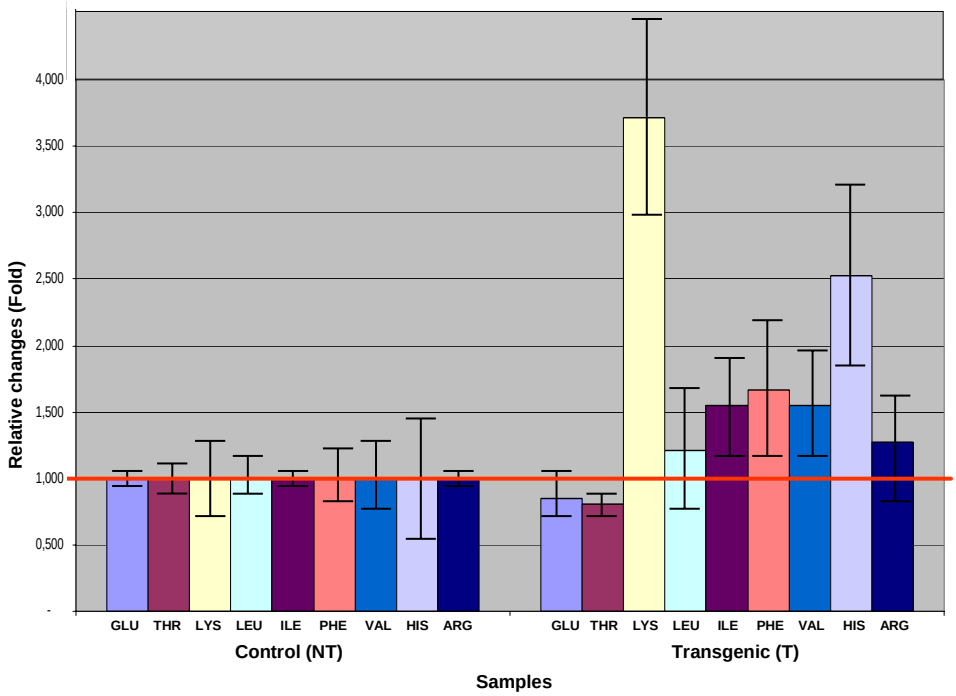

Figure 54: Relative changes in free amino acids in leaves of transgenic T_0 barley plants transformed with the *dapA* (DHDPS) gene under the control of *ubi1* promoter (mean value of 5 individual transgenic plants derived from 4 independent cell lines) compared to the control (NT) non-transgenic plants (mean value of 14 plants). The amino acid content was normalized to that found in the control plants which were given the value 1.

In the same way, the average of 5 individual transgenic plants derived from 5 independent cell lines transformed with the *lysC* gene driven by the *Ubi1* promoter was compared to the control non-transgenic plants (Table 26, Figure 55). 1.5-fold increase is calculated for lysine, threonine, phenylalanine, valine and a 2.5-fold increase for histidine.

Table 26: Relative changes in free amino acids in leaves of transgenic T_0 barley plants transformed with *lysC* (AK) gene in comparison to the control non-transgenic plants (NT)

Amino acid	Control (NT)	SD	Transgenic (T)	SD	Relative changes (Fold)
GLU	0.529	± 0.028	0.461	± 0.026	0.871
THR	0.063	± 0.007	0.094	± 0.007	1.492
LYS	0.014	± 0.004	0.022	± 0.003	1.571
LEU	0.014	± 0.002	0.013	± 0.002	0.928
ILE	0.011	± 0.001	0.015	± 0.004	1.363
PHE	0.012	± 0.002	0.018	± 0.007	1.500
VAL	0.022	± 0.005	0.032	± 0.006	1.454
HIS	0.017	± 0.008	0.044	± 0.016	2.588
ARG	0.041	± 0.002	0.026	± 0.006	0.634

Control (NT) = Mean value of 14 plants, SD = Standard Deviation
Transgenic (T) = Mean value of 5 individual plants derived from 5 independent cell lines:
[B3-L1 (1), B3-L3 (3), B3-L5 (1), B3-L7 (1), B6-L2 (2)]
Relative changes (Fold) = The amino acid content was normalized to that found in the control plants which were given the value 1.

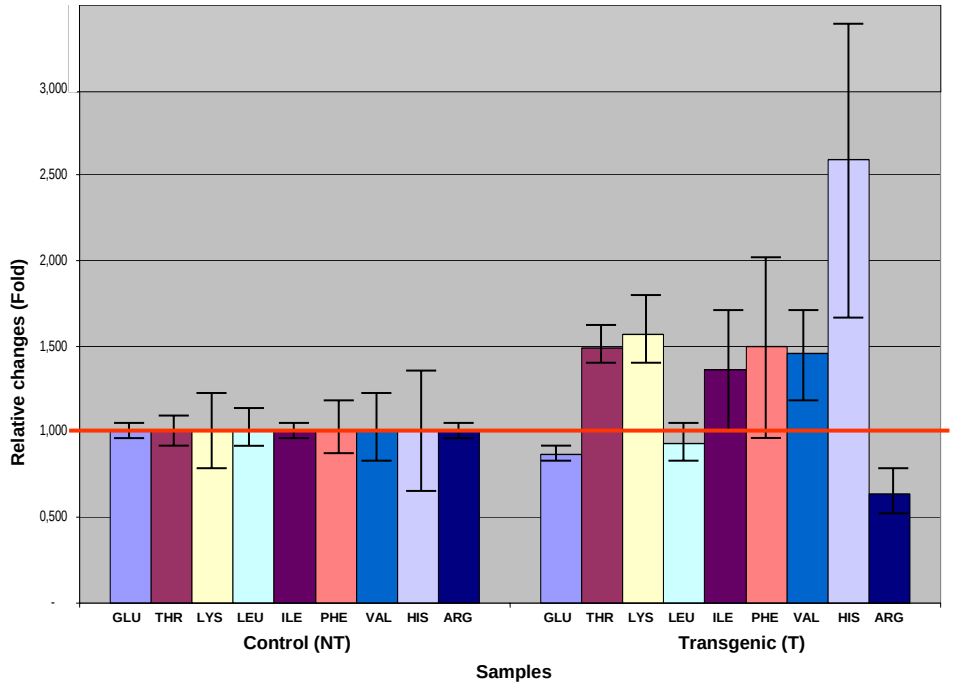

Figure 55: Relative changes in free amino acids in leaves of (T) transgenic T_0 barley plants transformed with the *lysC* (AK) gene under the control of *ubi1* promoter (mean value of 5 individual transgenic plants derived from 5 independent cell lines) compared to the control (NT) non-transgenic plants (mean value of 14 plants). The amino acid content was normalized to that found in the control plants which were given the value 1.

