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Review Article

Soybean genetic transformation: A valuable tool for the functional study of genes and the production of agronomically improved plants

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Abstract

Transgenic plants represent an invaluable tool for molecular, genetic, biochemical and physiological studies by gene overexpression or silencing, transposon-based mutagenesis, protein sub-cellular localization and/or promoter characterization as well as a breakthrough for breeding programs, allowing the production of novel and genetically diverse genotypes. However, the stable transformation of soybean cannot yet be considered to be routine because it depends on the ability to combine efficient transformation and regeneration techniques. Two methods have been used with relative success to produce completely and stably transformed plants: particle bombardment and the *Agrobacterium tumefaciens* system. In addition, transformation by *Agrobacterium rhizogenes* has been used as a powerful tool for functional studies. Most available information on gene function is based on heterologous expression systems. However, as the activity of many promoters or proteins frequently depends on specific interactions that only occur in homologous backgrounds, a final confirmation based on a homologous expression system is desirable. With respect to soybean biotech improvement, transgenic lines with agronomical, nutritional and pharmaceutical traits have been obtained, including herbicide-tolerant soybeans, which represented the principal biotech crop in 2011, occupying 47% of the global biotech area.

Keywords: transgenic plants, particle bombardment, *Agrobacterium* systems, functional analysis, genetic improvement.

Economic Importance of Soybean

The economic importance of soybeans worldwide as a source of oil and meal for human and animal consumption as well as for industrial uses, such as biofuel production, has made this crop a target for genetic improvement. Conventional soybean breeding programs are frequently handicapped by the restrictive variability of its germplasm. Plant genetic transformation provides an attractive advancement for soybean breeding programs, allowing the production of novel and genetically diverse plant materials. Transgenic plants also represent a priceless tool for molecular, genetic, biochemical and physiological studies by gene overexpression or silencing, transposon-based mutagenesis, protein sub-cellular localization and/or promoter characterization.

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Biotech soybeans are the principal biotech crop, occupying 47% of 160 million hectares of the global biotech area in 2011. Brazil is currently the second largest producer of soybeans in the world after the USA and is expected to become the first in the future (James, 2011). A study by Celeres has estimated that approximately 85% of the soybean growing area was planted with biotech seeds during the 2011/2012 season. Five biotech soybean products have already been approved for commercialization by the National Technical Commission on Biosecurity in Brazil. Considering the economic relevance of the soybean for our country and the continuing challenge to overcome biotic and abiotic stresses, biotech products can have a significant impact on accelerating Brazilian breeding programs.

Soybean Transformation Procedures

Soybean transformation was first reported in 1988 (Christou *et al*., 1988; Hinchee *et al*., 1988). Even after more than two decades, the stable transformation of soybeans cannot yet be considered to be routine because it depends on the ability to combine efficient transformation

and regeneration techniques. Two methods have been used with relatively greater success to produce completely and stably transformed plants: particle bombardment and the *Agrobacterium tumefaciens* system. Both systems are equally important in basic science and agricultural applications. In addition, the transformation of soybean roots by *Agrobacterium rhizogenes* has been used as a powerful tool for functional studies.

According to Somers *et al*. (2003), there are three main requirements to establish an efficient transformation system: (a) a source of totipotent cells that serve as recipients of the delivered DNA, (b) a means of delivering DNA into the target cells, and (c) a system for selecting or identifying the transformed cells. The main advances in soybean transformation protocols are reported below.

Particle bombardment and target tissues

Genetic transformation by particle bombardment, also called particle or projectile acceleration, biolistics or biobalistics, consists of the introduction of DNA into intact cells and tissues by accelerated microparticles driven at high speeds (Sanford, 1988). Accelerated particles are able to cross the cell wall and the cell and nuclear membranes. In the nucleus, exogenous DNA fragments are liberated and may be integrated into chromosomal DNA through the processes of illegitimate or homologous recombination, which depend exclusively on cellular components (Sanford, 1990; Kohli *et al*., 2003).

The main advantage of particle bombardment lies in the possibility of transfering genes to any cell or tissue type independently of genotype and without having to consider the compatibility between the host and bacterium, as required by the *Agrobacterium* system. Particle bombardment is also quicker and easier to use. However, this technique introduces multiple DNA copies that may be fragmented or recombined (Hadi *et al*., 1996).

