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Transformation of Soybean and Use of Transgenic Lines in Basic and Applied Research

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ABSTRACT

Research on transgenic soybean ensures the generation of new data for basic and applied research. Successful transformation of soybean was achieved by both particle bombardment and *Agrobacterium*-based methods. The introduction of transgenes is a powerful tool for increasing resistance to biotic and abiotic stresses. Thus, resistance to viral and fungal infection, nematodes and insects, tolerance to herbicides as well as drought and high temperature can be increased using transformation. In the case of the industrial use of soybean oil, the alteration of fatty acid composition widened the range of potential applications. Yield quality was also improved by changing the amino acid composition in order to fulfil the requirement necessary for soybean to be used as food or feed. The function-, organ- or developmental stage-specific expression changes of several genes were studied in transgenic soybean. Suppressed or increased expression of genes allowed the determination of the possible regulatory or functional role of their products. Up to now the desired traits have been manipulated in soybean mainly by modification of the expression of structural genes. However, in the case of abiotic stress tolerance, determined by several genes, even more success could be achieved in the future if the expression of whole regulons might be changed by the genetic manipulation of the corresponding transcription factors.

Keywords: *Agrobacterium*, metabolite production, particle bombardment, physiological studies, stress tolerance, transformation
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacaetic acid; **3AP**, 3-aminopyridine; **ABA**, abscisic acid; **BA**, benzyladenine; **BAP**, 6-benzylaminopurine; ***Bt***, *Bacillus thuringiensis*; **FN/L/S3/S3/ medium**, Finer–Nagasawa/Lite/3% sucrose/3 % sorbitol/ medium; **FNLOS3**, liquid FNLS3; **GFP**, green fluorescent protein; **GUS**, β-glucuronidase; **Hsf**, heat shock transcription factor; **Hsp**, heat shock protein; ***Imt***, inositol methyl transferase; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid; **SAAT**, sonication-assisted *Agrobacterium*-mediated transformation; **ShaM**, soybean histodifferentiation and maturation medium; **SPR**, surface plasmon resonance; **TDZ**, thidiazuron; **WST**, whisker-supersonic technique

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Invited Review

INTRODUCTION

Two plant families of great importance to agriculture world wide are the *Poaceae* and *Fabaceae*. The *Fabaceae* contains about 650 genera and 18,000 species. Legumes include many important crop species that contribute significantly to the protein intake of both humans and animals around the world especially in the developing countries where the available food and feed often is one-sided. Soybean is a member of the tribe *Phaseoleae*, the most economically important species of the legume tribes. Other legumes within this tribe include pigeon pea, common, lima and mung beans, cowpea, and Bambara groundnut (Hymowitz 2004). Soybean belongs to the genus *Glycine* (Hymowitz 1990). This genus is paleopolyploid, with 2n=40 as its base chromosome number, as compared to other phaseoloid legumes which are largely 2n=20 or 22 (Goldblatt 1981; Nielsen *et al.* 1989). The economic importance of this genus lies within the sub-genus *Soja*. Soybean (*Glycine max* (L.) Merr.) is a summer annual herb that has never been relocated into the wild (Hymowitz 1999). It is believed to be a cultigen from *Glycine ussuriensis* (Duke 1983). *G. max* has many popular names of which soybean and soyabean are the most common. The soybean genome contains one billion base pairs on 20 chromosomes and an estimated gene total of nearly 25,000 (Sinclair and Wynstra 1995). This paleopolyploid genome comprises about 1.1 Mb/C value, about seven and a half times larger than the genome of *Arabidopsis*, but less than half the size of the maize genome (Arumuganathan and Earle 1991), with high genetic and inter-genetic sequence conservation (Schlueter *et al.* 2006). According to Singh and Hymowitz (1988), the soybean genome is a partially diploidised tetraploid, a product of a diploid ancestor (n=11) that underwent aneuploid loss (n=10), polyploidisation (2n=20) and depolarisation (n=20) over time.

According to Hymowitz (1990), soybean is an ancient food crop of China and was domesticated during the Chou Dynasty which dated back to between the 7th and 11th C. B.C. As the Chou Dynasty expanded and trade increased, soybean migrated to South China and Korea (probably by the 1st C. A.D.). The movements of soybean were associated with the development, consolidation of territories and degradation of Chinese dynasties (Hymowitz 1990). The earliest soybean reference found in Japan was in the classic Kojiki (Records of Ancient Matters) which was completed in 712 A.D. It was later introduced into several countries (Indonesia, the Philippines, Vietnam, Thailand, Malaysia, Burma, Nepal and north India) and a number of landraces were developed. Soybean reached Europe in the 18th century. It was first cultivated in the Netherlands in 1737 and in France in 1739 and finally appeared in England in 1790. Soybean was introduced into the United States in the early 1800s and was grown as a minor forage crop for many years (Wilcox 1987). The development of the first major soybean-processing industry in Decatur, Illinois, USA in the early 1920s gave soybean cultivation a great impetus (Hymowitz 1990), and today soybean is a leading crop in most countries, ranking third behind maize and wheat (www.proteinresearch.net). By 1930, soybean-breeding programs had been initiated to hybridise plants and to select progeny better adapted than their parents (Wilcox *et al.* 1979).

Since soybean is self-pollinating and individuals are highly homozygous, the improvement and optimisation of soybean characteristics is very desirable. It is the world's main source of edible vegetable oils and high protein livestock feed (Wilcox 1987). Legume seeds are richer in protein than cereal grains. Soybean seeds contain between 35% to 55% protein on a dry weight basis (www.proteinresearch.net). Soybean is not only important as feed, but it is now generally recognized as the most economical source of protein for human consumption Soybean provides humans with a significant amount of their dietary protein requirements. In developing countries, increased cultivation of legumes is the best hope for combating projected shortages in food supplies, especially vegetable protein.

In the oilseed industry soybean is the leader with regards to oil production, providing more than half of the world's oil supply. In 2006, it was estimated that the global hectares of transgenic soybean represented 64% of global soybean production (James 2006). The world wide increase in the cultivation of soybean is mainly due to the substitution of fish meal by soy meal as a source of protein in animal feeds (www.agrimark.co.za).

Through the centuries plant species were bred and selective crossings performed resulting in the transfer of hundreds of genes to the offspring. Biotechnology has made it possible to transfer only the gene(s) of interest. In the literature biotechnology has been defined as "The applied use of living organisms or their components to make or modify products to improve plants or animals or to develop microorganisms for specific uses" (Industry Canada 1996).

PLANT REGENERATION FROM *IN VITRO* CULTURES AND DIFFERENT ORGANS

The development of procedures by which plants could be regenerated from single cells and organised tissues, with specific genes transferred to these plant cells, was the prerequisite for practical genetic engineering for soybean improvement. The limited genetic base in domestic soybean cultivars has restricted the traditional breeding methods for value added traits (Hinchee *et al.* 1996). The process for the production of transgenic soybean plants takes much longer and is more labour intensive than those of the model plant systems such as tobacco. *In vitro* techniques were thus applied with the aim of improving the desired traits of soybean. Two principle methods have been identified for soybean regeneration: somatic embryogenesis and shoot morphogenesis.

Somatic embryogenesis

Somatic embryogenesis is the process whereby embryos develop from either microspores or somatic tissue. Somatic embryos have both shoot and root axes and produce whole plants upon germination. Regeneration via somatic embryogenesis offers great potential for use in mass propagation and in transformation (Finer and McMullen 1991; Trick *et al.* 1997). Early attempts at soybean transformation focused on regeneration of embryogenic suspension cultures. Several studies with somatic embryos were conducted from 1973 to 1983, but developmental progress was made only as far as the torpedo stage (Kimball and Bingham 1973; Gamborg *et al.* 1983). Christianson *et al.* (1983) were the first to report successful embryogenic regeneration of soybean. They were able to regenerate one immature embryo from cultivar 'Mitchell'. Regeneration of complete plants was reported with the use of callus derived from immature embryos (Kerns *et al.* 1986). The somatic embryo methodology that exists for soybean (Parrott and Clemente 2004; Schmidt *et al.* 2005) is among the most advanced embryogenic systems available, and can be used to assist in the study of seed physiology and development.

To study seed-specific transgene expression, the ability to use transgenic somatic embryos has long been recognized (Liu *et al.* 1996; Mazur *et al.* 1999). Furthermore, transgenic somatic embryos may be used to efficiently study seed genomics, by either over-expressing or suppressing embryo-specific genes, without the need to recover an entire plant (Kinney 1998) or for reverse genetics approaches targeted towards seed-specific traits (Schmidt *et al.* 2005).

Immature cotyledon research experienced a period of growth with the discovery of the multi-cellular origin of somatic embryos. By using a medium supplemented with α-naphthalene acetic acid (NAA) Hartweck *et al.* (1988) were able to induce somatic embryos from the distal perimeter of cotyledon explants, but by using 2,4-dichloro-

phenoxyacetic acid (2,4-D) in the medium they were able to induce embryos from most of the epidermal surface of the cotyledons. A medium containing 2,4-D resulted in proliferated embryogenesis of somatic embryos in the apical region of primary somatic embryos (Finer 1988), and proliferated globular embryos were induced in embryogenic cell suspension cultures using a media containing 2,4-D and asparagine (Finer and Nagasawa 1988). The influence of cultivar genotype on somatic embryo capacity was reported by various authors (Parrot *et al.* 1989; Komatsuda *et al.* 1990; Bonacin *et al.* 2000).

