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Review Article

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Recent advances in the regeneration and genetic transformation of soybean

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Abstract: Soybean is one of the world's most important sources of vegetable oil and protein meal. The results of plant breeding efforts have greatly improved the crop characteristics but genetic engineering offers new possibilities. Genetic transformation has tremendous potential in developing improved soybean varieties with desired agronomic traits, which are otherwise difficult to achieve through traditional breeding. Development of efficient *in vitro* regeneration system of soybean through tissue culture, and transformation protocol is the prerequisite for the adoption of *de novo* biotechnological approaches aiming at genetic manipulation. Such an alternative approach, for the development of improved soybean varieties is to introduce exogenous gene in soybean genome using gene transfer technique. However, the successful development of transgenic soybean depends upon an efficient plant regeneration protocol and its suitability to transformation techniques. During the last thirty years, significant progress has been made in soybean biotechnology, particularly in the area of transgenic technology. This review provides a detailed account of the advances made in the regeneration and genetic transformation of soybean and their potential applications.

Keywords: Genetic transformation; Plant regeneration; Soybean; Transgenic; Tissue Culture

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The powerful combination of genetic engineering and conventional breeding programs permits useful traits encoded by transgenes to be introduced into commercial crops within an economically viable time frame. There is a great potential for genetic manipulation of crops to enhance productivity by increasing resistance to diseases, pests and environmental stress and by qualitatively changing the seed composition. The development and commercial release of transgenic soybean plants relies exclusively on two basic requirements, a method that can transfer a gene or genes into the soybean genome and govern its expression in the progeny. The two main gene delivery systems for achieving this goal are *Agrobacterium* - mediated transformation and particle gun bombardment. The other requirement is the ability to regenerate fertile plants from transformed cells. This is achieved by regenerating plants via organogenesis or somatic embryogenesis. Development of efficient *in vitro* regeneration system of soybean through tissue culture, and transformation protocol is the prerequisite for the adoption of *de novo* biotechnological approaches aiming at genetic manipulation. Such an alternative approach, for the development of improved soybean varieties is to introduce exogenous gene in soybean genome using gene transfer technique. However, the successful development of transgenic soybean depends upon an efficient plant regeneration protocol and its suitability to transformation techniques. Many researchers have used different parts of the soybean plant as an explant for successful regeneration. The explant used in various shoot regeneration protocols are stem node, hypocotyl segments, immature cotyledon, epicotyls, young embryonic axes, primary leaf node and cotyledonary node.

The transformation of soybean has been accomplished by several different methods, however; *Agrobacterium tumefaciens*-mediated transformation

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(Hinchee et al.1988; Parrott et al.1989a; Zhang et al.1999; Clemente et al.2000; Olhoft and Somers, 2001; Olhoft et al.2001; Ko et al.2003; Zeng et al.2004; Paz et al.2006) and particle bombardment (McCabe et al.1988; Christou et al.1989; Finer and McMullen, 1991; Aragão et al.2000; Droste et al.2002; Schmidt et al.2008) were efficiently used. Other remaining methods have also been optimized for soybean, but they are comparatively less efficient and hence have not often been used.

Considerable research has been conducted in tissue culture and transformation of the soybean. Soybean transformation has been reviewed by Trick et al.(1997), Somers et al.(2003), Parrott and Clemente, (2004), Dinkins and Collins, (2008), and Finer and Larkin, (2008). However, information is available on genetic transformation in soybean, a through coverage of recent progress in cellular and molecular biology in soybean is lacking which includes organogenesis, somatic embryogenesis and various transformation methods. We have reviewed regeneration and transformation methodology along with respect to desirable traits agronomic traits have been engineered in soybean.

Soybean Tissue Culture

Plant tissue culture or the aseptic culture of cells, tissues and organs, is an important tool in both basic and applied studies. It is founded upon the research of Haberlandt, a German plant physiologist, who in 1902 introduced the concept of *totipotency*: that all living cells containing a normal complement of chromosomes that are capable of regenerating the entire plant. Considerable research work has been undertaken in plant tissue culture in the 1950s and 1960s. Soybean has been used extensively in tissue culture since the 1960's.