Since 1988 when the first transgenic soybean plant was generated (Christou *et al*., 1988), many reports describing soybean transformation by particle bombardment using meristems as the target tissue have been published (McCabe *et al*., 1988; Christou *et al*., 1989; Christou and McCabe, 1992; Aragão *et al*., 2000; Vianna *et al*., 2011). Importantly, the original apical meristem in soybean is composed of multiple cell layers (L1, L2 and L3) (Christou *et al.*, 1990; Christou and McCabe, 1992), and all three layers are involved in the production of the whole shoot, with L1 being responsible for the epidermis and L2 and L3 being responsible for the production of the more internal tissues (Sussex, 1989). This composition explains why plants resulting from the transformation of shoot meristems are often chimeric (Sato *et al*., 1993), and thus the acquisition of transgenic progeny depends on the transformation of the internal cell layer. A significant increase in the recovery of fertile transgenic soybean plants was reported by Rech *et al*. (2008), who combined the bombardment of embryogenic axes, multiple shoot induction and a selection system based on the imazapyr herbicide.

Somatic embryogenesis (SE) in soybean was first reported by Christianson *et al*. (1983) and provides an alternative system to proliferate and regenerate tissues *in vitro.* SE consists of the development of embryos from microspores or somatic tissues in a process that generates a plant without involving gamete fusion (Williams and Maheswaran, 1986).

Initially, the transformation of primary somatic embryos originated chimeric plants (Parrott *et al*., 1989) due to their multicellular nature (Finer, 1988). The latter author showed that secondary somatic embryos could be proliferated directly from the apical or terminal portions of the older primary somatic embryos and that secondary embryogenesis could be achieved by keeping the tissue in a medium rich in 2,4-dichlorophenoxyacetic acid (2,4-D). Furthermore, Sato *et al*. (1993) proved that the proliferation of secondary somatic embryos occurred from single cells of primary or secondary embryos. The unicellular origin of secondary embryos makes this tissue ideal as the main target for genetic transformation because the risk of generating chimeras is eliminated (Sato *et al*., 1993).

Somatic embryos can be regenerated and proliferated on semi-solid media (Parrott *et al*., 1988; Finer, 1988) or in liquid suspension culture media (Finer and Nagasawa, 1988). Several studies of soybean transformation via particle bombardment using embryogenic tissues have been published (Finer and McMullen, 1991; Finer *et al*., 1992; Stewart Jr *et al*., 1996; Droste *et al*., 2002; Homrich *et al.,* 2008a; Wu *et al*., 2008; Li *et al*., 2009; Hernandez-Garcia *et al*., 2009; Xing *et al*., 2010).

The use of 2,4-D and long duration culture tends to produce genetic and epigenetic variations in many plant species (Larkin and Scowcroft, 1981). In soybean, a direct relationship between somatic embryogenesis and somaclonal variation has not been demonstrated thus far. However, a cytological examination of embryogenic tissues and the recovered plants revealed various chromosomal aberrations, with the soybean genotypes differing in their susceptibility to tissue culture-induced chromosomal instability (Singh *et al*., 1998). In addition, plants regenerated from old embryogenic cultures showed problems with sterility (Hadi *et al*., 1996) and a range of phenotypic abnormalities (Singh *et al*., 1998). Therefore, the establishment and transformation of young (< 1 year old) embryogenic cultures are imperative (Trick *et al*., 1997a).

Particle bombardment can be achieved through high- or low-helium pressure gene guns, enabling the penetration of the target tissue to be controlled very accurately so that the majority of the particles carrying the DNA can be directed to a specific cell layer. This feature is extremely important because different explants may require different acceleration conditions for optimum particle penetration (Christou *et al*., 1990). Meristems (composed of multiple

cell layers) require the employment of high-pressure equipment to reach the inner layers. In contrast, secondary somatic embryos (originated from single epidermal cells) require shallow penetration, which is achieved by low-pressure bombardment (Sato *et al*., 1993). Finer *et al*. (1992) developed a particle accelerator called the *Particle Inflow Gun* (PIG), which was successfully applied in soybean somatic embryo transformation (Droste *et al*., 2002; Homrich *et al*., 2008a, Hernandez-Garcia *et al*., 2009).

Agrobacterium system

Agrobacterium is a soil-borne Gram-negative phytopathogenic bacterium that naturally infects different plants (DeCleene and DeLey, 1976). These phytopathogens cause a variety of neoplasms, including crown gall disease (*A. tumefaciens* and *A. vitis*), hairy root disease (*A. rhizogenes*), and cane gall disease (*A. rubi*) on numerous plant species (Gelvin, 2010b). The origin of these sicknesses is interkingdom horizontal gene transfer. When virulent strains of *Agrobacterium* infect plant cells, they transfer one or more segments of DNA (transferred DNA or T-DNA) from the Ti (**T**umor **i**nducing) or Ri (**R**oot **i**nducing) plasmids into the host plant cells (Gelvin, 2003). This gene transfer process was recently reviewed (Gelvin, 2010a,b; Pitzschke and Hirt, 2010).