Some factors that influence *in vitro* growth rates of soybean embryos include growth media, explant orientation, synthesis and accumulation of storage proteins and desiccation period. Lippmann and Lippmann (1993) found a culture medium containing KNO_3, glutamine, plant growth regulators and sucrose to be optimal for growth of cotyledon stage embryos. A greater amount of embryos form when the cotyledons of 'J103' and 'McCall' were placed with the abaxial surface facing down on a medium supplemented with 25 mg/l 2,4-D and 3% sucrose (D25) (Hartweck *et al.* 1988). Santarem *et al.* (1997) observed an increase in the efficiency of somatic embryogenesis by adjusting the induction medium to pH 7.0 and solidifying it with gelrite. The explant of cultivars 'Jack', 'Thorne', 'Resnik' and 'Chapman' were also cultured with the abaxial side facing the medium. Stachyose was reported to be essential in the acquisition of desiccation tolerance that has been linked with synthesis and accumulation of storage proteins associated with the ability of the embryos to germinate (Blackman *et al.* 1992; Kermode 1995). The importance of abcisic acid (ABA)in water relations during maturation period (Xu *et al.* 1990) was studied. It was reported that ABA promoted embryo development and maturation when applied at globular stage of development (Tian and Brown 2000).

Many researchers have attempted to find the "magic" medium for optimum embryogenesis by changing components such as pH, auxin and hormone concentrations, and light intensities. Lazzeri *et al.* (1987) observed higher numbers of normal embryos on a modified MS medium supplemented with 10 mg/l NAA compared to a media supplemented with 2,4-D. Xu *et al.* (1990) found that osmotica maintain synthesis of developmental proteins. Sucrose concentration, osmotic pressure, nitrogen content, and ammonium to nitrate ratio were found to be some of the major factors controlling proliferation of soybean embryogenic cultures (Samoylov *et al.* 1998b). Routine methods for somatic embryogenesis are available (Parrott *et al.* 1995).

The basic medium and protocols for the histodifferentiation and maturation of soybean somatic embryos are those of Finer and Nagasawa (1988), Bailey *et al.* (1993a, 1993b), Samoylov *et al.* (1998a) and Walker and Parrott (2001). In the two-step process MSM6 method (Bailey *et al.* 1993a), embryos are firstly placed on a modified Finer and Nagasawa (FN) media consisting of solidified MS basal salts, 6% maltose and 0.5% activated charcoal, and transferred after 30 days to a similar media without the charcoal. The FNLS3 media (Samoylov *et al.* 1998a) consisted of basal Finer and Nagasawa Lite (FNL) salts and 3% sucrose. This had the advantage of producing large numbers of somatic embryos in a short period of time, however, few embryos converted into plants. This medium was modified by Walker and Parrott (2001) through the addition of 3% sorbitol to the medium, now called FNLS3S3 medium. Samoylov *et al.* (1998b) compared solid with liquid media, MS with FSL maltose with sucrose and with or without auxins. They found that the liquid medium FNLOS3, FNL with 3% sucrose, resulted in the highest recovery rate of cotyledon stage embryos. Bailey *et al.* (1993b) observed that no existing protocol is optimal for recovery of all soybean cultivars, but that small changes will always be needed to yield acceptable frequencies of plant regeneration from somatic embryogenesis. They found success in some cultivars by changing the relative humidity of the germination medium and/or length of the desiccation treatment. Bonacin *et al.* (2000) demonstrated genotype influence on somatic embryogenic capability, with the most embryogenic cultivars being 'BR-16', 'FT-Cometa' and 'IAS-5'. The optimum auxin concentration was found to be 10 mg/l NAA and 7.0 being the optimum pH value. Light intensity did not have any affect on somatic embryo production. Schmidt *et al.* (2005) experimented with modifications to the FNLS3S3 soybean embryo maturation medium (Walker and Parrot 2001) on cultivars 'Jack', 'Benning' and 'PL417138', by comparison of sucrose and maltose, asparagine and glutamine as well as MS and FNL macro salts. They also add ABA, as well Gelrite to the media. Their liquid medium, soybean histodifferentiation and maturation medium (SHaM), consisted of FNL basal salts, 3% sucrose, 3% sorbitol, 30 mM glutamine, 1 mM methionine and Gelrite. They observed 61% more plants with the use of maltose instead of sucrose, but also found that maltose grown embryos took 27% longer to reach physiological maturity.

Organogenesis

Shoots can be formed from a number of different tissues and can be excised and rooted to generate new plants (Trick *et al.* 1997). Success stories in organogenesis include the regeneration of pre-existing meristems from immature embryo axes and cotyledons (Chyuan and Yeh 1991). Organogenesis occurred with cotyledonary nodes from immature embryos of cultivar 'Williams 82' on a medium containing as high a concentration of 13.3 μM 6-benzylaminopurine (BAP) (Barwale *et al.* 1986). Organogenesis was possible with the cotyledonary nodes of seedlings that were cultured on a reduced inorganic salt MS medium containing 5 μM benzyladenine (BA) (Wright *et al.* 1986). Regardless of orientation, the excised epicotyl of cultivar 'Wayne' yielded an average of seven sections per explant on a media containing 3-aminopyridine (3AP) (Wright *et al.* 1987). They also found that while 2,4,5-trichlorophenoxyacetic acid was demonstrated to be essential for regeneration, addition of BA was found to enhance regeneration. Multiple shoot proliferation was obtained from shoot tips derived from immature zygotic embryos of cultivar 'Williams 82' (Sato *et al.* 1993) that were grown on a MS media supplemented with BAP, NAA and sucrose (McCabe *et al.* 1988). Organogenesis from hypocotyl explants of cultivars 'Ohsuzu', 'Kosuzu', 'Suzukari', 'Suzuyutaka', 'Tachiyutaka' and 'NT-98-236' was obtained by using B5 medium containing thidiazuron (TDZ) (Yoshida 2002). A method was developed for cotyledonary nodal callus induction in cultivars 'Williams 82', 'Loda' and 'Newton' on medium containing 2,4-D and sorbitol, and shoot bud differentiation on media supplemented with BAP and maltose (Sairam *et al.* 2003). Adventitious bud and shoot induction (Mante *et al.* 1989) were also reported. Another issue includes the isolation, encapsulation and culture of protoplasts (Widholm *et al.* 1992) and primary leaf tissue (Kim *et al.* 1990). The initial segmentation patterns of microspores and pollen viability in soybean cultured anthers were investigated (Cardoso *et al.* 2004), as well as a 2% frequency of anther production was observed in two cultivars 'IAS5' and 'RS7' that were subjected to callus and embryo induction on B5 medium containing 2,4-D and BA (Kaltchuk-Santos *et al.* 1997).

Changing of the composition of the media can influence efficient organogenesis, such as found with the addition of BAP (Buising *et al.* 1994). Kaneda *et al.* (1997) observed multiple shoot formation from cotyledonary nodes and hypocotyl segments cultured on basal medium with a low salt concentration, and supplemented with TDZ. Regeneration via organogenesis utilising immature embryos at various stages of development has been reported (Yeh 1990). The correlation between the floral bud morphological size index and microspore developmental stages was established for Brazilian soybean cultivars 'Decada', 'IAS5' and 'RS7' (da Silva *et al.* 2003). They reported that

buds of the same size of different cultivars did not have microspores at the same stage of development and neither were the microspores from different anthers of the same flower at the same developmental stage.

Although soybean plants have been seen as recalcitrant to regeneration, these citations show that with endurance, somatic embryogenesis and organogenesis are possible. The development of effective preservation and long-term storage techniques is a critical requirement in the ex-situ preservation of biodiversity. Cryopreservation is based on the reduction and subsequent arrest of metabolic functions of biological material stored at ultra-low temperatures. Cryogenic preservation of plant cells for extended periods without genetic change and the subsequent recovery of normal plant cells have important implications in plant breeding and genetic engineering. A successful method was described by Luo and Widholm (1996) whom utilized a pre-culture treatment in a medium containing 3% sucrose and 3% sorbitol for soybean suspension culture cells. The cells survived if frozen with 10% dimethylsulfoxide and 8% sucrose. Pollen of annual soybean stored at -20°C retained their germination viability for 4 months (Tyagi and Hymowitz 2003).

TRANSFORMATION OF SOYBEAN

Successful transformation of plants demands that certain criteria be met, such as target tissues that are competent for propagation and regeneration, the ability to recover fertile transgenic plants at a reasonable frequency as well as a simple, efficient, reproducible, genotype-independent and cost-effective process (Birch 1997; Hansen and Wright 1999). The goal of a transformation system is to transfer a gene from one organism to another. To evaluate the performance of the transgene it is imperative that genetic variation between the genetically modified organism and its parent be minimized. Expression of the transgene can be affected by numerous factors, such as the compatibility of the construct with the organism or the site of insertion into the genome (Gruber and Cosby 1993). Soybean transformation frequency was very low, possibly due to the small number of cells that had been found to be totipotent (Trick *et al.* 1997).

