Chapter 20

In vitro Regeneration and Genetic Transformation of Soybean: Current Status and Future Prospects

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http://dx.doi.org/10.5772/54268

1. Introduction

Soybean [*Glycine max* (L.) Merrill], grown for its edible seed protein and oil, is often called the miracle crop because of its many uses. It belongs to the genus Glycine under the family Leguminosae, and is widely cultivated in the tropics, subtropics and temperate zones of the world [1].

Soybean is now an essential and dominant source of protein and oil with numerous uses in feed, food and industrial applications. It is the world's primary source of vegetable oil and protein feed supplement for livestock. The global production of soybeans is 250-260 million tons per year. The US is the largest producer with 90.6 million metric tons. Other major countries such as Brazil, Argentina, China and India contributing 70, 49.5, 15.2 and 9.6 million metric tons, respectively [2]. The US, Brazil and Argentina are the major exporters of beans; while China and Europe are the major importers. The annual world market value is around 2 billion US dollars, which stands second in world food production.

Recent nutritional studies claim that consumption of soybean reduces cancer, blood serum cholesterol, osteoporosis and heart diseases [3]. This has sparked increased demand for the many edible soybean products. The priority for more meat in diets among the world's population has also increased the demand for soybean protein for livestock and poultry feed.

Soybean seeds are comprised of 40% protein, mostly consisting of the globulins β-conglycinin (7S globulin) and glycinin (11S globulin). The oil portion of the seed is composed primarily of five fatty acids. Palmitic and stearic acids are saturated fatty acids and comprise 15% of the oil. Soybean is rich in the unsaturated fatty acids like oleic, linoleic and linolenic,

which make up 85% of the oil. Soybeans are a good source of minerals, B vitamins, folic acid and isoflavones, which are credited with slowing cancer development, heart diseases and osteoporosis [4].

The productivity of soybean has been limited due to their susceptibility to pathogens and pests, sensitivity to environmental stresses, poor pollination and low harvest index. Among the abiotic stresses, drought is considered the most devastating, commonly reducing soybean yield by approximately 40% and affecting all stages of plant growth and development; from germination to flowering, and seed filling and development as well as seed quality [5]. It suffers from many kinds of fungal diseases, such as frogeye leaf spot and brown spot [6]. As demand increases for soybean oil and protein, the improvement of soybean quality and production through genetic transformation and functional genomics becomes an important issue throughout the world [7].

The main objectives of soybean improvement include increase in yield, development of resistance to various insects, diseases and nutritional quality. Commercial breeding is still very important for the genetic improvement of the crop. However, breeding is difficult due to the fact that the soybean is a self pollinating crop, and the genetic base of modern soybean cultivars is quite narrow [8]. Most of the current soybean genotypes have been derived from common ancestors; therefore, conventional breeding strategies are limited in capability to expand the soybean genetic base. Recent advances in *in vitro* culture and gene technologies have provided unique opportunities for the improvement of plants, which are otherwise difficult through conventional breeding. The technology of plant transformation is only moderately or marginally successful in many important cultivars of crops, which can be a major limiting factor for the biotechnological exploitation of economically important plant species and the wider application of genomics.

Although numerous methods have been developed for introducing genes into plant genomes, the transformation efficiency for soybean still remains low [9]. Since the first successful transformation of soybean was reported [10], two major methods have been used in soybean transformation: one is particle bombardment of embryogenic tissue and another is *Agrobacterium tumefaciens*-mediated transformation of the cotyledonary node. Both methods have limitations: the former is highly genotype-dependent, requires a prolonged tissue culture period and tends to produce multiple insertion events, while the latter is labour intensive and requires specially trained personnel to undertake the work [9]

For soybean *in vitro* regeneration, two principal methods have been identified: somatic embryogenesis and shoot morphogenesis. Each of these systems presents both advantages and disadvantages for production of transformed plants, and each can be used with both of the predominant transformation systems [11]. A better understanding of physiology and molecular biology of *in vitro* morphogenesis needs focal attention to reveal their recalcitrant nature.