4. DISCUSSION

4.1. Barley immature embryo culture and regeneration

A reliable and efficient regeneration system is a prerequisite for gene transfer into barley plants. A number of examples are given in the literature, describing regeneration systems in barley. Really successful plant regeneration could only be achieved starting with cells of immature embryos (Thomas and Scott 1985; Chang *et al.,* 2003; Eudes *et al.,* 2003). Although there are regeneration protocols available, the individual laboratory, growth cabinet and greenhouse conditions at different places normally prevent success by direct application. Therefore optimization steps are the first experiments to start with. Barley cv. Golden Promise donor plants were grown under controlled conditions in a growth cabinet and immature embryos were isolated roughly 20 days after anthesis. Auxin type, embryo size (= physiological stage) and the duration of the callus induction period were tested to optimize a regeneration protocol.

Somatic embryos are an important tool for a large-scale vegetative propagation. Therefore, regeneration via somatic embryogenesis is the key factor to achieve an efficient transformation system. In barley scutellum cells of immature embryos are the best target cells for production of embryogenic callus and somatic embryogenesis.
Both 2,4-D and Dicamba induced vigorously growing, embryogenic calli when added to the callus induction medium (CIM) with a concentration of 2.5 mg/l, but Dicamba was superior. It enhanced significantly callus quality and somatic embryogenesis resulted in the maximum level of 94.52 % regeneration. This agrees with reports from the literature (Lührs and Lörz, 1987; Przetakiewicz *et al.,* 2003).
Dicamba was successfully used as auxin substance in several barley transformation studies (Wan and Lemaux, 1994; Tingay, *et al.,* 1997, Travella, *et al*., 2005), whereas Trifonova *et al*., 2001 documented that plant regeneration from barley transgenic callus was difficult to obtain when 2,4-D was used.

The developmental stage of the explant is a crucial factor for the *in vitro* culture and regeneration of cereal plants (Lu *et al.,* 1984; Bebli *et al.,* 1988; Castillo *et al.,* 1998; Sahrawat and Chand 2001). Callus quality and finally regeneration capacity are strongly

dependent on the developmental stage of the explant. A parameter good to observe reflecting the physiological stage of the explant is the size of the immature embryo. In this study, the use of different sizes of immature embryos affected the quality of derived calli, number of regenerated plantlets per explant and the percentage of regeneration. The medium size (1.5-2 mm) had the highest regeneration potential and proved to be a suitable size for *in vitro* culture of barley (cv. Golden Promise). This result is in accordance with that previously reported by Wan and Lemaux, 1994, Tingay *et al.,* 1997, and Travella *et al.,* 2005. The optimal size of immature embryos varies with plant genotype or species. Chang *et al.,* (2003) reported that, using different sizes of immature embryos of barley (cv. Morex) the highest regeneration capacity was recorded using immature embryos in size range 0.5-1.5 mm.

To screen for the optimal callus induction duration and to assess the critical stage for embryogenic development, different periods of callus duration were compared. Callus duration for all four periods (2, 4, 6, and 8-week) markedly affected the percentage of regeneration and the average number of plantlets/ explant.

High frequencies of regeneration appeared with a 4-week treatment, although, the highest numbers of plantlets/explant were recorded after 6-week and longer time treatment. Long duration of callus induction may lead to an increase of the number of regenerated plantlets from callus lines, but on the other hand has a negative effect on regeneration percentage, meaning that callus cannot maintain its regeneration ability without changes over long periods. Von Arnold *et al.,* (2002) mentioned that, in most crops, the embryogenic potential decreases with prolonged culture and is eventually lost.

The period between 15 and 28 days after culture initiation has been identified as a critical period for somatic embryogenesis. Careful microscopic observations of cultured immature embryos revealed that, somatic embryo-like structures start to be frequently visible 18 days after culture initiation. They fully develop and germinate after 28 days of culture initiation (Figure 11, p. 35).

These findings may explain the low percentage of regeneration and low number of produced plantlets per explant with a 2-week callus induction period, and refer to the importance of keeping cultured immature embryos on callus induction medium for 4 weeks until transfer to the regeneration medium to induce germination of newly developed somatic embryos. On the other hand, when callus induction period is prolonged for more than 4 weeks it is possible through the subsequent developmental stages for some of the

somatic embryos, which have been kept on callus induction medium for a period of more than 4 weeks either to form mature somatic embryos (Figure 11C, p 35) or to reproduce callus which may result in a secondary somatic embryo. This will lead to an increase of the total number of regenerated plantlets/explant.

The overall conclusion of the work reported in this study is that a high efficient regeneration system of barley is achieved by culturing immature embryos (1.5-2 mm) for 4 weeks on callus induction medium containing 2.5 mg/l Dicamba.

4.2. Establishment of *Agrobacterium tumefaciens*-mediated barley transformation using reporter genes

Although there are a number of publications describing successful *Agrobacterium*-mediated transformation of barley (Tingay *et al.,* 1997; Murray *et al.,* 2001; Wu *et al.,* 2003; Travella *et al.,* 2005), the ability to routinly transform barley using *Agrobacterium tumefaciens* is currently restricted to a few laboratories worldwide; this is partly due to the need for experienced personnel and plant growth infrastructure and also to a lack of clearly-written transformation protocols. In this study *Agrobacterium*-mediated transformation is the method of choice due to several significant advantages for transferring DNA via *Agrobacterium*, including a reduction in transgene copy number, the stable integration with fewer rearrangements of long molecules of DNA and the ability to generate lines free from selectable marker genes (Jones, 2005; Cheng *et al.,* 2004; Travella *et al.,* 2005).