Transformation methods are classified into two main groups: Indirect gene transfer, where exogenous DNA is introduced by a biological vector and direct gene transfer, where physical and chemical processes are responsible for DNA introduction. A number of procedures exist for the introduction of foreign DNA into soybean. Particle bombardment (Falco *et al.* 1995; Hadi *et al.* 1996) is based on the acceleration of DNA-coated particles towards a plant cell. Electroporation (Christou *et al.* 1990; Chowrira *et al.* 1995; Hou and Lin 1996) is a technique that uses electrical discharges to create reversible pores in the plasma membrane, thus allowing the introduction of foreign DNA into cell tissue. Direct *Agrobacterium*-mediated transformation utilises *Agrobacterium* as the biological vector (Hinchee *et al.* 1988; Chee *et al.* 1989). The combination of an integrated tungsten particle bombardment and a T-DNA transfer via *Agrobacterium* transformation system was developed using proliferative embryogenic tissue (Droste *et al.* 2000). Sonication-assisted transformation (SAAT) involves subjecting the plant tissue to periods of ultrasound in the presence of *Agrobacterium* (Trick and Finer 1997). A whisker supersonic mediated gene transfer method (WST) using whisker of potassium titanate fibers accompanied with supersonic treatment was developed for soybean (Khalafalla *et al.* 2006). Chloroplast transformation differs from nuclear transformation in many ways. The transgenes represent high level expression, multigene engineering in a single event, maternal inheritance and lack of gene silencing (Dhingra and Daniell 2006).

Particle bombardment

The first stable transformation and recovery of soybean callus (Christou *et al.* 1988) and of meristems from embryonic axes of immature seeds (McCabe *et al.* 1988) was obtained by the physical bombardment of tissue with DNA-coated gold particles. McCabe *et al.* (1988) have transformed cultivars 'Mandarin Ottawa' and 'Williams 82' with plasmid pCMC1100 that includes a *nptII* and *gus* coding region under control of CaMV 35S promoter. They observed more than five copies of the NPTII fragment in the genome. Christou *et al.* (1988) reported the stable transformation of soybean calli with plasmid pCMC1022 containing the NPTII coding region under control of CaMV 35S promoter in 'Williams', 'Mandarin Ottawa' and 'Hardin'. The gene copy and level of NPTII activity varied widely between calli. The invention and optimisation of the particle bombardment technique for the genetic engineering of soybean became a reality when it was shown that whole plants could be derived from a single transformed cell using a *de novo* organogenic pathway (Christou *et al.* 1989) as well as by using meristems or immature zygotic embryos (Christou *et al.* 1992). They transformed embryonic axes of cultivar 'Williams' with pAcX1100P and pAcX1021P that contained the *gus* and *nptII* genes. Southern blot analysis revealed a copy number higher than two for *gus*. A commercial process was developed by the combination of a genotype-independent regeneration protocol and an electric discharge particle acceleration technique (Christou *et al.* 1990; McCabe and Christou 1993). Co-transformation was analysed by using five plasmids comprising four different markers. PCM1021 (NPTII), was used in conjunction with pMC1220 (chloramphenicol acetyl transferase (CAT)) and pCMC1100 (GUS) to establish a co-transformation frequency of unlinked genes at about 20% (Christou and Swain (1990). They observed a co-transformation frequency of linked genes of about 50% by using pCMC1220, pTVGUS and pTVBAR. Sato *et al.* (1993) reported stable transformation via particle bombardment using shoot tip cultures and somatic embryogenesis. Bombardment of shoot tips produced GUS positive sectors in 30% of the regenerated shoots. However, none of the regenerants that developed into plants produced GUS-positive tissue. The bombardment of embryogenic suspension cultures produced GUS-positive plants. This procedure, however, has draw backs such as reduced fertility or sterility, as well as high variability of transgenic events (Stewart *et al.* 1996). Particle bombardment for the transformation of chloroplasts derived from embryogenic tissue generated fertile transplastomic soybean expressing *Bacillus thuringiensis* Cry1Ab protoxin (Dufourmantel *et al.* 2005) that proved to be stable over 6 generations (Dufourmantel *et al.* 2006). Molecular analysis confirmed that the Cry1Ab protoxin is highly expressed in leaves, stem and seed tissue. Hazel *et al.* (1998) found that embryogenic tissue undergo a burst of mitotic activity shortly after transfer to fresh medium, thus any treatment to increase the mitotic index, especially when the cell lines are less than 6 months old, may facilitate higher micro-projectile bombardment transformation frequency of cell lines. Birch (1997) also report on the development and optimisation of micro-projectile systems for plant transformation.

According to Christou (1997), particle bombardment is the best method for commercial engineering of soybean in a variety-independent fashion. Various reports on the use of particle bombardment as a transformation technique of embryogenic suspension cultures as explants have been filed: Stable transformation with cultivar 'Merril' encoding for hygromycin resistance and GUS, displayed varied copy numbers in independent clones (Finer and McMullen 1991). They observed an average of three transgenic clones per bombardment. Parrott *et al.* (1994) reported one transgenic plant out of 195 regenerated. Transformation using embryonic suspension cultures were reported (Bond *et al.* 1995) using FNL media (El-Shemy *et al.* 2004) and plasmid

sGFP(S65T) which encodes for a green fluorescent protein (GFP) (Khalafalla *et al.* 2005). El-Shemy *et al.* (2004) isolated 44 independent transgenic soybean plants with one of two gene constructs, pHV and pHVS, contain the hygromycin phosphotransferase gene (hpt), a modified glycinin gene (V3-1) or sGFP(S65T). Multiple gene copies were observed in all cases. Embryogenic soybean cultures have also been transformed with a *Manduca sexta* chitinase (*msc*) gene using micro-projectile bombardment (Ornatowski *et al.* 2004).

Electroporation

Stable transformation of soybean cells has been achieved through the direct uptake of DNA into protoplasts that were permeabilised by electroporation (Lin *et al.* 1987; Christou *et al.* 1990). Articles have been published on the recovery of transgenic soybean plants using protoplast electroporation with plasmid DNA carrying the *nptII* selectable marker under control of the 35S promoter linked with a non-selectable mannityl opine synthesis marker (Dhir *et al.* 1992; Widholm *et al.* 1992). However, these claims were later retracted since the transformation could not be reproduced. *In planta* gene transfer by electroporation-mediated gene transfer using intact meristems has also been reported (Chowrira *et al.* 1995). Transient expression of a chimeric *gus* reporter gene was used to monitor the uptake and expression of the introduced DNA by electroporated nodal axillary buds *in vivo*.

Agrobacterium-mediated transformation

Agrobacterium-mediated gene transformation is the most widely used gene transfer technique in plants. This technique takes advantage of the pathogenicity of the soil dwelling bacteria, *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. *A. tumefaciens* has the ability to transfer a portion of its DNA, called T-DNA, into the genome of plant species. This has the effect of inducing those cells to produce metabolites fulfilling the bacterium's nutritional requirements (Gelvin 2003). *Agrobacterium*-mediated transformation takes advantage of this concept by replacing the T-DNA of the *Agrobacterium* with a foreign set of genes, thus, making the bacterium a vector capable of transferring the foreign genes into the genome of the plant cell. Soybean was considered a poor host for *A. tumefaciens* (Matthysse and Gurlitz 1982). However, it has been proved that gall formation takes place on some soybean genotypes following inoculation with the octopine type tumor-inducing (Ti) plasmid (Owens and Cress 1985). Out of the 27 genotypes tested, three *G. max* cultivars and one *G. soja* line were highly susceptible to pTiA6. A number of cultivars, including 'A5308', 'Duiker', 'Forrest', 'Hutton' and 'Impala', produced tumours in response to infection with *A. tumefaciens* strain A281 (McKenzie and Cress 1992) as was observed in cultivars 'Merril', 'Peking' and 'Brag' with strains A281 and A208 (Bond *et al.* 1996). It was indicated that the co-infection with a super virulent strain, pTiBo542, or the addition of a phenolic compound, 10 μM acetosyringone, could promote transformation of soybean cells (Owens and Smigocki 1988). Byrne *et al.* (1987) tested the response of different *Agrobacterium* strains on various soybean genotypes. They reported a large degree of variation between Ti and root-inducing (Ri) strains, as well as between the *G. max*, *G. soja* and *G. canescens* genotypes. The susceptible genotypes, *G. max and G. soja*, displayed a heightened response to nopaline strains of *A. tumefaciens* (A208) and *A. rhizogenes* (pRi8196). It was, thus, demonstrated that tumours form on soybean, especially cultivar 'Peking', in response to infection with *A. tumefaciens*, but not to the extent observed in other dicotyledonous plants such as tobacco. Recovery of transgenic plants at 1.7% transformation frequency was obtained when a partially disarmed (oncogenic) *Agrobacterium* strain pKYRT with a functional T_R-DNA sequence in order to stimulate embryogenesis, was used on immature cotyledons (Ko *et al.* 2004). Tumorigenesis of soybean is a quantitative trait (Bailey *et al.* 1993a) and the heritability estimates are higher than 50% (Mauro *et al.* 1995). This characteristic could easily be transferred to new genotypes. However, genotype differences for tumorigenesis are not necessarily a reflection of the frequency of integration or T-DNA expression (Facciotti *et al.* 1985), and it can later be manifested through oncogenic expression (van Wordragen *et al.* 1992). Predictions of gene integration and expression are, thus, more accurate using marker genes (van Wordragen *et al.* 1992).