Organogenesis

Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. Organogenesis is an indispensable tool for plant regeneration using tissue culture techniques and also for plant transformation. Organogenesis has been widely used for regeneration in *Glycine* species. Regeneration of plants via organogenesis has been accomplished from various tissue such as stem node (Saka et al.1980),protoplast (Wei and Xu,1988), hypocotyl segments (Dan and Reichert, 1988; Kaneda et al.1997; Yoshida, 2002), epicotyl (Wright et al.1987), embryonic axes (Liu et al.2004), primary leaf node (Kim et al.1990), half seed (Paz et al.2006; Verma et al.2011) and cotyledonary node (Cheng et al.1980; Barwale et al.1986b; Franklin et al.2004; Shan et al.2005; Ma and Wu, 2008; Verma et al. 2009). However, cotyledonary node remains the most desirable explants for tissue culture and has been used for most of the soybean genotypes.Numerous aspects of tissue culture condition that play an important role in plant regeneration are discussed below.

Plant growth regulator regime

Diverse plant growth regulators (PGR) have been used in regeneration of plants via organogenesis. Cytokinin, 6-benzylaminopurine (BAP) was commonly used PGR for miropropagation of plants. First study on organogenesis was reported by Cheng et al. (1980) from cotyledonary explants derived from germinated soybean seedling. In this study soybean seeds were directly evaluated for the optimum level of BAP in germination, which produced multiple buds from the axillary meristem. Wright et al. (1986) reported modified cotyledonary node explant from germinated seedling and Barwale et al.(1986a) used immature cotyledonary node, which produced *de novo* shoots in the presence of 5 µM of BAP. Incremental concentrations of BAP (5-10 µM) induced the greatest numbers of shoots from hypocotyl explant (Dan and Reichert, 1998). These high concentrations of BAP were sufficient to overcome apical dominance and produced multiple shoots or buds. Paz et al. (2006) also reported that 5 µM BAP was efficient for multiple shoot formation from half seed explant. BAP in combination with indole butyric acids (IBA) was used for improved regeneration frequency from embryonic axes (Liu et al.2004) and cotyledonary node (Ma and Wu, 2008; Verma et al.2009).

Another cytokinin, thidiazuron (TDZ) is a substituted phenylurea compound with both cytokinin and auxin-like effects (Mok el al., 1982, Visser et al.1992). TDZ is considered to be one of the most active cytokinins for shoot induction in plant tissue culture (Murthy et al.1998; Verma et al.2011). Little is known about mechanism of TDZ induced direct organogenesis in plants. TDZ has been suspected of promoting regulated morphogenesis in plants through the modulation of endogenous cytokinin and auxin (Capella et al.1983, Thomas and Katterman, 1986, Gill and Saxena, 1992). TDZ was responsible for higher regeneration capacity and multiple shoot formation efficiency than BAP (Kaneda et al.1997; Verma et al.2011). TDZ in combination with BAP was also used to improve regeneration percentage as well as mean number of shoots from cotyledonary node explants (Franklin et al.2004). The positive influence of pretreatment of seeds with TDZ or BAP on regeneration of shoots has been reported in soybean (Wright et al.1986; Yoshida, 2002; Shan et al.2005). Low concentration of TDZ led to fast shoot development and too high concentration resulted in abundance of compact calli (Shan et al.2005; Verma et al.2011).

From above mentioned studies it can be concluded that both BAP and TDZ are most effective cytokinin for shoot organogenesis in soybean and regeneration frequency and number of shoots depends upon the cytokinin concentration and explant interaction.

Other media constituents

Culture media composition is very important factor for any regeneration protocol. Regeneration percentage and number of shoots are also influenced by basal media composition. In a study, reduced salt-supplement with BAP in the medium induced maximum

in vitro response from cotyledonary node explants (Wright et al.1986). In another study, Kaneda et al. (1997) reported that low salts concentrations in combination with TDZ increased the frequency of shoot organogenesis from hypocotyl segment. Sucrose is the most widely used carbon source in soybean regeneration and is the main sugar translocated into phloem tissues. Various carbon sources have been used in soybean regeneration and sorbitol was found to be the most efficient for callus induction while maltose was found suitable for plant regeneration (Sairam et al. 2003). These differential responses of the explant to various sugars may be due to the ability of various developmental stages to metabolize different carbon sources.

Genotypic response

Regeneration in soybean is highly genotype dependent. Choice of explant also plays an important role in regeneration. There are reports that suggested that hypocotyl segment is highly genotype dependent than the cotyledonary node and embryonic axes explants. Kimball and Bingham, (1973) reported that cultivars Corsoy and Dunn yielded hypocotyl explant responses of 3-10%. In another study by Dan and Reichert, (1998) hypocotyl section from all cultivar were found amenable for regeneration, though the genotypic differences were also observed. Yoshida, (2002) reported that regeneration of hypocotyl ends showed significant variation among soybean genotypes. This genotypic variation in hypocotyl segement may be due to presence or absence of meristamatic tissues in regeneration point. Barwale et al. (1986b) showed that genotypic differences such as maturity groups, seed coat color and shoot-forming capacity at the cotyledonary node did not influence plant regeneration. In other regeneration study, cotyledonary node was most responsive explant and the regeneration frequency didn't vary among cultivars (Franklin et al. 2004; Sairam et al. 2003; Verma et al. 2009, 2011).