In the last 30 years, disarmed (non-tumorigenic) *Agrobacterium* strains have provided a means to produce genetically modified plants. To obtain engineered (binary) vectors derived from Ti or Ri plasmids, genes present in the T-DNA region are replaced by foreign DNA (Gelvin, 2010b). The advantages of *Agrobacterium*-mediated gene transfer over particle bombardment include the possibility of transferring relatively large segments of DNA, a lower number of transgene copies integrated into the plant genome, rare transgene rearrangement, a lower frequency of genomic DNA interspersion and reduced abnormal transgene expression (Gelvin, 2003; Kohli *et al*., 2003). Moreover, this system involves a low operating cost and simple transformation protocols (Brasileiro and Lacorte, 2000). However, plants differ greatly in their susceptibility to *Agrobacterium*-mediated transformation. These differences occur among species, cultivars or tissues (Droste *et al*., 1994; Gelvin, 2010b; Wiebke-Strohm *et al*., 2011). According our experience, this transformation system usually results in lower transformation rates compared with particle bombardment (Wiebke-Strohm *et al*., 2012).

Agrobacterium tumefaciens and target tissues

Agrobacterium was initially considered to be non-pathogenic to soybean (DeCleene and DeLey, 1976). However, later studies showed soybean was susceptible to this bacterium (Pedersen *et al*., 1983; Droste *et al*., 1994; Mauro *et al*., 1995). The addition of acetosyringone during bacterial infection, selection of the most appropriate *A. tumefaciens* strain and soybean cultivar, and development of super-virulent plasmids have contributed to the increased efficiency of soybean transformation (reviewed by Somers *et al*., 2003).

Recovery of the first transgenic soybean plants using *A. tumefaciens*-mediated transformation was reported by Hinchee *et al*. (1988), using cotyledonary nodes as the target tissue. Subsequently, advances in transformation techniques were achieved, and several research teams have reported the generation of transgenic plants using different target tissues, such as cotyledonary nodes (Paz *et al*., 2004; Liu *et al*., 2008), hypocotyls (Aragão *et al*., 2000; Wang and Xu, 2008), half-seeds (Paz *et al*., 2006), organogenic callus (Hong *et al*., 2007), and immature zygotic cotyledons (Parrott *et al*., 1989; Yan *et al*., 2000; Ko *et al*., 2003, 2004).

Although secondary somatic embryos remain the major target for soybean transformation via particle bombardment, the transformation of these tissues via *A. tumefaciens* has been proven to be challenging. Instead of the conventional *A. tumefaciens*-mediated transformation system, alternative methods have been proposed for this target tissue: the ``Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT)'' (Trick *et al*., 1997b, Trick and Finer, 1998) and the "combined DNA-free particle bombardment and *Agrobacterium* system (bombardment/*Agrobacterium* system)" (Droste *et al*., 2000; Wiebke *et al*., 2006, Wiebke-Strohm *et al*., 2011). The difference between these methods lies in the technique used to induce tissue wounding to provide an entry point for the bacteria: SAAT uses sonication, and the other system relies upon bombardment. Unfortunately, both systems are time-consuming and laborious and depend on the availability of specific equipment for routine application.

Despite these advances, only a few viable transgenic lines have been generated from the above experiments, showing that more appropriate and effective methods need to be developed to improve the efficiency of soybean transformation via *A. tumefaciens*.

Agrobacterium rhizogenes

The *A. rhizogenes*-mediated transformation leads to the development of the hairy-roots phenotype, consisting of roots that grow plagiotropically and rapidly and are highly branched in the absence of exogenous plant growth regulators (Collier *et al*., 2005). *A. rhizogenes* can co-transfer T-DNAs from the Ri plasmid and from a binary vector containing the gene of interest into the plant genome (Christey, 2001; Broothaerts *et al*., 2005). Hairy-roots can be clonally propagated in culture medium (Chabaud *et al*., 2006). In addition, "composite plants", *i.e.*, plants with wild-type shoots and transgenic roots, can be obtained (Collier *et al*., 2005). Each transgenic hairy-root represents an independent transformation event, and high numbers of transformants can be obtained and analyzed (Kereszt *et al*., 2007). The major advantage is a relatively short period (approxi-

mately 6-8 weeks) needed to screen potential genes and promoters in the stably transformed tissues (Cho *et al*., 2000).