An efficient selection system is an important parameter to select small numbers of stably transformed cells; this parameter is becoming an essential factor when multicellular targets like immature embryos are used. To establish an efficient transformation system of barley, two different vector-selection systems the binary vector pDM805 for bialaphos selection and pBWvec10 for hygromycin selection were optimized and compared.
The transformation efficiency using the binary vector pDM805 containing the *bar* gene for bialaphos selection proved to be too low for a routine transformation procedure. Although transgenic calli (tested by PCR for *bar* and *uidA* genes) have been generated under

bialaphos selection, the calli could regenerate scarcely, indicating a potent inhibiting effect of the selective agent Bialaphos on the regeneration process.

The first set of experiments using the transient expression of the *uidA* gene was performed in order to determine optimal conditions for a transformation protocol. The following factors were optimized:
Inoculum density, inoculation time, co-cultivation period, presence of acetosyringone, size of immature embryos, and preculture duration.

Optimal density of the inoculum was achieved with an optical density OD_{600} = 1.0 which correlated with the highest percentage of explants with GUS foci and a good survival rate. Although the treatment OD_{600} = 1.5 showed the highest number of GUS foci/explant, the survival rate was relatively low. Results of inoculation time treatments proved that 60 minutes is the optimal inoculation time that resulted in the highest percentage of explants with GUS foci in comparison to the other treatments. Inoculation of immature embryos for 60 minutes in *Agrobacterium* culture with OD_{600} = 1.0 proved to be the best condition in this study, while, Tingay *et al.,* 1997 suggested that optimal conditions are when immature embryos after immersion in inoculum $OD_{600=}$ 1.0 will be immediately transferred to the callus induction medium.

Co-cultivation of inoculated immature embryos for 3 days is a convenient period, which resulted in the highest percentage of explants with GUS foci and number of GUS foci/ explant. Co-cultivation periods longer than 3 days reduced the percentage of explants with GUS foci, the number of GUS foci/explant and dramatically decreased the survival rate. This result confirms reports of other researchers (Tingay *et al.,* 1997; Murray *et al.,* 2001; Wu *et al.,* 2003). Therefore, 3 days was considered as standard co-cultivation period in all subsequent experiments reported here.

Different concentrations (50, 100, 200, and 400 µM) of the phenolic compound, acetosyringone (AS) were added to the inoculum culture to enhance the expression level of the virulence genes. Although AS is shown in many studies to be essential for monocot transformation, such as rice (Hiei *et al.,* 1994), wheat (Guo *et al.,* 1998), maize (Ishida *et al.,* 1996) and barley (Guo *et al.,* 1998; Roussy *et al.,* 2001), in this study, no significant

changes in the percentage of explants with GUS foci and the number of GUS foci/explant were observed. In addition, a slight decrease of GUS foci percentage and number/explant was recorded with 200 and 400µM treatments.

This finding maybe due to the constitutive expression of the *vir* genes caused by the presence of the hypervirulent tumor-inducing plasmid pTiBo542. Consequently when using the hypervirulent *Agrobacterium* strain AGL1 (Lazo *et al.,* 1991) there is no need to add a phenolic inducer for T-DNA transformation. This is in accordance with reports on *Agrobacterium*-mediated transformation of barley in the absence of AS (Tingay *et al.,* 1997; Murray *et al.,* 2001, Travella *et al.,* 2005).

Embryo death after co-cultivation is a big hurdle towards improving the transformation efficiency. A main reason for embryo death is the sensitivity of immature barley embryos to *Agrobacterium* infection particularly embryos with size 0.5 and 1mm. The survival rate was very low for the immature embryos in size 0.5mm (26.39%) and 74.31% for the size 1mm while it was 91.44% for the size 1.5-2mm. Determination of transient expression of the *uidA* gene in different sized immature embryos revealed that the ability of immature embryos to survive and grow after co-cultivation with *Agrobacterium* was increased by increasing the size of the embryo. In addition the percentage of explants with GUS foci is increased by increasing the embryo size to reach the highest level (67.12%) with the size 1.5-2 mm. These findings suggest that the choice of explant size is a crucial factor for achieving an efficient transformation system.

The size of the immature embryo reflects a distinct physiological state and determines the number of cells in the tissue which are competent for T-DNA transfer by *Agrobacterium* as well as the ability to recover and divide after foreign DNA integration. The effect of barley embryo size on *Agrobacterium*-mediated transient expression of the *uidA* gene has not been investigated previously; nevertheless several reports on barley transformation (Tingay *et al.,* 1997; Murray *et al.,* 2001, Travella *et al.,* 2005) are in accordance with this study and prefer using immature embryos in size 1.5-2mm.

High level of transient GUS expression was recorded with preculture treatments of barley immature embryos. The percentage of explants with GUS foci and the average number of GUS foci/explant was increased significantly using the preculture duration from 2 to 4 days.

Highest percentage of explants with GUS foci (83.93 %) was recorded by applying 3 days of preculture duration. This correlated with the highest number of GUS foci/explant

(11.73) in comparison to the control treatment (without preculture) which resulted only in 61.53 % and 5.52 GUS foci/explant. Moreover the 3-day preculture period raised the survival rate from 87.6% to 94.44%. This treatment is therefore necessary for a high efficiency of *Agrobacterium*- mediated barley transformation.

This may be due to the effect of the auxin substance (Dicamba) in the callus induction medium on inducing cell division in the explant (half immature embryo). Starting the cell division program means that a cell will prepare itself and starts to divide into two cells. Consequently the physiological state of the cell in this case (after 3 days on CIM) will be completely different compared to cells in the explant after isolation from the developing seeds. The results of transient expression of the *uidA* gene revealed that this cell division state proved to be accompanied with an increase in the ability of the cell to be transformed by the T-DNA. In conclusion preculture of the immature embryos makes the cells more competent for T-DNA transfer by *Agrobacterium*.