The present review gives an overview on the problems associated with low transformation efficiency, and the research conducted to improve tissue culture and transformation efficiency of soybean during the past (Table 1&2) and also discuss the future prospects, demands of these technologies and upcoming new technologies in soybean improvement.

Year	Explant tissue	Major contribution	Reference
1973	Hypocotyl	Adventitious bud development	Kimball and Bingham, [13]
1980	Cotyledonary node	Shoot morphogenesis	Cheng et al. [14]
1986	Immature embryo	Plant regeneration from callus	Barwale et al. [18]
1986	Cotyledonary node	Multiple shoot formation	Barwale et al. [19]
1986	Cotyledonary node	Multiple shoot formation	Wright et al. [20]
1987	Epicotyl	Callus induction and shoot regeneration	Wright et al. [29]
1988	Cotyledonary node	Transfered *npt* II and *gus* gene by Agrobacterium mediated transformation	Hinchee et al. [10]
1988	Immature seeds	Developed transgenic soybean by Particle bombardment	McCabe et al. [25]
1989	Germinating seeds	Transfered *npt* II gene by Agrobacterium mediated transformation	Chee et al. [45]
1989	Immature seed	Particle bombardment of meristems	Christou et al. [62]
1990	Immature cotyledon	Plant regeneration from protoplast	Luo et al. [127]
1990	Cotyledon, cotyledonary node	Evaluated *Agrobacterium* sensitivity and adventitious shoot formation	Delzer et al. [44]
1990	Immature cotyledon, plumule, cotyledonary node	Analysed plant regeneration efficiency of various explants	Yang et al. [32]
1990	Immature embryo	Organogenesis and plant regeneration	Yeh,[128]
1990	Primary leaf node	Adventitious shoot formation	Kim et al. [27]
1991	Immature cotyledon	Plant regeneration from protoplast	Dhir et al. [129]
1992	Epicotyl and hypocotyl	Investigated the stimulative effect of allantoin and amides on shoot regeneration	Shetty, et al. [21]
1993	Shoot tip	Transfered *gus* gene via particle bombardment	Sato et al. [130]
1994	Primary leaf node	Investigated the synergistic effect of proline and micronutrients on shoot regeneration	Kim et al. [40]
1996	Cotyledonary node	Developed transgenic soybean resistance to bean pod mottle virus (BPMV)	Di et al. [131]
1997	Cotyledonary node and hypocotyl	Multiple shoot induction by TDZ	Kaneda et al. [22]
1998	Cotyledonary node	Evaluation of sonication assisted Agrobacterium mediated transformation (SAAT) for cotyledonary node	Meurer et al. [50]
1998	Hypocotyl	Adventitious shoot regeneration	Dan and Reichert, [33]

Year	Explant tissue	Major contribution	Reference
1999	Cotyledonary node	Assessed the use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean	Zhang et al. [61]
2000	Cotyledonary node	*Agrobacterium* two T-DNA binary system as a strategy to derive marker free transgenic soybean	Xing et al. [132]
2000	Cotyledonary node	Evaluated the effect of glyphosate as a selective agent for *Agrobacterium* mediated cotyledonary node transformation system	Clemente et al. [60]
2000	Embryonic axes	Used of Imazapyr as selection agent for selection of meristematic soybean cells	Aragao et al. [47]
2001	Cotyledonary node	Investigated the use of thiol compound to increase transformation frequency	Olhoft et al. [56]
2001	Cotyledonary node	Increased *Agrobacterium* infection using L-cystine	Olhoft and Somers, [16]
2001	Cotyledonary node	Developed transgenic soybean plants resistant to soybean mosaic virus (SMV)	Wang et al. [133]
2001	Cotyledonary node	Expressed oxalate oxidase gene for resistant to sclerotinia stem rot caused by *Sclerotinia sclerotiorum*	Donaldson et al. [65]
2003	Hypocotyl	Screened soybean genotype for adventitious organogenic regeneration	Reichert et al. [41]
2003	Cotyledonary node	Assessed the effect of genotype, plant growth regulators and sugars on regeneration from calli	Sairam et al. [1]
2003	Cotyledonary node	Used mixture of thiol compounds and hygromycin based selection for increased transformation efficiency	Olhoft et al. [57]
2004	Cotyledonary node	Assessed glufosinate selection for increased transformation efficiency	Zeng et al. [134]
2004	Cotyledonary node	Investigated the effect of seed vigor of explant source, selection agent and antioxidant on *Agrobacterium* mediated transformation efficiency	Paz et al. [15]
2004	Cotyledonary node	Transferred chitinase gene and the barley ribosome-inactivating protein gene to enhance fungal resistance	Li et al. [6]
2004	Mature and immature cotyledon	Shoot regeneration	Franklin et al. [31]
2004	Embryonic tip	Established regeneration and *Agrobacterium* mediated transformation system	Liu et al. [35]