A. rhizogenes-mediated transformation has become a powerful tool for gene functional and root biology studies due to its quick and simple methodology (Kereszt *et al*., 2007; Cao *et al*., 2009). In soybeans, the method has been used to characterize promoters (Hernandez-Garcia *et al*., 2010), the propagation of nematodes (Cho *et al*., 2000), symbiotic interactions (Hayashi *et al*., 2008), pathogenic interactions (Lozovaya *et al*., 2004; Li *et al*., 2010), and gene silencing via RNAi (Subramanian *et al*., 2005).

Unlike other plants species, there are no reports to date on successful plant regeneration from soybean hairy-roots. However, Olhoft *et al*. (2007) reported the recovery of complete and stable soybean transgenic plants from primary-node explants infected by a disarmed *A. rhizogenes* strain.

Selection System

Regardless of the gene delivery system used, the process of producing transgenic plants often requires an effective means for identifying and selecting transgenic cells and tissues. Selectable marker genes are able to lend resistance or tolerance to antibiotics or herbicides, allowing the selection of the transgenic material.

The hygromycin antibiotic has been successfully used as a selection agent and become the standard for the selection of soybean transgenic tissues, especially embryogenic tissues (Trick *et al*., 1997b; Trick and Finer, 1998; Droste *et al*., 2000; Droste *et al*., 2002; Wiebke *et al*., 2006; Homrich *et al*., 2008a; Hernandez-Garcia *et al*., 2009; Li *et al*., 2009; Wiebke-Strohm *et al*., 2011; Wiebke-Strohm *et al*., 2012) and cotyledonary node cells (Olhoft *et al*., 2003). Olhoft *et al*. (2003) demonstrated that hygromycin reduces both the number of non-transformed escapes and the time in culture.

Some studies have reported the use of different herbicides for selecting soybean transgenic tissues. The phosphinothricin acetyltransferase (PAT) proteins, which are encoded by the *bar* coding sequence from *Streptomyces hygroscopicus* or the *pat* coding sequence from *Streptomyces viridochromogenes*, are present in glufosinate-ammonium-tolerant plant varieties of various crops, such as corn, cotton, rice, oilseed rape, and soybean. The soybean transformation efficiency was maximized by using optimized levels of glufosinate during the selection of transgenic shoots recovered from cotyledonary nodes (Zhang *et al*., 1999; Zeng *et al*., 2004). Rech *et al*. (2008) developed a novel system to select transgenic meristematic cells from embryogenic axes after the introduction of an *Arabidopsis* mutant gene (*csr1-2*) to achieve resistance to imidazolinone herbicides. The success of this agent in the selection of transgenic embryogenic axes is attributed to its ability to translocate and be concentrated in the apical meristematic region of the embryogenic axes. Preliminary results in our lab showed that this marker/agent can also efficiently select transgenic somatic embryos (unpublished results). Rao *et al*. (2009) successfully developed a system to select somatic embryos using the *E. coli dap*A gene, which confers resistance to glufosinate, glyphosate, S-(2 aminoethyl)-L-cysteine and imidazolinones.

Functional Characterization of Soybean Genes

Considerable progress has been made in developing genomic resources for soybean, the model plant for legume studies (Gepts *et al*., 2005), including sequencing its genome (Schmutz *et al*., 2010). A large number of soybean genes have been identified, most with unknown function. Therefore, a major research priority in the post-genome sequencing era is determining the function of these genes, especially those involved in agronomic performance (Wesley *et al*., 2001; Kokkirala *et al*., 2010). The integration of genetic and genomic data from multiple legumes and other plant species provides support for soybean genome annotation and comparative functional genomics (Tran and Mochida, 2010). Although annotation may suggest a gene function, confirmation through biochemical or genetic studies is necessary.

The primary tool for dissecting a genetic pathway is the screen for the loss of gene function, disrupting the target pathway. However, the limitations of this method are the following: (i) genes that act redundantly are rarely identified, (ii) inferring function is difficult when genes act during multiple stages of the life cycle, and (iii) the loss of function of important genes is lethal (Weigel *et al*., 2000; Zhang, 2003).

Modern biotechnology has enabled the elucidation of gene function through the systematic modification of gene expression followed by quantitative and qualitative analyses of the gene expression products. The modulation of gene expression can be achieved by the integration of foreign DNA sequences in the plant genome, leading to either overexpression or gene silencing. Gene silencing is currently achieved through interference RNA (RNAi), a process of sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNA that is homologous in sequence to the target gene. Overexpression and silencing are complementary strategies to functionally characterize genes. The main advantage of overexpression is the low effect of other genes with functional redundancy (Zhang, 2003).