Many studies on wheat (Cheng *et al.,* 1997; Weir *et al.,* 2001; Wu *et al.,* 2003) reported the positive effect of preculture conditions on the transformation efficiency supporting the present finding for *Agrobacterium*-mediated barley transformation.

As a conclusion, to achieve a high efficient *Agrobacterium*-mediated transformation of barley immature embryos, the following conditions should be considered:

1- Using immature embryos in size (1.5- 2mm)
2- Preculture of explants for 3 days before inoculation with *Agrobacterium.*
3- Inoculation of explants with *Agrobacterium* culture OD_{600} = 1.0.
4- Inoculation of explants for 60 minutes in the *Agrobacterium* culture.
5- Co-cultivation of the inoculated explants with *Agrobacterium* for 3 days.

To produce stable transgenic barley plants expressing the *bar* and *uidA* genes 37 experiments using the *Agrobacterium* vector pDM805 were performed. 19 successful transformation experiments out of 37 experiments resulted in 40 independent transformed lines derived from 2647 half embryos, giving a transformation efficiency of 1.51 %. The remaining 18 experiments failed to produce any transgenic plant from the produced transgenic calli. These results revealed that, the pDM805 system is not reliable for routine transformation. The average transformation rate (1.51 %) was lower than the highest transformation efficiency (4.2 %) recorded by Tingay *et al.,* (1997) and than (2 %) that of

the most recent study conducted by Travella *et al.,* (2005). It was further observed that many of the transgenic calli (tested for *bar* and *uidA* genes by PCR) growing on selection medium, failed to regenerate suggesting that the selective agent bialaphos may interfere with barley regeneration. Therefore, it was decided to use another vector system. The pWBVec10 containing the hygromycin resistance gene *hpt* as a selectable marker was used in order to study the significance of the selection system and its impact on the transformation efficiency.

During the early transformation experiments using the hygromycin vector it was observed that the regeneration of transgenic calli is frequently higher than that with the bialaphos system. Consequently to study the effect of bialaphos on the regeneration process a set of experiments "Post Regeneration Selection" (up to 2 weeks regeneration without selection pressure) using the binary vector pDM805 was performed. Results of the 4 subsequent "Post Regeneration Selection" experiments revealed that bialaphos strongly inhibits the regeneration of barley transgenic calli and that in the absence of bialaphos the transgenic calli are able to regenerate to new plantlets. Thus the transformation percentage significantly increased with a maximum level of 6.66 % and in average 3.95% which is more than 2.5-fold in comparison to the average value of the 19 previous experiments (1.51%). Therefore the use of the Post Regeneration Selection protocol is a good way to overcome the inhibitory effect of bialaphos on the regeneration of transgenic calli for achieving a high transformation efficiency using the binary vector pDM805 or another vector based on bialaphos selection.

GUS expression in callus lines 4 weeks after inoculation with *Agrobacterium,* PTT (Basta) resistance leaf tests and positive GUS assays of leaf samples of T_0 transgenic plants showing the expression of *bar* and *uidA* genes, indicate stable transformation using the pDM805 vector. Moreover, analysis of 60 T_1 plants derived from 6 individual plants representing 6 T_0 independent cell lines using the PTT resistance test, GUS assay of leaf samples and PCR analysis demonstrated the expression and presence of *bar* and *uidA* genes in the progeny of T_0 plants. Both genes were inherited in the T_1 generation following the Mendelian pattern of single gene segregation (3:1). This result is in accordance with the previous reports of Tingay *et al.,* (1997) and Travella *et al.,* (2005).

In an attempt to investigate the effect of another selection system on the efficiency of barley transformation by *Agrobacterium*, the pBWVec10 vector system, developed by Wang *et al.,* (1997) was used. It contains the *hpt* gene for hygromycin resistance.

3 days preculture treatment was applied to determine its effect on the efficiency of the transformation using pWBVec10. The preculture treatment of half immature embryos produced a significant increase in the percentage of explants with GUS foci from 62.43 % to 76.38 % on average, as well as the average number of GUS foci/explant increased from 10.30 % to 17.51 %. A dramatic increase in the percentage of explants with GUS foci from 8.77 % to 71.22 % on average was recorded with intact scutellum of immature embryos. The number of GUS foci/explant increased from 12.51% to 16.27% on average and most of the GUS foci were scattered over the basal side of the scutellum in comparison to the control without preculture, which showed most of the GUS foci on the upper side of the scutellum close to the groove of the embryo axis. This change in the position of the infection may explain the effect of the preculture treatment on the cells of the scutellum at the basal side which are usually rare to be transformed directly by *Agrobacterium.* This is another indication, that a preculture treatment of barley immature embryos for 3 days is a vital prerequisite to achieve a high efficiency of transformation.

This study made evident that an efficient and reproducible transformation system for barley can be achieved using the binary vector pWBVec10. In 14 experiments, 1177 half-embryos were used generating 158 independently transformed lines, giving a transformation efficiency of 13.42 %. This result is higher than the highest efficiency (9.2 %) reported by Murray *et al.,* (2001) with the same vector.

The histochemical assay of GUS activity in 2-week old callus showed a strong stable expression of the *uidA* gene using the pBWVec10 vector. Moreover, GUS assay of leaf samples confirmed a stable expression of the *uidA* gene in T_0 transgenic plants.
Southern blot analysis was performed using genomic DNA isolated from some representative individuals of transgenic lines tested by PCR. The results confirmed the integration of the *uidA* gene into the barley genome with a low copy number (1 to 2 copies). This result is in accordance with many reports (Tingay *et al*., 1997, Travella *et al.,* 2005).