Year	Explant tissue	Major contribution	Reference
2004	Cotyledonary node	Established liquid medium based system for selection transformed plants	Yun, [58]
2005	Cotyledonary node	Developed repetitive organogenesis system	Shan et al. [23]
2005	Cotyledonary node	Expressed Escherichia coli K99 fimbriae subunit antigen in soybean to use as edible vaccine	Piller et al. [66]
2006	Cotyledonary node	*Agrobacterium* mediated transformation efficiency was improved by using half seed explant from mature seed	Paz et al. [24]
2007	Cotyledonary node	Investigated *Agrobacterium rhizogen* to transform soybean cotyledonary node cells.	Olhoft et al. [59]
2007	Cotyledonary node	Expressed synthetic *Bacillus thuringiensis* cry1A gene that confers a high degree of resistance to Lepidopteran Pests	Miklos et al. [135]
2007	Cotyledonary node and leaf node	Established organogenic callus induction and *Agrobacterium* mediated transformation	Hong et al ., [43]
2007	Half seed	Expressed jasmonic acid carboxyl methyltransferase in soybean to produce methyl jasmonate, which resulted in tolerant to water stress	Xue et al. [67]
2008	Hypocotyl	Used silver nitrate to enhance adventitious shoot regeneration after *Agrobacterium* transformation and developed transgenic soybean producing high oleic acid content by silencing endogenous GmFAD2-1 gene by RNAi	Wang and Xu, [7]
2008	Cotyledonary node	Improved transformation efficiency using surfactant Silwet L-77 during *Agrobacterium* infection and L-cysteine during co-cultivation	Liu et al. [136]
2008	Cotyledonary node	Developed rapid regeneration system using whole cotyledonary node	Ma and Wu, [2008]
2010	Cotyledonary node	Production of isoflavone in callus cell lines by expression of isoflavone synthase gene.	Jiang et al. [69]
2010	Cotyledon and embryo	Developed shoot regeneration from calli of soybean cv.Pyramid	Joyner et al. [39]
2011	Hypocotyl	Transgenic soybean with low phytate content	Yang et al. [70]
2011	Cotyledon	Developed transgenic soybean with increased Vitamin E content by transferring γ-tocopherol methyltransferase (γ-TMT) gene in to seedling cotyledon	Lee et al. [137]