Somatic embryogenesis

Somatic embryogenesis relies on plant regeneration though a process analogous to zygotic embryo germination. One of the most efficient methods for soybean regeneration is somatic embryogenesis, first described in by Christianson et al. (1983). Lazzeri et al.(1985) reported the use of immature cotyledons for the embryo induction, since then immature cotyledons have been used for regenerating into plantlets via somatic embryogenesis in most of the studies (Finer and Nagasawa, 1988; Bailey et al.1993; Ko and Korban, 2004; Lim et al. 2005; Hiraga et al. 2007; Klink et al. 2008). Embryonic axes (Loganathan et al.2010), callus (Phillips and Collins, 1981; Gamborg et al.1983; Yang et al.1991), microspores (Hu et al.1996) and embryogenic leaves (Rajasekaran and Pellow, 1997) have also been reported to regenerate through embryogenesis.

Plant growth regulator regime

Various auxins have used in inducing somatic embryogenesis in soybean. 2,4-dichlorophenoxyacetic acid (2,4-D) have efficiently been used for repetitive or proliferative embryogenesis in a number of studies (Ranch et al.1985; Schmidt et al.1994). However, the use of high concentrations of 2,4-D leads to development of abnormal somatic embryoes. Somatic embryos initiated on naphthalene acetic acid (NAA) were more advanced in embryonic morphology; but were not suitable in establishing repetitive suspension cultures (Parrott and Clemente, 2004). Liu et al.(1992) reported that somatic embryoes incubated in a medium containing NAA did not proliferate in suspension culture as well as those produced on a semi-solid medium containing 2, 4-D. Lazzeri et al. (1985) conducted a comparative study between 2, 4-D and NAA and reported that 2, 4-D induced higher frequencies of somatic embryogenesis, whereas NAA was superior for producing somatic embryo of normal shape. A number of current protocols have utilized 40 mg/L 2, 4-D for induction of somatic embryos (Ranch et at., 1986; Finer, 1988) and of 20 mg/L 2, 4-D for maintenance of embryos (Ranch et al. 1986; Wright et al.1991).

Other media constituents

Osmoticum plays very importance role in soybean somatic embryo's proper histodifferentiation and maturation. Significant differences have been reported among types and levels of osmoticum for their influence on number of mature embryos, maturation and their germination. Samoylov et al. (1998) reported that sucrose promotes faster embryo histodifferentiation and maturation, and allows the recovery of up to 50% or more mature, cotyledon-stage embryos within 3 weeks. However, Lazzeri et al. (1987) reported a decrease in mean number of somatic embryos per responding cotyledon as the sucrose concentration increased from 1.5 to 12%. In other studies also high numbers of somatic embryos were obtained on low sucrose concentrations (0.5 and 1%) (Komatsuda et al.,1991; Hofmann et al.,2004). In a study by Walker and Parrott, 2001, supplementation with 3% sorbitol resulted in a 9-fold increase in germination frequencies and a 13-fold increase in embryo conversion frequencies. Addition of glutamine and other amino acids to liquid medium during the histodifferentiation and maturation phase has also been reported to lead to larger embryos which reached physiological maturity in about 5 weeks but germinated in rapidly and vigorous fashion. 30 mM glutamine and 1 mM of methionine appears to be best combination for somatic embryo histodifferentiation and maturation (Schmidt et al.2005).

The presence of exogenous 2,4-D can interfere with proper embryo development, either by inhibiting histodifferentiation or by preventing the formation of bilateral symmetry. Schmidt et al.(1994) reported that the addition of activated charcoal to the medium to adsorb 2,4-D normalized development of somatic embryos. The effect of pH has not been found to have

any significant effect on percent initiation of somatic embryogenesis in soybean. In a number of studies, no differences in frequency of embryogenesis were recorded for pH levels ranging between 5.0 to 7.0 (Lazzeri et al.1987; Hofmann et al.2004). However, significant effects were observed for mean number of somatic embryos per responding explant. A number of workers like Lazzeri et al.1987; Hartweck et al.1988; Shoemaker et al.1991 have reported the use of a pH range of 5.7–5.9, while others viz; Bailey et al.(1993), Li and Grabau, (1996) recommended pH of 7.0 for the regeneration medium. In another study, Santarém et al.(1997) observed differences in initiation frequencies among different pH levels and reported that a pH of 7.0 produced the highest frequency of initiation with a number of soybean genotypes. Use of gellan gum in place of agar as the solidifying agent in the medium has also been reported to increase embryogenesis in soybean (Santarém et al.1997).