An alternative route to down-regulate gene expression is to couple T-DNA regions with transposon elements. The T-DNA integration into the plant genome can disrupt the coding region of a gene, resulting in gene inactivation. Because T-DNA insertions tend to reside in transcriptionally active regions, subsequent movement of the transposon throughout the genome would provide launching sites for further mutagenesis upon the activation of

transposition (Mathieu *et al*., 2009). In the long-term, the building of a transposon-mutant collection may provide an important resource for functional genomic studies.

Transient alterations in gene expression can also be generated by virus-induced gene silencing (VIGS) (Baulcombe, 1999; Burton *et al*., 2000), a method that exploits an RNA-mediated anti-viral defense mechanism. The advantage of VIGS is that entire cDNA libraries can be cloned into the viral vector instead of individual genes. VIGS can also be used to target genes that, when stably mutated or silenced, cause lethal effects on plant development (Burch-Smith *et al*., 2004). However, VIGS-mediated phenotypes are transient, and appropriate viral vectors that can infect and alter gene expression in desired species need to be developed (Tadege *et al*., 2005).

Methods for the subcellular localization of proteins provide an important tool to determine their functions (Kokkirala *et al*., 2010). The fusion of a gene of interest to a reporter gene or tag is a convenient and powerful method for protein subcellular localization (Nakagawa *et al*., 2007; Kokkirala *et al*., 2010). A large number of vectors are available for this use (Karimi *et al*., 2002; Nakagawa *et al*., 2007). Techniques for the subcellular localization of proteins are also effective in the detailed analysis of gene networks, including colocalization, complex formation, gene product interactions and protein relocation (Nakagawa *et al*., 2007).

The characterization of new promoters represents another relevant topic of research to provide new tools for transgene manipulation. The constitutive cauliflower mosaic virus 35S RNA promoter (CaMV 35S) is the most widely used in plant biotechnology (Odell *et al*., 1985). However, the promoter derived from the soybean polyubiquitin (*Gmubi*) gene is able to induce constitutive gene expression at levels up to five-fold higher than the traditional CaMV 35S promoter (Chiera *et al*., 2007; Hernandez-Garcia *et al*., 2009). The promoter of the *Gm*HSP90L gene, which encodes a heat shock protein, is four-fold more potent than the CaMV 35S promoter (Chiera *et al*., 2007). Thus, both are alternative promoters for soybean transformation.

In some cases, a high expression level of a recombinant protein can be detrimental to the plant due to toxicity. In other cases, the protein can be unnecessary in certain tissues and/or developmental stages. Therefore, regulating transgene expression by tissue-specific or inducible promoters would be advisable (Preiszner *et al*., 2001; Qinggele *et al*., 2007). Inducible promoters are activated by biotic or abiotic factors, and they should prevent gene expression during steps that interfere in the growth, regeneration or reproduction of transgenic plants (Boetti *et al*., 1999). Tissue-specific promoters have the potential to direct gene expression to specific plant tissues or organs where the accumulation of recombinant proteins is needed (Ma *et al*., 2003).

Most available information on genetic modification is based on heterologous expression systems (Chen *et al*., 2007; Xue and Zhang, 2007; Zhang *et al*., 2009b; Zhou *et al*., 2010). Studies with model plants produce results faster and have been well accepted by the scientific community. Sometimes the gene function is the same in both homologous and heterologous backgrounds (Mazarei *et al*., 2007). Frequently, however, the activity of many promoters or proteins depends on specific interactions that are only found in homologous backgrounds. In these cases, a final confirmation of gene/promoter function in a homologous expression system is desirable (Krajewska, 2009; Hernandez-Garcia *et al*., 2010). A relatively large number of reports on functional studies in the soybean were performed in heterologous systems. However, there are few reports in homologous systems (see Table 1).

Soybean Transformation in Agriculture

The first generation biotech crops offered a significant increase in yield and production by protecting crops from losses caused by pests, weeds, and diseases. The main advantages of biotech products available in the market include the following: (i) existence of farmer demand for techniques facilitating their work; (ii) reductions in production losses; and (iii) reduced amounts of chemicals used on crops or the use of less toxic products (Job, 2002; James, 2011). Based on the genesis of biotech crop commercialization from 1996 to 2011, herbicide tolerance and insect resistance have consistently been the dominant traits of interest. Herbicide-tolerant soybean remains the dominant crop in 2011, occupying 47% of the global biotech area (James, 2011). Brazil recently approved the first stacked soybean with insect resistance and herbicide tolerance for commercialization (www.ctnbio.gov.br).