Table 1. Major landmarks in soybean organogenesis and transformation

Explant Tissue	Year	Major Contribution	Reference
Embryonic axes	1983	Embryoids development and plant regeneration *via* suspension culture	Christianson et al.[77]
Immature cotyledon	1984	Somatic embryo Induction	Lippmann & Lippmann, [84]
Immature cotyledon	1985	Plant regeneration *via* somatic embryogenesis	Lazzeri et al. [138]
Immature embryo	1985	Somatic embryogenesis and assessment of genotypic variation	Ranch et al. [139]
Immature embryo, cotyledon and, hypocotyl from germinating seedling	1986	Somatic embryogenesis from callus	Ghazi et al. [140]
Hypocotyl and cotyledon	1986	Embryoids development in suspension culture	Kerns et al. [141]
Immature embryo and cotyledon	1987	Investigated the effect of nutritional, physical, and chemical factors on somatic embryogenesis	Lazzeri et al. [85]
Immature cotyledon	1988	Investigated the effect of auxin and orientation of explant on somatic embryogenesis	Hartweck et al. [142]
Immature cotyledon	1988	Analysed genotype dependency and High concentration of auxin on somatic embryo induction	Komatsuda and Ohyama, [143]
Immature cotyledon	1988	Investigated the interaction between auxin and sucrose during somatic embryogenesis	Lazzeri et al. [86]
Immature cotyledon	1988	Germination frequency of somatic embryo has been improved by reducing the exposure to auxin	Parrott et al. [87]
Immature cotyledon	1988	Developed rapid growing maintainable embryogenic suspension culture	Finer and Nagasawa, [82]
Immature cotyledon	1988	Histological analysis to investigate secondary somatic embryo formation.	Finer, [79]
Immature cotyledon	1989	Demonstrated the effect of genotype on embryogenesis	Parrott et al. [144]
Immature cotyledon	1989	Developed primary transformants expressing zein gene by agrobacterium mediated transformation	Parrott et al. [105]
Immature cotyledon	1989	Assayed somatic embryo maturation for conversion into plantlets	Buchheim et al. [94]
Immature cotyledon	1989	Investigated the developmental aspects of somatic embryogenesis	Christou and Yang, [145]
Immature cotyledon	1990	Screened soybean genotypes for somatic embryo production	Komatsuda et al. [146]
Immature cotyledon	1991	Transformed embryogenic cultures with *gus* and *hpt* gene *via* particle bombardment	Finer and McMullen., [64]

http://dx.doi.org/10.5772/54268

Explant Tissue	Year	Major Contribution	Reference
Immature cotyledon	1991	Analysed the interaction between genotype and sucrose concentration on somatic embryogenesis	Komatsuda et al. [147]
Immature cotyledon	1991	Demonstrated adventitious shoot formation from cotyledonary and torpedo stage embryo	Wright et al. [148]
Immature cotyledon	1992	Somatic embryo proliferation by somatic embryo cycling.	Liu et al. [83]
Immature cotyledon	1993	Improved germination efficiency of somatic embryos of cultivar H7190 by desiccation	Bailey et al. [101]
Immature cotyledon	1993	Demonstrated genotypic effect on induction, proliferation, maturation and germination of somatic embryo	Bailey et al. [96]
Immature cotyledon	1993	Investigated the factors affecting somatic embryogenesis	Lippmann & Lippmann, [149]
Immature cotyledon	1993	Soybean transformation by particle bombardment of embryogenic cultures	Sato et al. [130]
Immature cotyledon	1994	Developed transgenic soybean resistance to insect.	Parrott et al. [150]
Immature embryos	1995	Investigated the effect of glutamine and sucrose on dry matter accumulation and composition of somatic embryo.	Saravitz and Raper, [151]
Immature cotyledon	1996	Demonstrated the significance of embryo cycling for transformation	Liu et al.[152]
Immature cotyledon	1996	Transformed embryogenic cultures with 12 different plansmid *via* particle bombardment	Hadi et al. [115]
Immature cotyledon	1996	Developed transgenic soybean expressing a synthetic Bacillus thuringiensis insecticidal crystal protein gene (BtcrylAc) which is resistance to insects	Stewart et al. [46]
Immature cotyledon	1997	Investigated the effect of ethylene inhibitors on embryo histodifferentiation and maturation	Santos et al. [92]
Epicotyls and primary leaves	1997	Somatic embryogenesis and plant regeneration from cotyledon, epicotyls and primary leaves	Rajasekaran and Pello, [153]
Immature cotyledon	1997	Studied the effect of explant orientiation, pH, solidifying agent and wounding on induction of soybean from immature cotyledons	Santarém et al. [81]
Immature cotyledon	1998	Studied growth characteristics of embryogenic cultures for transformability	Hazal et al. [113]