The development of *Agrobacterium*-mediated transformation techniques was slow in the late eighties to early nineties, but in a few cases transgenic plants have been obtained. The first experiments describing successful recovery of transformed soybean plants using *Agrobacterium* were reported by Hinchee *et al.* (1988). They produced stable transgenics via shoot organogenesis from cotyledon explants of the cultivar 'Peking'. Cotyledon explants were inoculated with *A. tumefaciens* harbouring plasmids conferring kanamycin resistance and GUS activity or kanamycin resistance and glyphosate tolerance. This protocol, however, only yielded a 6% transformation efficiency. Cultivar 'Peking' was introduced to the USA in 1906. It has limited agronomic value (Mauro *et al.* 1995) but was selected for its susceptibility to *Agrobacterium* infection. This *Agrobacterium* procedure did not result in the recovery of transformed progeny in varieties other than 'Peking'. A number of other laboratories attempted to reproduce the system, but the regenerated plants were chimeric, and the transgenes were not transmitted to the progeny (Parrott *et al.* 1989; Christou 1997). Soybean protoplasts have been transformed at a low frequency by using *Agrobacterium* (Baldes *et al.* 1987). However, no transgenic plants were regenerated from these transformed protoplasts since no regeneration systems were available. Infection by needle inoculation of the plumule, cotyledonary node and adjacent cotyledon tissue of germinated seeds was reported (Chee *et al.* 1989). *In vitro* grown seedlings of 'Forrest' was inoculated with a co-integrate vector containing oncogenes of pTiA6 (Facciotti *et al.* 1985). Co-cultivation of immature zygotic cotyledons as explants with *Agrobacterium* were also reported (Ko *et al.* 2006). The somatic embryos were plated on selective media, followed by maturation and regeneration of individual somatic embryos into whole plants with an efficiency of 1.7%.

Agrobacterium-mediated success stories include a number of patents filed on the technology. Martinell *et al.* (2002, 2006) reported the transformation of individual cells in a freshly germinated soybean meristem as well as immature embryo axes, which can be induced directly to form shoots that give rise to transgenic plants. Their method does not involve a callus-phase. Wounded cotyledonary explants were transformed in the region of the axillary meristematic cells or cotyledonary node explants (Olhoft *et al.* 2005, 2006). The cotyledon was prepared for transformation by removing the hypocotyl region, splitting and separating the hypocotyl segment or by removing the epicotyl. A method for producing a stable transformed soybean was discussed where *Agrobacterium*-mediated gene delivery was made into the cells at the primary leaf base or in the area of the primary leaf break point (Khan *et al.* 2003). The shoot induction process facilitates the development or regeneration of transformed shoots from the targeted primary leaf base cells. *Agrobacterium* mediated transformation was also successful when using cotyledonary nodes as explants with transformation efficiency in 12 tested cultivars ranging from 2-6% if glufosinate was used as selective agent (Paz *et al.* 2004), Paz *et al.* (2006) described an improved cotyledonary node method using a "half-seed" explant for *A. tumefaciens*-mediated soybean transformation. They experienced a transformation efficiencies of 3.8% based on the number of transformed events that have been confirmed in the T1 generation by phenotypic assay using the herbicide

Liberty® (active ingredient glufosinate) and by Southern blot analysis.

The development of a method to obtain transformed plants, which is independent of the problems inherent to tissue culture, has been the dream of many laboratories. A non-tissue culture approach to *Agrobacterium*-mediated transformation using germinating seed of *Arabidopsis thaliana* was adapted by Chee *et al.* (1989), who succeeded in transforming soybean meristematic or mesocotyl cell tissues from germinated soybean seed with a needle inoculation *Agrobacterium*-mediated transformation technique. This method depends on the growth of preformed shoots. de Ronde *et al.* (2001) produced transgenic soybean plants by using germinated seed and a vacuum infiltration *Agrobacterium*-mediation method.

Other transformation methods

Trick and Finer (1997) developed a new and potentially more effective method for the delivery of *Agrobacterium* to plant target tissues that was termed sonication-assisted *Agrobacterium*-mediated transformation (SAAT). This method mechanically disrupts and wounds cells via brief periods of ultrasound in the presence of *Agrobacterium*. Immature cotyledons and embryogenic suspension cultures were inoculated and sonicated and co-cultured in a maintenance media containing acetosyringone (Trick and Finer 1998). This method was later applied with success on cotyledonary nodes (Meurer *et al.* 1998) and on an embryonic tip regeneration system (Liu *et al.* 2004). SAAT resulted in efficient transformation of the total tissue surface, unlike particle bombardment where DNA-coated particles are delivered only to one side of the target tissue and with limited penetration (Trick and Finer 1998).

The WST method was used to deliver the pUHG plasmid into soybean embryogenic tissues by using whisker of potassium titanate fibers with an average diameter of 0.5 μm and length ranging in length from 3- 50 μm (Khalafalla *et al.* 2006). Hygromycin resistant transgenic lines were developed using this system.

General comparison of transformation methods

It has been claimed in the past that the DNA integration patterns in transformed plant tissue obtained via particle bombardment tend to be highly variable with multiple insertion events and that fragmented copies of the introduced gene constructs are common (Hadi *et al.* 1996). However, recent detailed structural analysis of transgene loci have indicated that particle bombardment does not generate more rearranged or broken transgene copies than *Agrobacterium* mediated methods (Altpeter *et al.* 2005). A major advantage of particle bombardment is that the delivered DNA can be manipulated to influence the quality and structure of the resultant transgene loci and is not limited by cell type or genotype (Altpeter *et al.* 2005). In contrast to particle bombardment, *Agrobacterium*-mediated transformation results in lower copy number integration (Tinland *et al.* 1994). Multiple gene co-transformation is possible using particle bombardment (Altpeter *et al.* 2005). The ratio of plasmids in co-transformation influenced the number of transgenic plants which can be recovered, as was observed by Hadi *et al.* (1996) with the simultaneously insert of 12 transgenes into soybean callus. Seventy-three percent of the transgenic callus lines had integrated all 12 plasmids. Particle bombardment is the most efficient method to achieve plastid transformation (Dufourmantel *et al.* 2005, 2006).

Transgenic plants obtained through the SAAT procedure (Trick and Finer 1998) were sterile as a result of the use of long-term embryogenic suspension cultures as also previously described by Hadi *et al.* (1996). The sterility is a function of the tissue culture process and not of the transformation process. The transformation efficiency of WST was compared to particle bombardment in two independent experiments by using same genotypes, plasmid pUHG (SK), containing hpt (hygromycin) and sGFP genes, and tissue culture systems (Khalafalla *et al.* 2006). They demonstrated that WST resulted in transgenic plants containing higher copy number for both the genes, but as high a transient expression of sGFP (S65T) as that obtained with particle bombardment. The WST method is, thus, as efficient as particle bombardment for soybean transformation.

Some aberrations observed in bombarded transgenic soybean include stunted plant growth, leathery dark green leaves, partiality to total sterility, chromosomal deletions, duplications, trisomics and tetraploidy (Singh *et al.* 1998) as observed after cytological examination of suspension cultured derived lines 'A2242' and 'A2872'. Plants regenerated from relatively old suspension cultures also showed a range of phenotypic abnormalities. Transgenic plants derived by particle bombardment have a tendency to have multiple integration events (Reddy *et al.* 2003) or rearrangement of the transgenes and are, therefore more susceptible to gene silencing (El-Shemy *et al.* 2004).The latter observed more gene silencing in transgenic plants with more complex transgene integration than with plants with low copy number.

IMPROVEMENT OF RESISTANCE TO ABIOTIC STRESSES AND HERBICIDES

Adverse environmental conditions such as extreme low and high temperatures as well as drought and salinity, significantly affect growth and development and, thus, crop productivity. Subsequently, improvement of tolerance against abiotic stresses is very important for agriculture and genetic transformation of soybean is a powerful tool for this purpose.