Effect of explants

Number of explants viz; hypocotyl segement, cotyledon, embryonic axes and microspores have been used for somatic embryogenesis in soybean. Beversdorf and Bingham, (1977) for the first time initiated suspension culture from hypocotyl sections of soybean and induced embryo-like structures in the suspension culture. The embryo-like stuctures diffrentiated into roots, but none developed into plantlets. Somatic embyogenesis have also been reported in suspension cultures of hypocotyl derived callus (Phillips and Collins, 1981; Gamborg et al.1983). For the first time, embryonic axes were used by Christianson et al.(1983) in developing somatic embryos. The first recovery of a plant from microspores cultured on 2,4-D was reported by Yin et al.(1982). Subsequent to these, many reports of soybean somatic embryogenesis were published, where immature cotyledon were used as explant (Lippmann and Lippmann, 1984, Lazzeri et al.1985, Ranch et al.1985; Parrott et al.1988). Numbers of studies have reported immature cotyledon as the most efficient explants for inducing somatic embryogenesis in soybean.

Genotypic response

Somatic embryogenesis from immature cotyledons has been consistently reported to be genotype dependent by a number of workers (Parrott et al.1989b; Komatsuda and Ko 1990; Shoemaker et al.1991). Ranch et al. (1985) reported the genotypic differences for somatic embryogenesis in 14 genotypes of soybean. They also worked out the correlation of embryogenic competence of these genotypes to their maturity durations and observed no correlation between these two. However Parrott et al.(1989b) chronicled large genotypic effect on ability of immature soybean cotyledons to undergo auxin-stimulated somatic embryogenesis. In this study, all the lines that showed good regeneration potential were found to have highly regenerative ancestral lines 'Manchu' or 'A.K. Harrow' in their pedigree. When 'Manchu' was crossed with 'Shiro', a genotype showing extremely poor regenerations, F_1 hybrid cotyledons showed intermediate regeneration capacity.

Other factors

Other parameters like explants orientation and exogenous culture conditions also contribute to efficient regeneration system via somatic embryogenesis. Orientation of the immature cotyledons on the medium has been reported to have a very strong effect on somatic embryogenesis. Hartweck et al. (1988) reported that immature cotyledons with adaxial side up in the medium produced higher number of somatic embryos. Light intensity showed significant variation on somatic embryogenesis of soybean (Ranch et al.1986; Lazzeri et al.1987).

Soybean transformation

Several methods such as *Agrobacterium*-mediated transformation of excised plant tissues (Horsch et al.1985), particle bombardment (Sanford, 1988), electroporation (Formm et al.1985), silicon carbide fiber (Kaeppler et al.1990), liposome-mediated transformation (Caboche, 1990) and *in planta Agrobacterium-mediated* transformation via vacuum infiltration of plants (Bechtold et al.1993) have been used to developed genetically transformed plants. The transformation of soybean has been accomplished by several different methods however, *Agrobacterium tumefaciens*-mediated (Hinchee et al.1988; Parrott et al.1989a; Zhang et al.1999; Clemente et al.2000; Olhoft and Somers, 2001; Olhoft et al.2001; Ko et al.2003; Zeng et al.2004; Paz et al.2006; Rani et al.2012; Song et al.2013) and particle bombardment (McCabe et al.1988; Christou et al.1989; Finer and McMullen, 1991; Aragão et al.2000; Droste et al.2002; Schmidt et al.2008) has been more efficiently used. The other methods have also been tried in soybean, but the recovery of fertile transgenic plants was very low, therefore less efficient and not often used.