Explant Tissue	Year	Major Contribution	Reference
Immature cotyledon	1998	Established sonication-assisted A*grobacterium* mediated transformation of soybean immature cotyledon	Santarem et al.[48]
Immature cotyledon	1998	Established sonication-assisted Agrobacterium mediated transformation of embryogenic suspension culture tissue	Trick and Finer, [108]
Immature cotyledon	1998	Improved proliferation efficiency of embryogenic cultures by modifying sucrose and nitrogen content in medium	Samoylov et al. [89]
Immature cotyledon	1998	Developed liquid medium based system for histodifferentiation of embryogenic cultures	Samoylov et al. [154]
Immature cotyledon	1998	Studied soluble carbohydrate content in soybean somatic and zygotic embryo during development.	Chanprame et al. [155]
Immature cotyledon	1999	Studied the factors influencing transformation of prolific embryogenic cultures using bombardment	Santarem and Finer, [116]
Immature cotyledons	1999	Developed transgenic plants with bovine milk protein, β-casein	Maughan et al. [114]
Immature cotyledons	1999	Transformed GFP into embryogenic suspension culture with the aim to improve transformation and regeneration strategy	Ponappa et al. [156]
Immature cotyledons	2000	Improved somatic embryo development and maturation by application of ABA	Tian and Brown, [157]
Immature cotyledon	2000	Screened genotypes for proliferative embryogenesis	Simmonds and Donaldson, [97]
Immature cotyledons	2000	Studied physical factors influencing somatic embryo development from immature cotyledons.	Bonacin et al. [99]
Immature cotyledon	2000	Investigated the factors affecting *Agrobacterium* mediated transformation soybean	Yan et al. [109]
Immature cotyledon	1989	Investigated maturation of somatic embryo for efficient conversion into plantlets	Buchheim et al. [94]
Immature cotyledon	2000	Developed and evaluated transgenic soybean expressing a synthetic cry1Ac gene from *Bacillus thuringiensis* for resistance to variety of insects	Walker et al. [158]
Immature cotyledon	2001	Effect of polyethylene glycol and sugar alcohols on soybean somatic embryo germination and conversion	Walker and Parrott, [90]

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Explant Tissue	Year	Major Contribution	Reference
Immature cotyledon	2000	Developed integrated bombardment and *Agrobacterium* transformation method	Droste et al.[159]
Immature cotyledon	2001	Screened soybean from different location in the US for uniform embryogenic response	Meurer et al. [103]
Immature cotyledon	2001	Studied the effect of osmotica for their influence on embryo maturation and germination	Walker & Parrott, [90]
Immature cotyledon	2001	Developed transgenic plant expressing 15-kD zein protein under β-phaseolin seed specific promoter	Dinkins et al. [125]
Immature cotyledon	2001	Somatic embryogenesis in Brazilian soybean cultivars	Droste et al. [160]
Immature cotyledon	2002	Somatic embryogenesis and particle bombardment for south Brazil cultivars	Droste et al. [100]
Immature cotyledon	2002	Histological analysis of developmental stages of somatic embryogenesis	Fernando et al. [161]
Immature cotyledon	2002	Screened soybean genotypes for somatic embryo induction and maturation capability	Tomlin, [162]
Immature cotyledon	2003	Investigated the effect of proliferation, maturation and desiccation on somatic embryo conversion	Moon and Hildebrand, [88]
Immature cotyledon	2004	Improved transformation efficiently using *Agrobacterium* strain KYRT1 carrying pKYRTI	Ko et al. [111]
Immature cotyledon	2004	Developed transgenic plant containing phytase gene that store (produces) more phosphrous in seed.	Chiera et al. [163]
Immature cotyledon	2004	Developed fertile transplastomic soybean	Dufourmantel et al.[117]
Immature cotyledon	2004	Transferred *chi* and *rip* gene to enhance fungal resistance	Li et al. [6]
Immature cotyledon	2004	Improved transformation efficiency using *Agrobacterium* strain KYRT1	Ko and Korban, [80]
Immature cotyledon	2004	Analysed media components and pH on somatic embryo induction	Hoffmann et al. [80]
Immature cotyledon	2005	Developed transgenic soybean expressing maize γ-zein protein	Li et al. [124]
Immature cotyledon	2005	Modified soybean histodifferentiation and msaturation medium with the aim to improve the protein and lipid composition of somatic embryo	Schmidt et al. [164]
Immature cotyledon	2005	Analysed the effect of carbon source and polyethylene glycol on embryo conversion	Korbes et al. [91]