Heat tolerance

In the control of the response of plants to high temperature stress heat shock transcription factors, regulating heat shock proteins, play an important role. Over-expression of the endogenous soybean heat shock transcription factor gene, *GmHsfA1*, having a constitutive expression profile in the different tissues examined, led to the activation of the heat shock protein GmHsp70 under normal temperature and to the higher expression of the corresponding gene, *GmHsp70*, under high temperature in soybean (Zhu *et al.* 2006). Besides GmHsp70, over-expression of the *GmHsfA1* gene may influence the expression levels of several other proteins, however, this hypothesis has yet to be investigated. The transgenic soybean plants exhibited enhanced thermotolerance compared to the wild type. Although the manipulation of the expression of regulatory proteins may have a much greater effect on stress tolerance compared to that of structural genes, over-expression of a gene involved in proline synthesis increased the tolerance of soybean to combined heat and drought stress (de Ronde *et al.* 2000, 2001). These results are described in more detail later in this review.

Drought tolerance

Drought tolerance of soybean was genetically manipulated by transformation of germinating seed with the *Arabidopsis* gene coding for L-Δ(1)-pyrroline-5-carboxylate reductase, the last enzyme of proline biosynthesis. This was achieved by using an *Agrobacterium*-mediated vacuum infiltration procedure. Proline plays an important role in the osmotic adjustment during water shortage (de Ronde *et al.* 2000, 2001, 2004a, 2004b). A heat-inducible promoter was used in order to ensure the controlled switching on of the gene. Under natural conditions drought stress often coincides with high temperature, therefore for more accurate simulations of field stress effects and activation of the promoter, withholding of water was carried out at supra-optimal temperatures. In the sense orientation the transgene increased

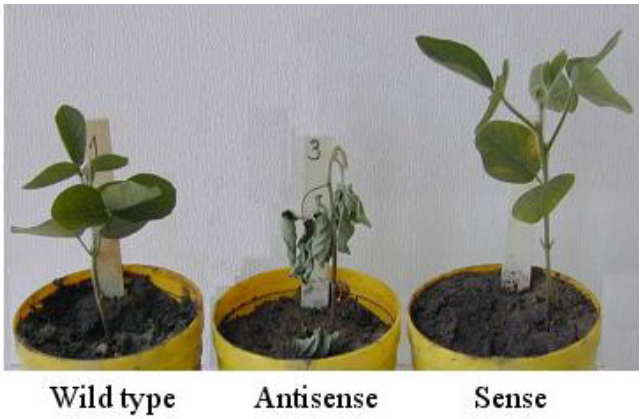

Fig. 1 Recovery of wild type and transgenic soybean after drought stress at supra-optimal temperature. The soybean plants were transformed with a construct containing a heat-inducible promoter and the gene coding for L-Δ(1)-pyrroline-5-carboxylate reductase, the last enzyme of proline biosynthesis, in sense and antisense direction. Plants were grown without watering for 10 days (35/25°C), rewatered once, further cultivated for additional 10 days without watering (35/25°C) and finally applied with an optimal amount of water for 10 days (25/15°C, recovery).

proline content and drought tolerance, while in the antisense orientation it resulted in reduced proline content and reduced drought tolerance compared to the wild type plants (**Figs. 1, 3B**). Proline degradation was also recorded in these experiments with the highest proline dehydrogenase activity being measured in antisense transformants and the lowest in the sense transformants (de Ronde *et al.* 2004a). In addition, antisense plants had lower seed production compared to the wild type (de Ronde *et al.* 2001). Changes in proline synthesis in transgenic plants also affected the carbohydrate levels (de Ronde *et al.* 2004a). In antisense plants an increase in sucrose content was observed, while in the sense plants a rise in reducing sugars was observed. Manipulation of proline levels also influenced the amino acid composition of soybean, since the synthesis of the different amino acids is interconnected and probably coordinately regulated (Simon-Sarkadi *et al.* 2005, 2006a). Drought stress resulted in a greater increase in the level of the proline precursor glutamate (**Fig. 2**) in sense transformants compared to the antisense transformants and wild type plants (**Fig. 3A**). Glutamate is also a precursor of polyamines (**Fig. 2**), thus, it is not surprising that the higher proline levels in the transformants compared to the wild type plants coincided with a smaller difference in alterations of spermine content between these two genotypes, except at day 4 of stress (**Fig. 3**). The greatest increase in glutamate was observed at the end of the stress treatment in the antisense plants without a corresponding change in proline or spermine content when compared to the other two genotypes (**Fig. 3A, 3C**). However, in the antisense transformants a sharp increase in the concentration of homo-glutathione, synthesized from glutamate, was observed at this sampling point (**Figs. 2, 3D**). In the sense transformants, smaller changes in homo-glutathione concentration were detected compared to that in the wild type plants. These results demonstrate that manipulation of proline affects the level of several other metabolites that are interconnected with proline synthesis (Kocsy *et al.* 2005; Simon-Sarkadi *et al.* 2005, 2006a, 2006b).

Use of stress-inducible promoters to increase stress tolerance

Stress-inducible promoters, as in the case of drought stress, are important for improvement of stress tolerance, since it is not advantageous for the plants to waste energy in the transcription of defense genes under optimal growth conditions as when a constitutive promoter is used. The stress-inducible promoter of soybean alcohol dehydrogenase was investigated using the glucuronidase reporter gene following transformation of soybean cotyledons by an *Agrobacterium*-mediated method (Preiszner *et al.* 2001). The transcription was only induced by anoxia, but not by cold, wounding or ABA, therefore, this promoter can be used to control the expression of transgenes for the improvement of tolerance to flooding or hypoxia. Using the same reporter gene, the inducibility of the soybean small heat shock protein gene promoter, *GmHSP 17.5E*, was compared in dif-

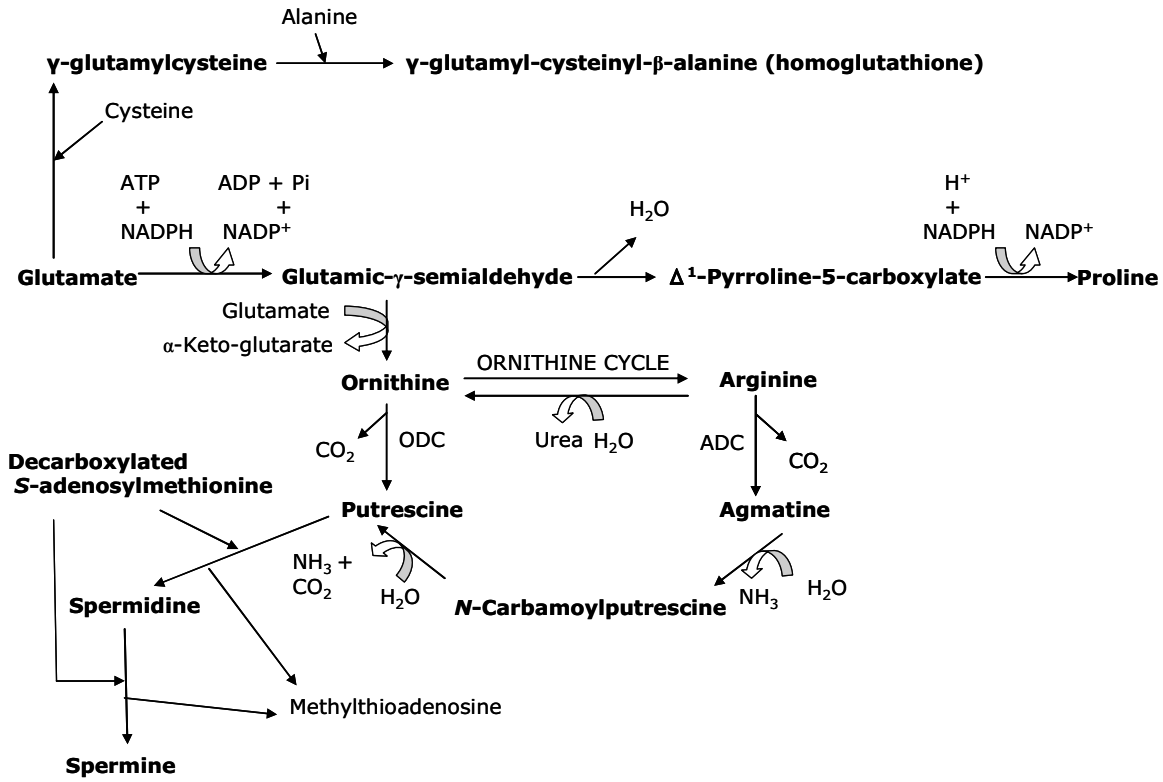

Fig. 2 Interconnection of proline, polyamine and homoglutathione synthesis. ADC, arginine decarboxylase; ODC, ornithine decarboxylase.