***Agrobacterium*-mediated Transformation**

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most widely used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants and also in studies on gene expression. *Agrobacterium tumefaciens* naturally infects the wound sites in dicotyledonous plants causing the formation of the crown gall tumors. The first evidence indicating this bacterium as the causative agent of the crown gall goes back to more than ninety years (Smith et al.1907). The first record on transgenic tobacco plant expressing foreign genes appeared at the beginning of the last decade, although many of the molecular characteristics of this process were unknown at that time (Herrera-Estrella, 1983). Soybean initially was considered a non host for *A. tumefaciens* (De Cleene and De Ley, 1976). Subsequently, it was shown that

tumors form on soybean in response to infection with *A. tumefaciens,* but not to the extent observed in other dicotyledon like tobacco *(Nicotiana tabacum* L.) (Pedersen et al.1983; Wyndaele et al.1985; Hawes and Pueppke, 1987). In soybean wide genotypic differences for tumorigenic response have been documented by various workers such as Owens and Cress, (1985); Byrne et al. (1987); Hinchee et al. (1988) and Delzer et al. (1990). It is now accepted that most, if not all, soybean cultivars are amenable to *Agrobacterium*-mediated transformation, but that the transformation efficiency varies significantly among cultivars. Various efforts have been made to overcome problems associated with host/tissue specificity of *Agrobacterium* as well as the low transformation efficiency. These include modifying the virulence of *Agrobacterium tumefaciens* strains (Hood et al.1993; Torisky et al.1997), sonication of explant tissues to increase the number of infection sites (Santarém et al.1998; Trick and Finer, 1998), and addition of *thiol* compounds to the co-cultivation medium (Olhoft and Somers, 2001; Olhoft et al.2001). In a comparative study to evaluate virulence of different strains of *A. tumefaciens* on soybean explants, strain KYRT1 was reported to be more virulent than other commonly used strains, including Chry5c, EHA105, GV3850, GV3101, LBA4404 and NTL4 (Torisky et al.1997; Meurer et al.1998; Ko et al.2003; Dang and Wei, 2007).

Transformation inefficiencies can be partially overcome by the addition of chemical inducer, acetosyringone, to induce expression of the *vir* genes (Stachel et al.1985; Delzer et al.1990), by the use of *Agrobacterium* strain that constitutively express the *vir* genes (Hansen et al.1994), by varying incorporation of L-cysteine in co-cultivation (Olhoft and Somers, 2001) and co-culture temperature (Fullner and Nester, 1996). *Agrobacterium*-mediated transformation of soybean tissue was first reported by Facciotti et al. (1985), but they could not recover transgenic plant. Hinchee et al. (1988) reported recovery of first fertile transgenic soybean plant using *Agrobacterium*-mediated transformation. In this study cotyledonary node explants derived from germinated seedling of soybean were inoculated with *A. tumefaciens* pTiT37SE harboring pMON9749 or pMON894, harboring neomycin phosphotransferase II gene as selectable marker. Kanamycin in the range of 200-300 mg/L was used for selection of transgenic shoots and it was recorded that kanamycin severely restricted growth though did not always completely inhibited callusing and regeneration from control cotyledonary explants. Their transformation procedure was further modified by Townsend and Thomas (1993; 1994) through addition of acetosyringone in *Agrobacterium* cell inoculum and changing the temperature during co-cultivation. Di et al. (1996) could produce transgenic plants containing bean pod mottle virus coat protein gene for viral resistance obtained by same modification in cotyledonary node transformation. In this study five transgenic plants were recovered using kanamycin as a selective agent for selection of transgenic shoots at 50 mg/L in root inducing medium, while transgenic shoots initially selected at 200 mg/L kanamycin recorded ineffective selection. Rani et al.2012 also reported that lethal concentration of kanamycin for selection of transgenic shoots did not always gave true transformants. Liu et al. (2004) reported *Agrobacterium*-mediated transformation protocol using embryonic tip explant. In this study kanamycin was used as selective agent and transformation frequency varied from 8.0 to 15.8 %. In another study on selection of transgenic shoots at elongation level, Wang and Xu, (2008) found 75 mg/L kanamycin as adequate concentration and could achieve transformation frequency up 9.3%.