Histochemical assay of GUS activity in the progeny of T_0 transformed plants proved the presence and expression of *uidA* gene in T_1 plants. Furthermore, PCR analysis for *hpt* and *uidA* genes using genomic DNA of T_1 plants confirmed the inheritance of both genes in the T_1 generation and exhibited a segregation ratio of 3:1. This result is in agreement with several reports (Murray *et al.,* 2001; Tingay *et al.,* 1997; Travella *et al.,* 2005)

Using the *bar* gene as a selectable marker proved to be unsatisfactory for achieving a high efficient barley transformation system due to the negative effect of the selective agent bialaphos on the regeneration of new plantlets from the transgenic calli. Only when a second protocol (Post Regeneration Selection) was developed to avoid the effect of bialaphos on regeneration, a reasonable transformation rate could be obtained (highest rate = 6.66%; average rate = 3.95%). But finally, the use of the *hpt* gene as a selectable marker proved to be the best method for a high efficient barley transformation system. The obtained results of 14 subsequent experiments with a transformation rate ranging between 4% and 23 % and on average 13.42% refer to the superiority of the hygromycin system over the bialaphos system for achieving a high efficient barley transformation system. Therefore it was decided to use the backbone of the binary vector pWBVec10 for the construction of new transformation vectors including the target genes *lysC* and *dapA*.

4.3. Transformation of barley with “high lysine” vectors

In order to express the high lysine genes in the developing endosperm the *D-hordein* promoter was chosen as a tissue-specific promoter. Hordein promoters including *D-hor* were characterized by (Brandt *et al*., 1985; Forde *et al*., 1985; Rasmussen and Brandt 1986; Sørensen *et al.,* 1996).

Many reports confirmed the strong activity of the endosperm-specific promoter *D-hordein*, for example Cho *et al.,* (1999) reported that expression of the *uidA* gene driven by B_1- or *D-hordein* promoter was observed in stably transformed barley plants co-transformed with the selectable herbicide resistance the *bar* gene, and they concluded that B_1- and *D-hordein* promoters can be used to engineer and subsequently study stable endosperm-specific gene expression in barley.

Horvath *et al.,* (2000) reported that production of a protein-engineered thermotolerant (1,3-1,4)-β-glucanase with the α-amylase promoter yielded ≈1 $\mu g \cdot mg^{-1}$ soluble protein, whereas 54 $\mu g \cdot mg^{-1}$ soluble protein was produced on average in the maturing grain of 10 transgenic lines with the vector containing the gene for the (1,3-1,4)-β-glucanase under the control of the *D-hordein* gene (*Hor3-1*) promoter.

Cho *et al.,* (2002) concluded from their GFP transformation experiments that GFP expression driven by the *D-hordein* promoter was more stable in its inheritance pattern in T_1 and T_2 progeny than that driven by the *Act1*promoter of the rice actin 1 gene or than *bar* gene expression driven by the maize ubiquitin promoter (*ubi1*).

Choi *et al.,* (2003) reported results of an evaluation of the B_1- or *D-hordein* promoter driving the *uidA* and the *sgfp* genes and showed a long-term stability of transgene expression driven by both promoters in transgenic barley plants.

A reproducible production of T_0 transgenic plants expressing the *lysC* gene under the control of the *D-hor* promoter resulted in 41 individual plants derived from 5 independent transformed lines. In addition, 50 individual plants of 8 lines carrying the *lysC* driven by the *ubi1* promoter, and 36 plants of 26 lines carrying *lysC* driven by the 35S promoter were produced. The transformation rate of 8 transformation experiments using different vectors of the *lysC* gene was on average 5.30%.

Transformation experiments for introducing the *dapA* gene into barley genome were additionally carried out and resulted in 32 individual T_0 plants derived from 9 independently transformed lines carrying the *dapA* gene driven by the *D-hor* promoter. Furthermore 20 individual plants of 17 lines carrying the *dapA* driven by the *ubi1* promoter, and 42 plants of 23 lines carrying *dapA* driven by the 35S promoter were produced. The transformation rate of 13 successful experiments using different vectors of the *dapA* gene was on average 2.97%. During the performance of transformation experiments with *lysC* and *dapA* it was observed that the transformation efficiency was lower in comparison to the vector pWBVec10 (13.42%). As shown in Tables 20 and 21, p. 72, 73, many experiments were stopped due to low transformation efficiency which was monitored through parallel transformation experiments using the pWBVec10 vector. It appeared that the plant material (half immature embryos) used in these experiments were not suitable for transformation. This may be due to the seasonal effect on plant material (immature embryos) derived from plants planted in the growth chamber and greenhouse

which can have a negative influence on the regeneration capacity of barley cv. Golden Promise as reported by Dahleen, 1999 and Sharma *et al.*, 2005.
Co-transformation experiments were carried out to introduce both genes into the barley genome, this resulted in 2 lines containing 5 individual plants carrying only the *lysC* gene driven by the *ubi1* promoter, while none of these plants carried the *dapA* gene driven by the *D-hor* promoter. PCR analysis of the transformed T_0 plants was performed and some representative plants of each construction were used for southern blot analysis. The investigated southern blots of the transformants confirm the integration of the transgenes *lysC* or *dapA* into the genome of barley with one to four copies. Multiple-copy integrants are observed commonly with cereal transgenic plants (Wan and Lemaux 1994; Tingay et al., 1997; Srivastava *et al.,* 1999).

An interesting single copy integration pattern of the *lysC* gene was detected in 4 individual plants derived from 4 independent cell lines (Figure 38B, p.77). All detected fragments from single digestion of genomic DNA with NotI which cuts one time inside the T-DNA of the plasmid pSMW20 were similar in size (2.740 kb), this size being equal to the size of the fragment cut from the plasmid by double digestion using NotI and BamHI. Another restriction enzyme (BglII, non-cutter of the T-DNA or the backbone of the plasmid vector) was used to investigate this pattern of integration. The fragment of the plasmid pSMW20 digested with BglII was detected on the membrane close to the 10 kb fragment of the DNA marker size as a supercoiled fragment, while 3 fragments of 3 samples were detected in size of about 18 kb and one fragment in size of about 16 kb. This result of a similar integration pattern (single copy of the transgene detected in genomic DNA fragments similar in size) of the transgene *lysC* in 3 independent transformation events may refer to a preferable integration site for the transgene in the genome of barley. This would need further investigation. Genetic analysis and *in situ* hybridization to T-DNA insertions suggest that transgenes integrate into the plant genome by illegitimate recombination typically at one or two loci, sited at random over the genome (Ambros *et al.*, 1986; Thomas *et al*., 1994). However, although integration sites appear randomly distributed over chromosomes, several experiments with promoter-less marker genes have indicated that transgenes integrate predominantly in transcriptionally active regions. This holds true for transgenes introduced by either *Agrobacterium*-mediated transformation (Gheysen *et al.,* 1998; Koncz *et al.,* 1989; Tingay *et al.,* 1997; Topping *et al.,* 1991; Travella *et al.,*