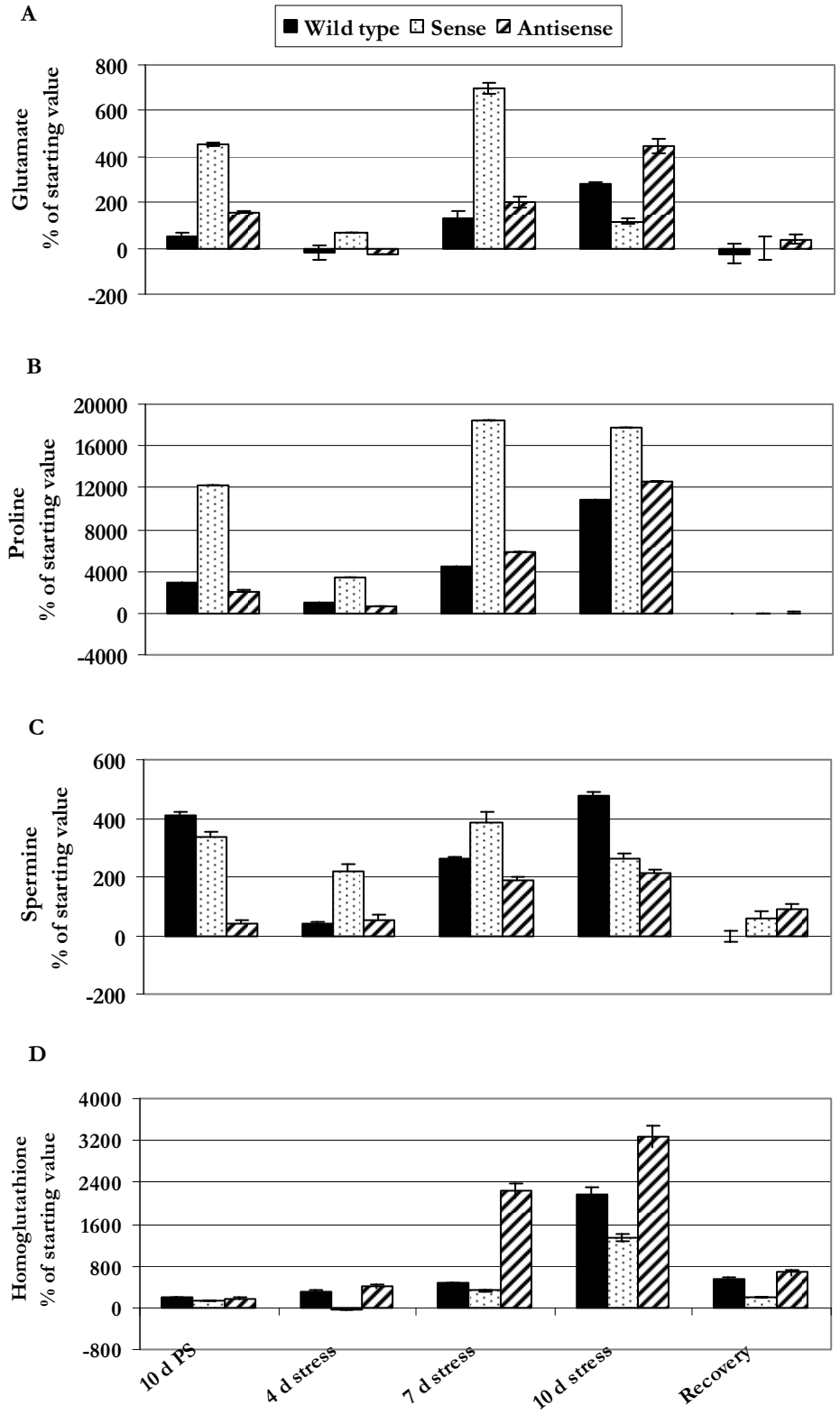

Fig. 3 Effect drought stress at supra-optimal temperature on relative changes in glutamate (A), proline (B), spermine (C) and homoglutatione (D) content. Starting values (Glu: 245.4, 167.5, 227.5; Pro: 90.4, 56.5, 91.0; Spm: 36.7, 25.5, 39.6; hGSH: 61.7, 38.6, 58.9 µg (g fresh weight)$^{-1}$ for wild type (W), sense (S) and antisense (A) transgenic soybean) were taken as 100%, and the stress-induced changes as their percentages are shown. Plants were grown without watering for 10 days (PS, preliminary stress), rewatered once, further cultivated for additional 10 days without watering (stress) and finally applied with an optimal amount of water for 10 days (recovery).

ferent organs of *Arabidopsis* using transient assays after micro-projectile particle bombardment (Crone *et al.* 2001). Following heat shock, gene expression was uniform in vegetative organs, but not in the reproductive organs. This was explained by the more complex regulation of the stress response in the floral organs compared to that in vegetative tissues. Using the same promoter:reporter gene construct in soybean, a similar difference in expression between the different organs can be anticipated. The observed tissue-specific gene expression can be used for protection of the more stress-sensitive organs.

Adaptation to extreme mineral concentration

Too low or too high concentrations of certain minerals can also result in disturbed growth and development of plants.

Expression of *Arabidopsis* ferric chelate reductase in soybean enhanced Fe^{3+} reduction in roots and shoots, led to reduced biomass loss, chlorosis and increased chlorophyll concentrations under iron deficiency compared to the wild type plants (Vasconcelos *et al.* 2006). However, in this study a constitutive promoter was used and the expression of the transgene under non-iron stress conditions resulted in decreased plant productivity. Phosphorous availability is also important for normal metabolism in plants. Improved mobilization of phosphorous from phytate (75% of total P is stored in this compound) in seed was ensured by overexpression of a soybean phytase gene (Chiera *et al.* 2004).

Herbicide resistance

Besides the unfavorable environmental conditions, the herbicides used to kill weeds can also reduce the growth and development of cultivated plants, therefore the generation of herbicide-resistant genotypes by genetic transformation is of importance to agriculture (Wenzel 2006). The herbicide glufosinate was successfully used as a selective agent following *Agrobacterium*-mediated transformation of soybean (Zeng *et al.* 2003). One of the first practical applications of genetic engineering of soybean was the development of tolerance to glyphosate, the active component in the herbicide Roundup (Padgette *et al.* 1995). Glyphosate-resistant soybean expressing an insensitive 5-enolpyruvylshikimic acid-3-phosphate synthase gene is commercially available and is useful for weed control (Pline-Srnic 2005). The deployment of glyphosate-tolerant soybean has permitted a switch to a more environmentally friendly herbicide, since it has facilitated the adoption of no-till agricultural practices. Production of glyphosate-resistant crops can result in lower soil erosion rates, less water runoff, increased soil moisture, increased carbon sequestration and less CO_2 emissions due to the reduced use of fossil fuel (Fawcett and Towery 2002). The implication of this technology was monitored over a period of several years in the field (Clemente *et al.* 2000; Duke *et al.* 2003; Arregui *et al.* 2004). The concentration of glyphosate and its metabolite, aminomethylphosphonic acid, was monitored in soil and water, in which no residues were observed, as well as in leaves, stems (1.9-4.4 mg residue kg^{-1} leaf or stem) and seeds (0.1-1.8 mg residue kg^{-1} seed) of glyphosate-resistant soybean sprayed with the herbicide (Arregui *et al.* 2004). Although application of glyphosate can reduce nitrogen fixation in early growth stages due to the sensitivity of the nitrogen fixing symbiont *Bradyrhizobium japonicum* to the herbicide, a reduction in yield was not observed (Zablotowicz and Reddy 2004). Similarly, in another experiment glyphosate formulations reduced nodule development and caused injury of the plants. However, the glyphosate-resistant soybean plants recovered from the herbicide stress (Reddy and Zablotowicz 2003). No differences were observed in host plant suitability to green cloverworm (*Hypena scabra*) between glyphosate-resistant and wild type soybean (Morjan and Pedigo 2002). Glyphosate field contamination is minimal compared to other herbicides and no risks to food or feed safety were observed (reviewed by Cerdeira and Duke 2006). The use of glyphosate at legal concentrations resulted in the amount of the compound and its degradation products to be within the established tolerance levels in seed. Its toxicity was tested by oral application to rats where no negative effects were found. Resistance to the herbicide phosphinothricin was recently reported following the introduction of the resistance genes into soybean by inoculation of wounded germinating half-seeds with *Agrobacterium* (Xue *et al.* 2006). Atrazine resistance was also introduced into soybean using the *psbA* gene from *Solanum nigrum* (Yue *et al.* 1990).

It can be seen from the cited studies that genetic transformation of soybean can successfully be used to increase tolerance to adverse environmental conditions as well as to herbicides which in turn reduces yield loss.

IMPROVEMENT OF RESISTANCE TO BIOTIC STRESSES

Different pathogens reduce plant growth or even result in the death of the host organism, therefore, the successful protection of plants against these pathogens are of extreme importance for stabilization of yield quantity and quality. Several publications demonstrate increased resistance of transgenic soybean against viruses, fungi, insects and nematodes.