In general cotyledonary-node explant originally reported by Hinchee et al. (1988) and kanamycin as selective agent has been successfully used in a number of studies to obtain fertile transgenic plants, but high frequencies of *escape plants* have also been reported. CN protocol was further improved by use of herbicide selection agents. Zhang et al. (1999) used *bar* gene which encodes for phosphinothricin acetyltransferase (*PAT*) that detoxifies *glufosinate*. In this study *bar* gene was used as selectable marker gene in soybean transformation, wherein germ-line transformation events were recovered at frequencies up to 3% using a selection regime of 5 mg/L *glufosinate* during the shoot initiation stage and 2 mg/L during shoot elongation. Clemente et al. (2000) reported herbicide selectable marker gene *CP4* for efficient selection of transgenic soybean. In their study *glyphosate* was used at levels varying from 0.05 mM to 0.15 mM. This herbicide is a relatively stringent selective agent at low doses and resultes in minimal accumulation of phenolic compounds that are typically observed when utilizing the aminoglycoside kanamycin as the selective molecule. In yet another study, 6 mg/L glufosinate used during shoot induction and shoot elongation stages yielded higher final transformation efficiency ranging from 2.0% to 6.3%, while *bialaphos* at varying doses of 4 to 8 mg/L recorded 0% to 2.1% transformation efficiency (Paz et al.2004). In another, Zang et al.(2004) evaluated four different selection schemes at levels 8/5, 8/8, 10/5, and 10/10 mg/L of *glufosinate* during the first/second shoot initiation stages and compared it with standard treatment of 5/5 mg/L *glufosinate* without the addition of L-*cysteine* into the co-cultivation medium. Transgenic plants were recovered in all selection schemes, but the optimal selection scheme was found to be with *glufosinate* at 8 mg/L across the first and second shoot initiation stages and 3–4 mg/L during shoot elongation. Recovery of transformants at 8/8 mg/L *glufosinate* was consistent with an average transformation frequency of 5.9%, which was higher than previously reported herbicide selection schemes.

Improved soybean transformation protocol using half-seed explants (an alternative cotyledonary explant that is derived from mature seed of soybean following an overnight imbibition), wherein *bar* gene was

employed as selectable marker, transformation frequency increased as compared to 1.5 fold cotyledonary node explants (Paz et al.2006). Similar observation also reported using the half seed explant with kanamycin (Rani et al. 2012). Dang and Wei, (2007) modified embryonic tip transformation protocol of Liu et al.(2004), by optimizing parameters for efficient T-DNA delivery and PPT based effective selection strategies. They could enhance transformation frequency and it ranged from 4.29 to 18.0% on the basis of PCR positive plants. In a recent modification to soybean transformation system, one day old germinated cotyledonary node cells of half seed were wounded mechanically by using a multi-needle consisting of 30 thin fibers and 5 and 3 mg/L PPT were used for selection of transgenic cells (Xue et al.2006). The transformation frequency reached up to 12 percent in this study.

In cotyledonary node explants, wounding and A. tumefaciens infection typically resulted in extensive enzymatic browning and cell death in wounded area. Olhoft and Somers, (2001) reported that addition of L-cysteine in co-cultivation medium prevented necrosis and significantly increased T-DNA transfer into cotyledonary cells. Incorporation of L-cysteine in solid co-cultivation medium resulted in a five fold increase in stable T-DNA transfer in newly developed shoot primordia. In their another study, thiol compounds, L-cysteine, dithiothreitol (DTT), and sodium thiosulfate, appeared to improve T-DNA delivery by inhibiting the activity of plant pathogen and wound-response enzymes such as peroxidases (PODs) and polyphenol oxidases (PPOs) (Olhoft et al.2001).

The increase in the frequency of transformed cells obtained by the addition of thiol compounds to the solid co-cultivation medium was independent of soybean genotypes and Agrobacterium strains, as well as of binary vectors.

However, to further improve selection schemes, which give rise to higher transgenic shoot regeneration, Olhoft et al.(2003) reported that hygromycin B based regime was most efficient for the selection of transgenic shoots in soybean and transformation efficiency ranging from 0.7 to 16.4% could be obtained. Liu et al.(2008) reported that adition of surfactant (Silwet L-77) to infection medium coupled with hygromycin based selection strategies led to transformation efficiencies ranging from 3.8 to11.7% in Chinese soybean varieties. Recently twenty soybean genotypes that originated from different soybean production regions in China were screened for stable transgenic efficiency. Three genotypes, Yuechun 04-5, Yuechun 03-3, and Tianlong 1, showed comparable stable transgenic efficiencies with that of the previously reported American genotypes Williams 82 and Jack (Song et al.2013)

Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT)