2005; Ziemienowicz *et al.,* 2001) or direct gene transfer methods (Salgueiro *et al.*, 1998). Although integration occurs by illegitimate recombination, it is also believed that partial, short homologies (not full homologous recombination) between the introduced foreign DNA and the recipient genomic DNA are responsible for initiating integration (Gheysen *et al.*, 1991; Hamada *et al.*, 1993), which is then completed by DNA repair processes at the transgene-genomic DNA junctions.

The pattern of transgene integration, which includes the locus and the insertion number of the transgene in the host genome, is highly variable not only between transformation methods, but also between experiments and among plant species. In general *Agrobacterium*-mediated transformation tends to produce lower insertion numbers than particle bombardment or protoplast based methods. This fact is clearly illustrated in maize and rice (Aldemita and Hodges, 1996; Christou *et al.,* 1991; Datta *et al.,* 1999; Hiei *et al.,* 1994; Ishida *et al.,* 1996). In wheat, the majority of transgenic plants produced by particle bombardment contain a large number of insertions (Barro *et al.,* 1998; ; Becker *et al.,* 1994; Nehra *et al.,*1994; Rasco-Gaunt *et al.,* 2001; Stoger *et al.,* 1998; Weeks *et al.,* 1993). Plants produced by *Agrobacterium* (Cheng *et al.,* 1997) appear to contain between 1 and 5 transgene insertions, 35% of these plants carry a single copy of the gene and 44% are single locus insertions. Rasco-Gaunt *et al.,* (2001) recovered 24% single copy and about 50% single locus transformants from particle bombardment.

In cereals, there have been relatively few studies analyzing transgene insertion loci in detail. Takano *et al.,* (1997) analyzed transgene integration sites in three transgenic rice plants produced by protoplast transformation. They found large-scale rearrangements in the recipient genome at the integration site, occurrence of filler DNA and microhomologies of five base pairs between plasmid and target DNA. Interestingly, they also found a common AT-rich repetitive sequence structure in all three junctions of the target genome. This adenine and thyamine (AT)-rich sequences had several characteristics of a SAR (scaffold-attachment region) and preliminary analysis revealed SAR activity of this fragment. Gheysen *et al.,* (1987) suggested that AT-rich sequences are preferred targets for DNA integration

Inheritance of *lysC* and *dapA* genes was confirmed in the progeny of T_0 transgenic plants using PCR analysis. 129 T_1 transgenic plants carrying the *dapA* gene driven by the *D-hor* promoter were detected, as well as 6 plants of the *ubi1-dapA* construction and 25 plants of

the 35S-*dapA* construction, while 52 plants of the *lysC* gene driven by the 35S promoter were detected.

4.4. Amino acid composition in T_0 transgenic plants

Analysis of amino acids composition in leaves of 50 T_0 transgenic plants carrying the *lysC* and *dapA* genes driven by the constitutive 35S and *ubi1* promoters was performed. As control leaves of 14 individual wild type plants (control) at the same developmental stage were analyzed for their amino acid composition. Due to insufficient amounts of seeds derived from the transgenic T_0 plants transformed with the *lysC* or *dapA* driven by the endosperm-specific *D-hor* promoter, the analysis of amino acid composition was not yet performed in the seeds of T_0 plants. The seeds of T_0 transgenic plants were planted to produce the T_1 transgenic plants. Consequently amino acids analysis will be done in the seeds derived from the transgenic T_1 plants (results cannot be included in this study).

The content of free lysine was elevated between two- and five-fold in 6 individual T_0 transgenic plants derived from 4 independent cell lines transformed with the *dapA* driven by the *ubi1* promoter, while the percentage of free lysine was increased up to 2.5-fold in one plant transformed with the *dapA* gene driven by the 35S promoter in comparison to the non-transformed control plants (Figure 45, p. 88). These results confirmed the influence of the transgene *dapA* on the production of free lysine in the T_0 transgenic plants and also refer to the higher activity of the *ubi1* promoter in barley compared to the 35S promoter, which is known for its limited activity in cells of cereal plants.
A slight increase in the level of free lysine was measured in plants transformed with the *lysC* gene. It was up to 2-fold in one transgenic plant transformed with the *lysC* gene driven by the 35S promoter and up to 1.9-fold in 2 plants transformed with the *lysC* driven by the *ubi1* promoter. These results are partially in accordance with the results reported by Brinch-Pedersen *et al.,* (1996): leaves of T_0 transgenic plants generated by particle bombardment with integrated *lysC* or *dapA* both driven by the 35S promoter exhibited a 14- and 16-fold increase of free lysine, respectively. These results in comparison to the results in the present work are relatively high. But taking into consideration the effect of the transformation technique on the copy number of the transgene in the genome of the transgenic plant, it will appear that this high values may result from the high copy number of the transgens inserted in the genome of these plants in comparison to the plants

produced here using *Agrobacterium* which proved to mediate a low copy number having a high potential for single integration of the transgene. In transgenic tobacco plants expressing the *dapA* driven by the 35S promoter the level of free lysine was 25- to 50-fold higher than the wild type plants (Kwon *et al.,* 1995).