Virus resistance

Transgenic soybean plants resistant to soybean dwarf virus were generated by the introduction of an inverted repeat viral coat protein gene into soybean somatic embryos by micro-projectile bombardment (Tougou *et al.* 2006). Soybean plants conferring high resistance to soybean mosaic virus were produced by introduction of the coat protein gene from the virus (Wang *et al.* 2001; Steinlage *et al.* 2002; Furutani *et al.* 2006). Mosaic virus-resistant transgenic plants had lower infection rates and significantly higher yields in field experiments (Steinlage *et al.* 2002). Protection against *Bean pod mottle como virus* was achieved by *Agrobacterium*-mediated transformation of cotyledonary nodes with the coat protein precursor gene of the virus (Di *et al.* 1996) or by particle bombardment of somatic embryos with the capsid polyprotein gene (Reddy *et al.* 2001). Homozygous progeny exhibited a resistant phenotype in both methods. In addition, Reddy *et al.* (2001) observed systemic resistance of the transgenic lines (obtained by the insertion of 1-3 copies of the transgene by particle bombardment of somatic embryos), since after incubation of leaves with the virus, the non-inoculated leaves were symptom-less and accumulated little or no virus. These lines could potentially be useful in generating commercial cultivars resistant to *Bean pod mottle como virus*.

Resistance against fungi

The resistance of soybean against fungi was also successfully increased by genetic transformation. Introduction of the gene coding for an oxalate-degrading enzyme, oxalate oxidase, has resulted in the reduced growth of white mold (*Sclerotinia sclerotinium* (Lib.) de Bary) in laboratory experiments, since oxalate is an important pathogenic factor for the fungus (Donaldson *et al.* 2001; Cober *et al.* 2003). Oxalate oxidase expressing plants were produced by *Agrobacterium*-mediated cotyledonary node transformation. One to two copies of the gene of the enzyme were inserted into the plant genome, with partial or complete gene silencing occurring in some of the T_2 population (Donaldson *et al.* 2001). Increased resistance of transgenic lines against white mold was confirmed in field trials that were conducted at 3 different sites (Cober *et al.* 2003). In non-infected trials, no significant differences were observed between the parental and transgenic lines for seed yield, seed maturity, seed weight as well as seed protein and oil content. *Agrobacterium*-mediated transformation of cotyledon explants of two soybean cultivars with a chitinase gene from bean, exhibitted increased resistance against *Rhizoctonia solani* as indicated by the comparison of mycelial growth in wild type and transgenic plants (Salehi *et al.* 2005). In another approach, a chitinase gene and a ribosome-inactivating protein gene were stacked in order to increase insect resistance of transgenic plants (Li *et al.* 2004). Transformation of soybean by particle bombardment of somatic embryos with the fungal elicitor-induced *ELI12* gene from parsley, resulted in the accumulation of crepenynic and dehydrocrepenynic acids in the seed (Cahoon *et al.* 2003). Natural products synthesised from these acids display not only antifungal, but also insecticidal and nematicidal properties.

Insect resistance

Transgenic soybean lines maintaining increased resistance to insects under field conditions are grown on a commercial scale (Babu *et al.* 2003). Use of endotoxin genes, such as the one from *Bacillus thuringiensis* (*Bt*) and plant-derived genes coding for proteinase inhibitors at the desired expression levels, to control insect pests still require considerable resources. Transgenic soybean (with 1-2 copies of the transgene) generated from somatic embryos by micro-projectile bombardment using a synthetic *B. thuringiensis* insecticidal crystal protein gene (*Bt Cry1Ac*) were protected from corn earworm (*Helicoverpa zea*), soybean looper (*Pseudoplusia includens*) tobacco budworm (*Heliothis virescens*) and velvet bean caterpillar (*Anticarsia gemmatalis*) damage (Stewart *et al.* 1996; Walker *et al.* 2000; Parrott and Clement 2004). Less than 3% defoliation was observed on transgenic plants, compared to 20% on a lepidepteran-resistant breeding line and 40% on a susceptible cultivar, due to corn earworm infection. Soybean plastid transformants, obtained by particle bombardment of embryogenic tissue, expressing the *B. thuringiensis* insecticidal protoxin Cry1Ab demonstrated increased resistance against the velvet bean caterpillar (Dufourmantel *et al.* 2005). High levels of protoxin were detected in leaves, stems and seed, but not in the roots. Transgenic lines of soybean expressing a synthetic *Cry1Ac* gene (*tic107*) from *B. thuringiensis* were completely protected against *Anticarsia gemmatalis* (Hubner), *Pseudoplusia includens* (Walker), *Epinotia aporema* (Walsingham), *Rachiplusia nu* (Guenee), and *Spilosoma virginica* (F.) in greenhouse tests (Macrae *et al.* 2005). These results were also confirmed in field trials against native populations of *A. gemmatalis* and *P. includens*.

Resistance against nematodes

The resistance of soybean against nematodes was also successfully increased by genetic engineering. The soybean cyst nematode (*Heterodera glycines*) results in a great reduction in soybean production. In root tissue of transgenic soybean transformed with an RNAi expression vector containing inverted repeats of a gene coding for the sperm protein of soybean cyst nematode, a 68% reduction in egg deposits was observed in T_0 plants and a 75% reduction in their progeny (Steeves *et al.* 2006). This study demonstrates the efficiency of the RNAi-based strategy for control of soybean cyst nematode infection. In another study an effective suppression of the population densities of the nematode *Hoplolaimus columbus* was demonstrated using transgenic soybean cultivars resistant to glyphosate after treatment with the herbicide in field experiments (Koenning 2002). Fumigation increased soybean yield, but there was a variation among cultivars in the response to *H. columbus*. This study indicated a cross tolerance to the different environmental stresses.

As was the case with abiotic stress, resistance to biotic stress can also be successfully increased by genetic transformation of soybean.

MANIPULATION OF METABOLITE COMPOSITION

The over- or under-production of certain metabolites could be useful for production of food or feed, or industrial raw materials fulfilling specific requirements.

Manipulation of fatty acid composition

Soybean oil is an important raw material for several industrial products. The modification of its fatty acid composition by genetic manipulation expands its application possibilities (reviewed by Cahoon 2003). Down-regulation of *FAD2* (the enzyme that converts the monounsaturated oleic acid to the polyunsaturated linoleic acid) expression increased the oleic acid content to 80% of total oil (Kinney 1997). High oleic acid content improves the oxidative stability of the oil which is important for lubricants. In contrast to high oleic acid content, an increase in the polyunsaturated linoleic acid content by over-expression of the *FAD3* gene (the enzyme converts linoleic acid to linolenic acid) resulted in a low oxidative stability which is desirable for drying oils used in coating applications (Cahoon 2003). By genetic manipulation, not only can the ratio of fatty acids existing in soybean be manipulated, but the production of new fatty acids originally not present in soybean can be achieved. Expression of a Δ(12)-oleic acid desaturase-related fatty acid conjugase gene from *Calendula officinalis*, coding for an enzyme catalyzing the formation of conjugated double bonds in polyunsaturated fatty acids, led to the accumulation of calendic acid in soybean (Cahoon *et al.* 2001, 2006). Oils with high calendic acid content can also be used as drying oils. The transgenic production of industrially valuable epoxy and hydroxylated fatty acids in soybean seed by expression of the gene coding for the cytochrome P_{450} enzyme from *Euphorbia lagascae* seed, was also investigated (Cahoon *et al.* 2002). Transformation of soybean with a bifunctional Δ12/ω3 desaturase from *Fusarium* species increased the amount α-linolenic acid, as well as the ratio of α-linolenic acid to linoleic acid, in the seed many-fold compared to the wild type (Damude *et al.* 2006). Introduction of the Δ5 desaturase and fatty acid elongase meadowfoam (*Limnanthes* spp.) genes into somatic soybean embryos resulted in the production of long chain fatty acids (Δ5 eicosenoic acid and a diene) which could be used in the synthesis of cosmetics and lubricants (Marilia *et al.* 2002). The fatty acid composition could also be modified in order to enhance the nutritional quality of soybean seed (Murphy 2006). Arachidonic acid, which is important for infant brain development, inflammatory responses, blood pressure regulation and cell signalling, was produced in transgenic soybean (generated by particle bombardment) following seed-specific expression of the genes coding Δ6 desaturase, fatty acid elongase and Δ5 desaturase from a filamentous fungus, *Mortiella alpine*, and down-regulation of the endogenous Δ15 desaturase gene (Chen *et al.* 2006). Stearidonic acid, having pharmaceutical potential, was produced in seed of soybean transformed with the borage Δ6 desaturase and *Arabidopsis* Δ15 desaturase genes (Eckert *et al.* 2006). Marker-free transgenic soybean producing stearidonic acid and γ-linolenic acid in the seed were also generated with an *Agrobacterium*-mediated transformation method (Sato *et al.* 2004). Besides the fatty acids existing in nature, foreign fatty acids could also be produced in soybean by the introduction of genes of rationally designed fatty acid modifying enzymes (Cahoon and Shanklin 2000). The aims of these studies were to induce the accumulation of foreign fatty acids without reduction of the agronomic quality of the transgenic seed. Such traits can more readily be increased by genetic engineering than by traditional breeding.