Explant Tissue	Year	Major Contribution	Reference
Immature cotyledon	2006	Improved fatty acid content	Chen et al. [119]
Immature cotyledon	2006	Investigated the ontogeny of somatic embryogenesis	Santos et al. [165]
Somatic embryo	2006	Developed transgenic soybean resistance to dwarf virus	Tougou et al. [120]
Immature cotyledon	2006	Investigated the influence of antibiotics on embryogenic cultures and *Agrobacterium tumefaciens* suppression in soybean transformation	Wiebke et al. [166]
Immature cotyledon	2006	Developed transgenic soybean for increased production of ononitol and pinitol	Chiera et al. [167]
Immature cotyledon	2007	Developed transgenic soybean resistant to dwarf virus	Tougou et al. [168]
Immature cotyledon	2007	Improved somatic embryogenesis in recalcitrant cultivars by back cross with a highly regenerable cultivar Jack	Kita et al. [104]
Immature cotyledon	2007	Evaluated Japanese soybean genotypes for somatic embryogenesis	Hiraga et al. [102]
Immature cotyledon	2007	Soybean seed over expressing the *Perilla frutescens* γ -tocopherol methyltransferase gene	Tavva et al. [123]
Immature cotyledon	2007	Improved protein quality in transgenic soybean transformed with modified Gy1 proglycinin gene with a synthetic DNA encoding four continuous methionines.	EI-Shemy et al. [169]
Immature cotyledon	2007	Analysed the effect of Abscisic acid on somatic embryo maturation and conversion.	Weber et al. [170]
Immature cotyledon	2007	Developed transgenic soybean resistance to soybean mosaic virus	Furutani et al. [121]
Immature cotyledon	2008	Used a new Selectable Marker Gene Conferring resistance to Dinitroanilines	Yemets et al. [171]
Immature cotyledon	2008	Developed strategy for transfer of multiple genes *via* micro projectile-mediated bombardment	Schmidt et al. [172]
Immature cotyledon	2009	Assessed the effect mannitol, abscisic acid and explant age on somatic embryogenesis in Chinese soybean cultivars	Yang et al. [98]
Somatic embryo	2009	Developed transgenic soybean with increased oil content	Rao and Hildebrand, [118]

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Explant Tissue	Year	Major Contribution	Reference
Embryonic tip	2010	somatic embryogenesis and plant regeneration from the immature embryonic shoot tip	Loganathan et al. [173]
Immature cotyledon	2010	Developed transgenic soybean with more tryptophan content in seed	Ishimoto et al. [122]
Immature cotyledon	2010	Screening of Brazilian soybean genotypes for embryogenesis	Droste et al. [174]
Immature cotyledon	2011	Demonstrated Metabolic engineering of soybean seed coat for the production of novel biochemicals	Schnell et al. [126]
Immature cotyledon	2011	Investigated developmental profile of storage reserve accumulation in soybean somatic embryos	He et al. [175]
Immature cotyledon	2011	Improved transformation efficiency by Micro wounding with DNA free particle bombardment followed by Agrobacterium mediated transformation.	Wiebke et al. [112]
Immature cotyledon	2012	Developed vacuum infiltration assisted *Agrobacterium* mediated transformation for Indian soybean cultivars.	Mariashibu et al. [176]