Many countries and companies are now fast-tracking the development of new soybean biotech crops featuring (i) superior nutritional traits, (ii) improved yield by enhanced tolerance to stresses, (iii) the capability to produce therapeutic products and vi) the capability of being used as biomass for biofuels (Job, 2002; McGloughlin, 2008).

Herbicide-tolerant soybean plants

Roundup Ready (RR) crops (registered trademarks of Monsanto Technology LLC) are tolerant to the herbicide glyphosate (N-[phosphonomethyl] glycine). Glyphosate tolerance was obtained through the expression of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (*epsps*) gene from *Agrobacterium* spp. strain CP4. The introduced glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic amino acids and other metabolites that are essential for plant development and growth in the presence of glyphosate (Padgette *et al*., 1995). Extensive research efforts have led to the development of a second-generation glyphosate-tolerant soybean product. Although both transgenic events produce identical CP4 EPSPS proteins, the

Table 1 - Functional studies of soybean genes by overexpression, silencing, transposon-based mutagenesis, protein sub-cellular localization and/or promoter characterization.

References	Approach	Description
Nunes *et al*. (2006)	Gene silencing RNAi	Correlation between myo-inositol-1-phosphate (*Gm*MIPS1) gene expression and seed development
Lee *et al*. (2005)	Gene silencing RNAi	The role of the thioredoxin gene in nodule development and the maintenance of the symbiotic state
Govindarajulu *et al*. (2009)	Gene silencing RNAi	The role of GS52 ecto-apyrase gene during the nodulation process
Zhang *et al*. (2009a)	Gene silencing VIGS	Transient silencing of the actin gene resulting in a reduced number of soybean mosaic virus (SMV) infection foci
Pandey *et al*. (2011)	Gene silencing VIGS	Screening of 140 genes for their ability to compromise resistance to *Phakopsora pachyrhizi*
Mathieu *et al*. (2009)	Gene silencing Transposon-based mutagenesis	Dissociation (Ds) transposon of maize: an effective tool for the mutagenesis of soybean
Kim *et al*. (2001); Yanxiang *et al*. (2006); Wu *et al*. (2008); Zhang *et al*. (2009b); Zhou *et al*. (2010)	Overexpression	Examples of soybean gene overexpression in a heterologous background
Mazarei *et al*. (2007)	Overexpression	Characterization of the soybean ethylene-responsive element-binding protein (*Gm*EREBP1) encoding gene as a transcription factor, which induces the expression of defense-related genes
Chen *et al*. (2009)	Overexpression	*Gm*DREB3 overexpression increasing the tolerance to cold, drought and salt
Hur *et al*. (2009)	Overexpression	The role of the soybean aldo/keto reductase 1 gene (*Gm*AKR1) in the regulation of nodule formation
Dhaubhadel *et al*. (2008); Chung *et al*. (2009); Yang *et al*. (2010); Yi *et al*. (2010); Li and Dhaubhadel (2011); Mazarei *et al*. (2007); Huang *et al*. (2009)	Subcellular localization	Examples of the subcellular localization of soybean gene products using heterologous systems
Preiszner *et al*. (2001)	Promoter characterization	Characterization of the soybean *Adh* gene promoter in transgenic hairy roots
Subramanian *et al*. (2004)	Promoter characterization	Study of soybean isoflavone synthase promoters in response to the plant/nitrogen-fixing bacteria interaction
Hernandez-Garcia *et al*. (2009)	Promoter characterization	Characterization of the soybean polyubiquitin gene promoter (*Gm*Ubi) in transgenic soybean plants
Chen *et al*. (2009)	Promoter characterization	Characterization of the *Gm*DREB3 promoter in response to cold stress
Chiera *et al*. (2007)	Promoter characterization	Characterization of a soybean heat shock protein 90-like (*Gm*HSP90L)
Qinggele *et al*. (2007)	Promoter characterization	Isolation and analysis of BCSP666, a promoter fragment with seed-specific activity
Cho *et al*. (1995)	Promoter characterization	Construction of expression cassettes containing regions of the soybean lectin gene promoter useful for driving foreign gene expression to modify embryo-specific traits

second-generation RR soybean has a yield advantage compared with the first in the same elite genetic background (Lundry *et al*., 2008; Levy-Booth *et al*., 2009).