Sonication- assisted *Agrobacterium*- mediated transformation (SAAT) as an efficient *Agrobacterium*-based transformation technology was reported by Trick and Finer, (1997). This method consists of subjecting the target plant tissue to brief periods of ultrasound while immersed in an *Agrobacterium* suspension. Ultra sound waves cause microwounds to form on the surface and deep within the plant tissue. Wounding due to sonication creates entry points for the bacteria and may stimulate the production of signaling molecules involved in T-DNA transfer process (Finer and Larkin, 2008). SAAT overcomes certain barriers such as the host specificity and the inability of *Agrobacterium* to reach proper cells in the target tissues. It also enhances DNA transfer in diverse plant groups including dicots, monocots, and gymnosperms. It is likely that the enhanced transformation rates using SAAT result from micro-wounding both on the surface and deep within the target tissue. Therefore, unlike other transformation methods, this system also has the potential to transform meristematic tissue buried under several cell layers. SAAT increase transient transformation efficiency in several different plant tissue including leaf tissue, immature cotyledons, somatic and zygotic embryos, roots, stems, shoot apices, embryogenic suspension cells and whole seedling (Trick and Finer, 1997). Transgenic soybean plants were successfully generated by using SAAT approach; however, recovered plants were fully sterile (Trick and Finer, 1997, 1998). Santarém et al. (1998) has optimized various parameters for transient *GUS* expression in soybean cultivars such as selection of binary vectors, optical density of *Agrobacterium* during infection, duration of sonication treatment, co-culture conditions, length of explant pre-culture and addition of acetosyringone during co-culture.

Particle Bombardment

Biolistic transformation was initially welcomed as an alternative method for generating transgenic plant. Particle bombardment utilizes high velocity metal particles to deliver biologically active DNA into plant cells. The technology was first reported by Klein et al. (1987). In their experiments, transient expression of exogenous RNA or DNA was demonstrated in the bombarded epidermal cells of onion (*Allium cepa*). The concept of particle bombardment (also known as biolistics, microprojectile bombardment, gene gun, etc.) has been described in detail by Sanford, (1990). Following these experiments, the technique was shown to be a versatile and effective way for the creation of transgenic organisms including microorganisms, mammalian cells and a large number of plant species. Somatic embryos and embryonic axes explants have been reported to be the most amenable to particle bombardment-mediated transformation in soybean.

The first transgenic soybean plants created using the particle bombardment was reported by McCabe et al. (1988). In this study embryonic axes excised from immature seeds of soybean cultivars were used. Approximately 2 percent of shoots derived from this meristem were chimeric for the expression of the introduced gene. Christou et al.(1989) in their study on

particle bombardment based genetic transformation of soybean demonstrated co-transformation of tandem markers and showed that both the markers were inherited as closely linked genes in subsequent generations. The recovery of clonal plants derived from single cells has also been observed (Christou et al.1989) but the frequency of such events was low. Christou and McCabe, (1992) reported that L2 related events were involved in germ-line transformation, however L1 and L3 related events were not involved in germline transformation.

Utilization of *imazapyr* herbicidal molecule in the culture medium combined with use of the mutant Acetohydroxyacid synthase (*ahas)* gene as a selectable marker introduced by microparticle bombardment into the soybean meristematic region created a highly efficient selection system for meristematic cells (Aragao et al. 2000).

Recovery of transgenic plants through bombardment of somatic embryogenic culture was first reported by Finer and McMullen, (1991). Sato et al. (1993) reported stable transformation via particle bombardment in two soybean regeneration systems from shoot apex and embryogenic tissues. In this report, these transformation systems results obtained appeared to be directly related to differences in the cell types which were responsible for regeneration and their accessibility to particle penetration. Stewart et al. (1996) employed two shots of bombardment under 650 psi pressure and obtained 3 stably-transformed clones from 10 gram of initial bombarded tissues. Droste et al. (2002) reported production of fertile transgenic plants from bombarded embryogenic tissues of soybean. In another study by Schmidt et al. (2008), six transgenes (marker or reporter gene) were co-transferred to soybean genome and expression and inheritance was observed up to T_2 generation. This system allows transfer of multiple genes for the manipulation of complex agronomic traits and the introduction of novel biosynthetic pathways.

Both *Agrobacterium*-mediated and particle bombardment-mediated transformation suffer from certain limitations. *Agrobacterium tumefaciens,* suffer from severe host specificity which limits the scope of its use, while particle bombardment-mediated transformation is genotypic independent but results in multiple copy integration of transgenes.

In planta Transformation and other methods

In planta transformation method can avoid the constraints imposed by genotype specificity during transformation and regeneration, and eliminate tissue culture-induced genetic variation. *Agrobacterium* suspension was directly injected into axillary meristemic region of germinated seedling and transgenic progeny were recovered with 0.07% transformation rate (Chee et al.1989). The delivery of foreign DNA into plants via the pollen-tube pathway has also been reported (Zhao et al.1995) and many studies have shown that exogenous DNA can be introduced into soybean via the pollen tube pathway transformation (Zhao et al.1995; Liu et al.2009). Recently Liu et al. (2009) reported the transfer of a minimal linear marker-free and vector-free smGFP cassette into soybean via ovary-drip transformation.