The level of free threonine was reduced in T_0 transgenic plants transformed with *dapA* driven by the *ubi1* promoter which exhibited an increase in the level of free lysine (Figure 45, p. 88). This may have resulted from the feed-back inhibition of the aspartate kinase (AK) by a high level of free lysine (Figure 1, p.2). In contrast, the transgenic plants transformed with *lysC* under the control of the *ubi1* promoter exhibited a slight increase of free threonine (up to 1.6-fold).
No changes were detected in transgenic plants transformed with the *lysC* gene driven by the 35S promoter. The same result was obtained by Brinch-Pedersen *et al.,* (1996), they reported no increase in the amount of threonine in transgenic barley plants and they postulated that an increase in synthesis of threonine could be rapidly compensated for by an accompanying increase in reactions involved in the degradation or conversion of threonine. This result is in contrast to tobacco plants expressing the *lysC* gene driven by the 35S promoter, which exhibited a 2- to 9-fold increase in the level of free threonine compared to control plants (Shaul and Galili, 1992).

The relative changes of free amino acids in transgenic plants exhibited an increase in free lysine and threonine, but this increase was also accompanied by an increase in the level of other essential amino acids such as leucine, isoleucine, phenylalanine, valine, and most prominent histidine (Figures 52 and 53, p. 95). These results are in accordance with the reports of Brinch-Pedersen *et al.,* 1996 and Shaul and Galili 1992, which point to the influence of the overproduction of lysine and threonine on the level of other free amino acids related to the aspartate pathway such as isoleucine and valine or even other amino acids like histidine and phenylalanine.

The level of free lysine exhibited a significant 3.7-fold increase when the mean value of 5 individual T_0 transgenic plants transformed with *ubi1-dapA* was compared to the mean value of 14 non-transgenic control plants (Table 25 and Figure 54, p. 96). A minor increase (1.5-fold) of free threonine was determined when the mean value of 5 T_0 transgenic plants transformed with *ubi1-lysC* was compared to the control (Table 26 and Figure 55, p. 97).

These results give evidence for the constitutive expression of the transgenes *dapA* and *lysC* in the T_0 transgenic barley plants which result in remarkable increases in the level of lysine and with lesser extent of threonine in leaves. It will be interesting to analyze the amino acid levels in those plants, which have been transformed with the high lysine genes under the control of the *D-hor* promoter. These plants are selected and grown in the greenhouse for seed production. Kernels of the T1 generation will then be measured for amino acid content. So far there is no report for barley, where elevated lysine levels could be detected in grains. Therefore tissue specific expression of these two genes in barley is aimed at for the first time in this study. In seeds of transgenic tobacco plants transformed with the *lysC* gene a high significant increase up to 7-fold in the free threonine content was determined (Karchi *et al.,* 1993; Galili 1995). Falco *et al.,* 1995, 1997 reported that a dramatic increase up to 100-fold in free lysine was determined and the total lysine was doubled in seeds of canola plants transformed with the *dapA* gene of *Corynebacterium,* and in soybean co-expression of both genes resulted in a several hundred-fold increase in free lysine and up to 5-fold in total lysine.

On the contrary endosperm specific expression of the maize lysine-feedback-insensitive dihydrodipicolinate synthase (DHPDS) gene using the rice glutelin (*GluB-1*) promoter in transgenic rice plants did not result in an elevation of free lysine in mature rice seeds, while using the CaMV 35S promoter the level of lysine was significantly increased in mature rice seeds in comparison to the control plants (Lee *et al.,* 2001) and they concluded that the presence of the foreign DHPDS gene driven by the *GluB-1* promoter leads to an increase of free lysine in the developing seeds which is accompanied by an increase of lysine ketoglutarate reductase (LKR) activity, resulting in enhanced lysine catabolism. However, over-expression of the DHPDS gene in a constitutive manner overcame lysine catabolism and sustained a high level in mature rice seeds.

In conclusion, a high efficient *Agrobacterium*-mediated transformation system of barley cv. Golden Promise was developed, which can be applied to introduce useful genes into barley commercial cultivars. Based on this efficient transformation system it seems that it is possible to generate marker free plants carrying the target genes through PCR screening as reported in potato plants by De Vetten *et al.,* 2003.

It might be possible that the level of lysine and threonine will be increased in the endosperm but due to the fact that most of the storage proteins in the endosperm of barley like in all cereals are poor in lysine and threonine, these free amino acids cannot be incorporated into proteins and will be degraded during grain maturation. In this case the level of lysine and threonine in mature seeds will be low as in the wild type. Therefore, improvements will be based on the results of amino acid analysis in the grains of T_1 plants at developing and mature stage. If the level of lysine and threonine is increased only in the developing grains, it might be necessary to re-transform these transgenic plants with a gene of an endosperm storage protein rich in lysine and threonine to serve as a sink for free lysine and threonine in the endosperm such as barley protein Z_4 (Brandt *et al*., 1990) or the maize elongation factor EF-1α (Habben *et al.,* 1995; Sun *et al*., 1997).

Another approach to elevate the level of lysine and threonine in the endosperm of barley may be achieved through introducing *dapA* and *lysC* genes and simultaneously knocking out the gene encoding the bifunctional enzyme lysine ketoglutarate reductase /saccharopine dehydrogenase LKR/SDH in order to decrease the catabolism of lysine, which may cause a synergistic increase in lysine in the endosperm of barley, as reported in the seeds of *Arabidopsis* plants by Zhu *et al.,* 2001; Zhu and Galili (2003).

In conclusion the combination of different strategies can be adopted as an approach for improving the nutritional value of barley grains through introducing a set of genes into the genome of barley, the set including genes for amino acid biosynthesis and knockout of genes responsible for the catabolic pathways as well as introducing genes for a seed storage protein rich in lysine and threonine.

5. SUMMARY

The nutritional quality of plant seeds is mainly determined by their content of essential amino acids provided in food for human or in feed for monogastric animals. Lysine is one of the most limiting essential amino acids in cereals. Metabolic engineering through transformation using chimeric genes (*lysC* and *dapA*) encoding feed-back-inhibition insensitive forms of the key enzymes of the aspartate-family biosynthetic pathway aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) respectively, is an effective method to elevate the level of free lysine and threonine in plant species.