Recently, the National Technical Commission on Biosecurity in Brazil approved the commercial release of a new class of genetically modified soybeans: the Soybean CV127 ("*Cultivance")*, which is tolerant to herbicides of the imidazolinone chemical class. The CV127 soybean has been genetically modified by researchers from Embrapa (The Brazilian Agricultural Research Corporation) to express an altered *csr1-2* gene from *Arabidopsis thaliana*, supplied by the German company BASF (Rech *et al*., 2008). The AtAHASL protein encoded by *csr1-2* is structurally and functionally identical to the native AtAHASL, except for a serine to asparagine substitution at residue 653 (S653N) that results in tolerance to imidazolinone herbicides (Sathasivan *et al*., 1991).

Insect-resistant soybean plants

Bacillus thuringiensis (*Bt*) is an entomopathogenic bacterium widely used as a biopesticide to control pest insects. *B. thuringiensis* produces proteins (δ-endotoxins) that are stored around spores, forming crystals (Peferöen, 1997) that exert specific toxic activity against lepidopteran, dipteran, and coleopteran larvae (Hongyu *et al*., 2000).

The transgenic expression of Bt proteins is reportedly very effective for controlling insect pests in several major crop plants, especially corn and cotton (James, 2011). A

stacked soybean (Bt/RR) was recently approved for commercialization in Brazil, and other soybean Bt lines have been developed. Parrott *et al*. (1994) reported that the expression of a native *cry1Ab* gene prevented *Anticarsia gemmatalis* larval feeding and growth. A transgenic line expressing high levels of a synthetic *cry1Ac* gene caused complete *A. gemmatalis* larval mortality and significantly reduced *Pseudoplusia includens* and *Helicoverpa zea* larval survival and feeding in laboratory bioassays (Stewart Jr *et al*., 1996) and in artificially infested field cages (Walker *et al*., 2000). Transgenic soybean lines expressing a synthetic *cry1A* gene exhibited a virtually complete efficacy against several lepidopteran pests in screenhouse and field trials (Macrae *et al*., 2005; McPherson and Macrae, 2009). Transgenic soybean lines expressing a *cry*1A synthetic gene with a high degree of resistance against the lepidopteran pests *Pseudoplusia includes*, *Helicoverpa zea* and *Anticarsia gemmatalis* was reported by Miklos *et al*. (2007). Similarly, Homrich *et al*. (2008a) described the development of a transgenic soybean expressing a synthetic *cry1*Ac gene. *In vitro* and *in vivo* bioassays indicated that the transgenic plants were highly toxic to *A. gemmatalis.* The results from an additional study showed that *cry1*Ac transgene did not affect the agronomic performance and yield (Homrich *et al*., 2008b).

Other transgenic soybean lines with agronomic, nutritional and industrial interest

Soybean growth, productivity and seed quality are affected by a wide range of abiotic and biotic stresses. Drought is considered the main abiotic stress, reducing soybean yield by approximately 40% and affecting all stages of plant growth and development (Manavalan *et al*., 2009).With regards to biotic stresses, Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* Sydow and Sydow, is one of the most severe diseases in soybean culture, resulting in 10-90% crop losses in different regions (Yorinori *et al*., 2005).

Genetic engineering using genes encoding components of stress-related metabolic pathways has shown the potential to enhance drought resistance in the soybean. Transgenic soybean plants overexpressing the *Arabidopsis* Δ1-pyrroline-5-carboxylate synthase (P5CR) gene showed greater tolerance to drought and heat stresses due to an increased free proline level (De Ronde *et al*., 2004a,b; Kocsy *et al*., 2005). Alternatively, tolerance to stresses could be achieved by modulating the expression of stress-induced transcription factors (TF), which in turn would regulate the expression of a large number of relevant downstream genes (Agarwal et al., 2006). Different TFs related to stress have been identified. DREBs belong to the ethylene-responsive factors (ERF) family of TFs and play a crucial role in providing tolerance to multiple stresses. A drought-sensitive BR16 soybean cultivar was transformed with the *AtDREB1A* gene under the control of a drought-inducible promoter (rd29A) from *A. thaliana*. The modified plants had more chlorophyll, higher stomatal conductance, and higher photosynthetic and transpiration rates. Several genes related to the drought response were highly expressed in these plants when submitted to a severe water deficit treatment. The results indicated that overexpression of *AtDREB1A* in soybeans may enhance drought tolerance (Polizel *et al*., 2011).