Electroporation

Electroporation is a technique that utilizes a high intensity electric pulse to create transient pores in the cell membrane thereby facilitating the uptake of macromolecules like DNA. Christou et al. (1987) reported soybean transformation using electroporation and showed stable integration of genes in the calli, but did not succeed in regenerating plants. Later, transgenic plants were regenerated from calli derived from electroporated protoplasts (Dhir et al.1992). Chowrira et al. (1995) reported electroporation of intact nodal meristems which circumvented the soybean tissue culture process completely.

Agronomically important genes transferred to soybean

Recent advances in transformation technology have resulted in the routine production of transgenic soybean plants for the introduction of not only marker genes but also agronomically important genes for quality improvement, resistance to drought, fungal pathogen and insect pests. The Roundup Ready (RR) soybean developed by Monsanto was among the first transgenic crops to reach market in 1996. A new transformation event known as RR 2 Yield transgenic soybean has been developed with high yield potential. For oil quality improvement, high oleic soybean has also been developed by DuPont Inc (1996). Agronomically important genes incorporated into soybean via *Agrobacterium* and particle bombardment is presented in Table 1.

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Table 1 Agronomically important genes transferred into soybean

Target tissue	Gene	Transformation method	Selectable marker	Phenotype	Reference
Somatic embryo	Cry1Ac	PB	*HPT*	Resistance against corn earworm (*Helicoverpa zea*), soybean looper (*Pseudoplusia includens*), tobacco budworm (*Heliothis virescens*), and velvetbean caterpillar (*Anticarsia gemmatalis*)	Stewart et al.(1996)
Somatic embryo	Maize 15 kDa zein protein gene	PB	*HPT*	Increased methionine and cysteine content	Dinkins et al.(2001)
Somatic embryo	CP-SMV	PB	*HPT*	Resistance against SMV	Furutani et al.(2006)
Somatic embryo	Bean-chitinase gene (*chi*) and ribosome-inactivating protein gene (*rip*)	AT & PB	*NPTII*	Bioassay not done	Li et al.(2004)
Cot-node	FAD3	AT	*PAT*	Significant reduction in linolenic acid (18:3) content, ranging from 1.0% to 3.1%	Flores et al.(2008)
Cotyledon	Cry1Ac	AT	*CP4 EPSPS & NPTII*	Protection against *soybean looper, soybean podworm*, and *velvetbean caterpillar*	Miklos et al.(2007)
Somatic embryo	SbDV-CP	PB	*HPT*	Exhibited resistance response against SbDV	Tougou et al.(2006)
Immature cotyledon	SMV - HC-Pro	AT	*HPT*	Bioassay not done	Lim et al.(2005)
Hypocotyl	SMV-CP-3'-UTR	AT	*NPTII*	Resistance against SMV virus	Wang et al.(2001)
Embryonic axes	Cry1Ac	AT	*PAT*	Resistance to cotton bollworm	Dang and Wei, (2007)
Cot-node	BPMV-CP-P	AT	*NPTII*	Resistance phenotype against BPMV	Di et al.(1996)
Embryogenic cells Cotyledon	CRC	PB	*HPT*	Enhanced accumulation of isoflavones in seed	Yu et al.(2003)
Embryogenic cells Cotyledon	CPs	AR	*NPTII*	Reduced soybean cyst nematode infection in treated plant	Marra et al.(2009)
Embryogenic cells Cot-node	β-casein	PB	*HPT*	Expression of a milk protein in soybean	Maughan et al.(1999)
Embryogenic cells Cot-node	γ-TMT	AT	*PAT*	41-fold increase in a-tocopherol	Lee et al.2011

AR: *Agrobacterium rhizogenes*; **AT:** *Agrobacterium tumefacien*; **BPMV:** bean pod mottle virus; **CP:** Coat Protein; **CPs:** cysteine proteinase; **CRC:** maize transcription factors C1; **CP-P:** coat proteinprecursor; ***EPSPS*:** 5-enolpyruvylshikimate-3-phophate synthase; **FAD3:** omega-3 fatty acid desaturase; **HC-Pro:** helper component protease; ***HPT*:** Hygromycin phosphotransferase; ***NPTII*:** Neomycin phosphotransferase II; ***PAT* :** Phosphinotricin-N-acetyltransferase; **PB:** Particle bombardment; **SbDV:** Soybean dwarf virus; **SMV:** Soybean mosaic virus; **γ-TMT:** γ-tocopherol methyltransferase.

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