The aim of this study was to produce transgenic barley plants carrying the mutated *E. coli* genes *dapA* and *lysC*. Both genes have been constructed under the control of two different promoters, the endosperm-specific *D-hordein* (*D-hor*) promoter and the constitutive promoter (*ubi1* or 35S) for enhancing the biosynthesis of lysine and threonine in barley plants, specifically in the developing endosperm.

To achieve this aim, an efficient *Agrobacterium*-mediated barley transformation system was established using two binary vectors based on two different selection systems (the binary vector pDM805 carrying the *bar* gene for bialaphos selection and the binary vector pWBVec10 carrying the *hpt* gene for hygromycin selection). Optimization of the transformation protocols led to a high transformation rate with a maximum average of 13.4 % using the vector pWBVec10. Subsequently the backbone of the pWBVec10 vector was used in construction of novel transformation vectors including the target genes under the control of the *D-hor*, *ubi1* and 35S promoter.

Successful transformation experiments resulted in the regeneration of fertile T_0 plants. Molecular analysis was performed to verify and select the transgenic T_0 plants. 103 cell lines with 226 individual T_0 plants were selected; 99 plants derived from 54 cell lines carry the *dapA* gene, and 127 plants from 49 cell lines carry the *lysC* gene.

The percentage of 13 free amino acids was determined in leaves of 50 transgenic T_0 barley plants. The level of lysine increased up to 5-fold in T_0 plants transformed with the *dapA* gene driven by the constitutive *ubi1* promoter, while simultaneously the level of threonine was slightly reduced. Plants transformed with the *ubi1-lysC* gene exhibited an increase of lysine up to 2-fold and threonine up to 1.6-fold.

The grains of the T_0 plants transformed with *lysC* or *dapA* driven by the endosperm-specific *D-hor* promoter were planted to produce T_1 plants. Consequently further studies must be done in order to determine the content of lysine and threonine in developing and mature grains of selected 212 individual T_1 plants, 160 plants carry the *dapA* and 52 plants carry the *lysC* gene.

6. ZUSAMMENFASSUNG

Die Nährstoffqualität pflanzlicher Samen und Früchte in der Nahrung von Mensch und monogastrischen Tieren ist wesentlich durch den Gehalt an essentiellen Aminosäuren bestimmt. Lysin ist eine der am limitiertesten essentiellen Aminosäuren in Getreidearten.
„Metabolic engineering“, die Veränderung des Stoffwechsels über die Transformation chimärer Gene, die für „feed-back-Hemmung“ insensitive Schlüsselenzyme (Aspartat-kinase, AK, und Dihydrodipicolinatsynthase, DHDPS) des Biosynthesewegs der Aspartat-Familie kodieren, ist eine effektive Methode um den Gehalt an freiem Lysin und Threonin in Pflanzenarten zu erhöhen.
Es war das Ziel dieser Arbeit, transgene Gerstenpflanzen zu entwickeln, welche die *E.coli* Gene *dapA* und *lysC* für die Expression von DHDPS und AK enthalten. Beide Gene wurden unter die Kontrolle des endosperm-spezifischen *D-hordein (D-hor)* Promotors gesetzt, um spezifisch die Lysin- und Threoninsynthese im sich entwickelnden Endosperm von Gerstenkörnern zu steigern. Alternativ wurde transgene Gerste erzeugt, die mit Hilfe des Mais-*Ubiquitin1 (ubi1)* und des 35S Promotors DHDPS und AK konstitutiv exprimieren.

Um dieses Ziel zu erreichen wurde ein effizientes Agrobacterium-Gersten-Transformationssystem etabliert. Dazu wurden zwei binäre Vektoren mit zwei verschiedenen pflanzlichen Selektionsmarkern verglichen – pDM805 enthält das *bar* Gen für Bialaphos-Selektion, pWBVec10 das *hpt* Gen für Hygromicin-Selektion. Die Optimierung der Transformationsprotokolle für beide Vektoren resultierte in einer hocheffizienten Transformationsmethode mit pWBVec10, mit der eine maximale mittlere Transformationsrate von 13,4 % erzielt werden konnte. In der Folge wurde das Vektorgerüst von pWBVec10 für die Konstruktion neuartiger Transformationsvektoren verwendet, welche die Zielgene unter der Kontrolle des *D-hor*, *ubi1* und 35S Promotors enthalten sollten. Insgesamt wurden 10 Transformationsvektoren entwickelt, je 5 Vektoren für jedes Gen.

Erfolgreiche Transformationsexperimente resultierten in der Regeneration vitaler T_0-Pflanzen für jedes Konstrukt. Die transgenen Pflanzen wurden mittels molekularer Analyse verifiziert und selektiert. Dies ergab 103 Zelllinien mit 226 individuellen T_0-Pflanzen, 99 Pflanzen aus 54 Zelllinien enthalten das *dapA* Gen, 127 Pflanzen aus 49 Zelllinien das *lysC*

Gen. Daraus wurden 212 individuelle T_1-Pflanzen selektiert, davon 160 mit dem *dapA* Gen und 52 mit dem *lysC* Gen.

In Blättern von 50 T_0-Gerstenpflanzen wurde der prozentuale Gehalt 13 freier Aminosäuren bestimmt. Lysin zeigte einen bis zu fünffachen Anstieg in T_0-Pflanzen, transformiert mit dem *dapA* Gen unter der Kontrolle des konstitutiven *ubiquitin1* Promotors, während gleichzeitig Threonin leicht reduziert wurde. In mit dem *ubi1-lysC* Gen transformierten Pflanzen stieg der Gehalt an Lysin um das 2 fache, Threonin erhöhte sich 1,6 fach. Aus Samen der regenerierten und selektierten transgenen T_0-Pflanzen mit jeweils endospermspezifisch *D-hor* Promotor gesteuerten *lysC* und *dapA* Genen wurden transgene T_1-Pflanzen gezogen. Samen dieser Pflanzen werden künftig ebenfalls auf erhöhte freie und gebundene Lysin- und Threoninwerte getestet.

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