Manipulation of the concentration of certain amino acids

The concentrations of certain amino acids were also altered in transgenic soybean. The lysine content in seed of soybean was increased by circumventing the feedback regulation of its synthesis by transformation with the bacterial genes of aspartokinase and dihydrodipicolinic acid synthase (Falco *et al.* 1995). The nutritional quality of soybean seed could be improved by increasing the ratio of sulfur containing amino acids, methionine and cysteine. This was achieved by insertion of the zein gene from maize under control of the seed-specific β-phaseolin promoter (Dinkins *et al.* 2001). Following transformation of soybean with the zein gene, Kim and Krishnan (2004) found increased methionine content only in the alcohol-soluble protein fraction, but not in the seed flour. The increase in methionine and cysteine was achieved without changes in the protein composition (Dinkins *et al.* 2001). Contrary to this observation,

genetic manipulation of proline content (de Ronde *et al.* 200, 2001, 2004a, 2004b) affected the concentration of several other amino acids (Simon-Sarkadi *et al.* 2005).

Production of proteins

Transgenic soybean can also be used efficiently for the production of different proteins important for food or feed quality, or of importance to the pharmaceutical industry (Kinney 2003). Before using a gene construct for transformation, the ability of the recombinant proteins to assemble into functioning three dimensional structures should be checked using *in vitro* translation systems as described in the case of molecularly manipulated glycine subunits with increased methionine content (Sammour 2006). Down-regulation of genes coding for vegetative storage proteins using an antisense construct had no negative effect on yield, protein, oil and amino acid composition in field trials. Therefore, these storage proteins could be replaced by genetic engineering for other agronomically important proteins (Staswick *et al.* 2001). By testing the purification efficiency of recombinant proteins from soybean seed accumulating β-glucuronidase, a 100% recovery rate was achieved which demonstrates the suitability of this system for the production of proteins (Robic *et al.* 2006). Suppression of the β-conglycinin subunits by gene silencing in the seed resulted in the accumulation of another storage protein, glycinine, which accumulated in the endoplasmic reticulum-derived vesicles instead of Golgi-derived vesicles (Kinney *et al.* 2001). By transformation with the soybean seed lectin promoter fused to a gene of bovine milk protein, β-casein, accumulation of β-casein in the seed was achieved (Philip *et al.* 2001).

Production of pharmaceutically useful compounds

Pharmaceutically useful compounds can also be produced using transgenic soybean. The promoter of the glycinin gene coding for a soybean seed storage protein was successfully used to ensure the seed-specific production of human basic fibroblast growth factor (Ding *et al.* 2006). The accumulation of the growth factor reached 2.3% of total soluble protein and it was biologically active. Soybean could also be used for the production of secretary IgA antibodies, the production of which is significantly cheaper in plants compared to steel tank bioreactors using mammalian cells or micro-organisms (Larrick *et al.* 2001). Another example of a pharmaceutical application of transgenic soybean is the increased production of isoflavones by the combination of transcription factor-driven (maize C1 and R transcription factors) gene activation and suppression of the competing (flavonone 3-hydroxylase) pathway (Yu *et al.* 2003). Isoflavones are plant estrogens which can slow or reverse the symptoms of osteoporosis.

Manipulation of carbohydrate metabolism

Carbohydrates play an important role in stress tolerance and in metabolism, therefore, genetic manipulation for controlling of their concentration is of importance in plant breeding. Cyclitol production was manipulated in pinitol-producing glycophytic soybean by introduction of the *Imt* (inositol methyl transferase, converts *myo*-inositol to ononitol) gene from the halophytic ice plant (*Mesembryanthemum crystallinum*) into embryogenic tissue by particle bombardment (Chiera *et al.* 2006). In transgenic soybean embryos, ononitol and pinitol (produced from ononitol) concentrations were higher compared to the wild type. However, in the leaves of mature plants no differences were observed.

Manipulation of antioxidant levels

The manipulation of antioxidant levels in soybean could improve stress tolerance and in the case of a seed-specific expression of the transgene, could have significant health benefits. α-Tocopherol efficiently prevents the peroxidation of membrane lipids, however, in soybean seed its main precursor, the less bioactive γ-tocopherol, is present. Seed-specific expression of γ-tocopherol methyltransferase from *Perilla frutescens* in soybean resulted in a significant increase in α-tocopherol content and reduced lipid peroxidation during germination compared to the wild type (Tavva *et al.* 2007). Transformation of soybean with two *Arabidopsis* genes involved in α-tocopherol synthesis (seed-specific expression) resulted in a more than eight-fold increase in α-tocopherol and a five-fold increase in vitamin E content of seed which is of importance to nutritional value and food quality (van Eenennaam *et al.* 2003).

The cited examples demonstrate that genetic manipulation of soybean can be used to alter the concentrations of certain metabolites in order to fulfil the requirements of food and feed production, as well as in the industrial applications of soybean.

STUDY OF DEVELOPMENT

Over- or under-production of various metabolites in transgenic soybean can assist in the understanding of their roles during different developmental stages. The role of a sucrose-binding protein was studied in plants containing the corresponding gene in the antisense orientation (Waclawosky *et al.* 2006). Significant reductions in photosynthesis and stomatal conductance were observed in these lines, but this was restricted to the reproductive phase. Sucrose content decreased both in source and sink leaves, while a reduction in starch concentration was observed only in the sink leaves. In another study the role of a soybean receptor-like kinase (GmSARK) in the regulation of leaf senescence was demonstrated (Li *et al.* 2006). RNAi-mediated knocking out of the *GmSARK* gene retarded leaf senescence and the disintegration of chloroplast structure, while over-expression of this gene accelerated senescence. The RNAi-method was also successfully used to silence the *myo*-inositol-1-phosphate gene and to demonstrate the involvement of the corresponding protein in seed development, since in transgenic plants the formation of seed was absent (Nunes *et al.* 2006). Organogenesis of legume root nodules could also be investigated using transgenic soybean (Kouchi *et al.* 1999). The promoter of the early nodulin gene from rice was fused to the *gus*-reporter gene and the expression of the gene was demonstrated in peripheral cells of soybean nodules (developed on hairy roots). Another example for the use of transgenic soybean for developmental studies is the demonstration of the transposition of the maize controlling element Ac in transgenic soybean (*Agrobacterium*-mediated transformation) calli, leaves, stems and roots (Zhou and Atherly 1990).

Transgenic soybean plants provide the opportunity to elucidate new insight in physiological and genetic processes during plant development.

CONTROL OF POSSIBLE DISADVANTAGEOUS AFFECTS OF GENETIC TRANSFORMATION

Before using transformed plants for food, feed or industrial raw material production, it is important to check the possible side-effects of transgenes on metabolism. A feeding study on salmon that receiving a diet prepared from glyphosate-resistant and wild type soybean, demonstrated that there were no differences between the groups of fish as far as growth, body composition, relative organ weights, plasma nutrient concentrations and enzyme activities (Sanden *et al.* 2006) were concerned. Allergenicity of glyphosate-resistant soybean was tested in two sensitive human groups, children with food and inhalant allergies and individuals with asthma-rhinitis. No difference in the reaction to transgenic and wild type soybean was observed (Batista *et al.* 2005). Transgene-induced gene silencing suppressed the

accumulation of Gly m Bd 30 K protein, an immuno-dominant allergen in soybean (Herman *et al.* 2003). The unfavorable consequences of genetic engineering was demonstrated after introduction of the 2S albumin gene from Brazil nut into soybean, since the allergenic effect of the nut (observed only in an extremely low percentage of the population) was also observed in soybean (Nordlee *et al.* 1996).

The studies cited show that in most cases, there are no adverse effects of transgene expression, but that unfavorable consequences of genetic transformation can also be observed in certain instances.

SCREENING FOR THE PRESENCE OF TRANSGENES

For safety and economic reasons it is necessary to clearly distinguish transgenic from non-transgenic plant material. A multiplex-PCR method coupled with oligonucleotide microarrays proved to be appropriate for a rapid and cost-saving screening of transgenic soybean with a 0.5% detection limit (Xu *et al.* 2006). A quantitative-competitive PCR test was successfully used for detection of the transgene providing glyphosate resistance in soybean (Dinelli *et al.* 2006). The peptide nucleic acid microarray approach was also successfully applied for the detection of the transgene in glyphosate-resistant soybean (Germini *et al.* 2004). Another methodology for the rapid determination of transgenes is the bio-specific interaction analysis with surface plasmon resonance (SPR) and biosensor technology which allows for real-time monitoring of the hybridization between oligonucleotide or PCR-generated probes and target single-stranded PCR-products derived from DNA of transgenic soybean (Gambari and Feriotto 2006).

These methodologies provide sensitive protocols for detection of transgenes in transgenic soybean-derived products. This is of extreme importance for food and feed production, and in industrial applications of the transgenic plants.

CONCLUSIONS

Efficient plant regeneration and transformation (biolistic and *Agrobacterium*-mediated) methodologies have been established for soybean which allow for organ-specific or stress-inducible expression of transgenes. Genetically modified soybean with increased stress and herbicide tolerance as well as modified seed protein, fatty acid and other metabolite content, is available for agricultural and industrial purposes. It could be expected that in future entire regulons will be modified by the introduction of transcription factor genes as described in the case of isoflavones (Yu *et al.* 2003) and heat shock proteins (Zhu *et al.* 2006). However, the extensive control of possible unfavourable side effects is necessary, since the expression of a number of genes could be altered.

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