The members of the WRKY TF superfamily play a key role in regulating the pathogen-induced defense responses (Dong *et al*., 2003) and abiotic stress responses (Fowler and Thomashow, 2002; Mare *et al*., 2004) and are involved in various physiological processes, including senescence, trichome development and secondary metabolite biosynthesis (Eulgem *et al*., 2000). A previous study on the global gene expression of compatible and incompatible interactions led to the identification of several WRKY TFs that were differentially regulated during infection (Van de Mortel *et al*., 2007). A total of 64 soybean WRKY TFs were silenced using VIGS to test their involvement in plant resistance (Pandey *et al*., 2011). The screen resulted in the identification of three *WRKY* TFs (*GmWRKY36*, *GmWRKY40*, and *GmWRKY45*) that compromised plant resistance when silenced.

From a consumer perspective, the focus on value-added traits, especially nutrient improvement, is of the greatest interest. McGloughlin (2008) reported examples of transgenic soybean plants with nutritionally improved traits, including modifications of the protein quality and level, essential amino acids, oils and fatty acids, functional secondary metabolites and mineral availabilities. More recently, both oil content and quality have drawn significant attention, and efforts have been made to increase oxidative stability, enhance ω-3 fatty acid content and increase the total oil amount in soybean seeds (Clemente and Cahoon, 2009). Soybean biotech with high oleic acid levels has already been granted regulatory approval for commercialization (CERA, 2010).

Regarding soybean plants as production factories for therapeutic products, Piller *et al*. (2005) demonstrated the possibility of expressing an immunogenic subunit antigen in soybean as the first step toward the development of a plant edible vaccine for cattle. In addition, the human growth hormone (hGH) and human coagulation factor IX (hFIX) were produced in genetically engineered soybean seeds (Cunha *et al*., 2010a,b).

Soybean oil represents the most widely available feedstock for biodiesel due to its enhanced biodegradation, increased flashpoint, reduced toxicity, lower emissions and increased lubricity (Kinney and Clemente, 2004). However, because of the high proportion of polyunsaturated fatty acids, soybean oil is oxidatively unstable, and an oxidized biofuel can compromise engine performance (Canakci *et al*., 1999). To maximize the fuel characteristics of the biodiesel, Duffield *et al*. (1998) suggested the devel-

opment of an oil that is high in oleic acid and low in saturated fatty acids. The implementation of biotechnology tools to directly target the perturbation of oil metabolism in soybean has been shown to produce a high-oleic-acid phenotype (Mazur *et al*., 1999). Buhr *et al*. (2002) described the development of transgenic soybean events in which the expression of two genes was simultaneously down-regulated in seeds, generating soybean oil with reduced palmitic acid and increased oleic acid contents. One of those events was evaluated as a feedstock for biodiesel (Graef *et al*., 2009). The extruded oil showed improved cold flow and enhanced oxidative stability.

Conclusions

Since the first studies on transformation, significant advances have been achieved despite soybean recalcitrance to regeneration and transformation. The difficulty of transforming soybeans is evidenced by the few reports on soybean gene expression in a homologous system. New phenotypes obtained by the transgenic approach are useful for functional gene studies and crop improvement. The first generation of commercialized soybean biotechnology products were crops focusing largely on input agronomic traits, such as herbicide tolerance and insect resistance. The present and future focus is on the continuing improvement of agronomic traits, value-added output traits such as improved nutrition and food functionality, and plant factories for therapeutics and industrial products. In the near future, functional studies may indicate new proteins and promoters with biotechnological interest that can be applied to soybean improvement through genetic engineering.

Transgene integration into plant genomes occurs randomly using any of the transformation methodologies currently available for the soybean. Recently, a novel approach was designed to recognize a target sequence in the genome of any eukaryote and direct the insertion of transgenes into this locus. Direct-DNA delivery methods combine high-fidelity DNA recognition/cleavage by engineered zinc-finger nucleases (ZFNs) and homology-directed repair at the specified break sites (a widely conserved biological pathway). This approach enables the targeted mutagenesis of an endogenous gene, targeted gene addition at an endogenous locus and/or targeted genome editing at an endogenous locus (Wright *et al*., 2005; Shukla *et al*., 2009). ZFN-mediated transformation has already been reported for some plant species, such as tobacco (Wright *et al*., 2005; Cai *et al*., 2008), *Arabidopsis* (Lloyd *et al*., 2005) and maize (Shukla *et al*., 2009). Direct-DNA delivery methods can be extended to the genome modification of any plant species that is amenable to tissue culture and regeneration, including soybean. This approach, combined with rapid advances in genome sequencing technologies and bioinformatics and the increasing efficiency of DNA delivery methods, establishes an efficient and precise strategy for plant genome engineering.

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