Table 2. Major landmarks in soybean somatic embryogenesis and transformation

2. Organogenesis and transformation

Organogenesis is characterized by the production of a unipolar bud primordium with subsequent development of the primordium into a leafy vegetative shoot. A successful plant regeneration protocol requires appropriate choice of explant, definite media formulations, specific growth regulators, genotype, source of carbohydrate, gelling agent, other physical factors including light regime, temperature, humidity and other factors [12]. Plant regeneration by organogenesis in soybean was first reported by Kimball and Bingham, [13] from hypocotyl sections followed by Cheng et al.[14] by culturing seedling cotyledonary node segments. Transfer of T-DNA into cotyledonary node cells by *Agrobacterium* mediated transformation was first reported by Hinchee et al. [10]. Advancement in soybean transformation appears to be slow compared to some of the recent improvement in cereal transformation (Paz et al. 2004). Olhoft et al. [16] stated that the efficiency of soybean transformation has to be improved 5-10 times before one person can produce 300 transgenic lines per year. Soybean transformation efficiency has been improved by optimizing the selection system, enhancing explant-pathogen interaction and improving culture conditions to promote regeneration and recovery of transformed plants.

2.1. Organogenesis

The successful application of biotechnology in crop improvement is based on efficient plant regeneration protocol. Soybean has been considered as recalcitrant to regenerate *in vitro*. Tissue culture responses are greatly influenced by three main factors viz. whole plant physiology of donor, *in vitro* manipulation, and *in vitro* stress physiology [17]. After the first report of adventitious bud regeneration from hypocotyl sections by Kimball and Bingham, [13] researchers have used different parts of the soybean plant as explants for successful shoot morphogenesis in soybean. These include cotyledonary node [10,14,18-24], shoot meristems [25], stem-node [26,27] epicotyls [28], primary leaf [29], cotyledons [30,31], plumules (32), hypocotyls [22,33,34], and embryo axes [25,35]. Plant regeneration *via* organogenesis from cotyledonary node was found to be the most convenient and faster approach in soybean. However, much improvement is needed for the cotyledonary node regeneration system. This limitation is mainly due to low frequency of shoot regeneration, long regeneration period and explant growth difficulties, which prevent the plant from being regeneration-competent[36].

The nutritional requirement for optimal shoot bud induction from different explants has been reported to vary with mode of regeneration. Media compositions have a key role in shoot morphogenesis, the basal medium MS [37] is most commonly used for soybean organogenesis and the medium B5 [38] are useful in some approaches. Benzylaminopurine (BA) has been the most commonly used plant growth regulator either alone or in combination with a low concentration of cytokinins, kinetin or thidiazuron (TDZ) [22, 39]. TDZ was reported to induce multiple bud tissue (MBT) from cotyledonary node axillary meristem which then gives shoots in the presence of BA [23]. The efficiency of shoot bud formation were enhanced by supplementing media with proline, increased level of MS micro nutrients [40], and ureide in the form of allantoin and amides [21].

Adventitious shoot regeneration from cotyledonary node or leaf node is based on proliferation of meristems. Use of pre-existing shoot meristems in transformation procedures can increase the chance of chimerism, so identifying tissues that can produce shoots in the absence of such pre-formed organs would be important [41]. Adventitious soybean shoots have been induced from hypocotyls [13]; cotyledons [18, 20], primary leaves [29] and epicotyls [28]. Hypocotyls of seedlings have been used as explants for adventitious shoot regeneration by Kaneda et al. [22]. Explants cultured on media supplemented with TDZ induced adventitious shoots more efficiently than BA. Histological analysis of adventitious shoot regeneration from the hypocotyl shows shoot primordias, formed from parenchymatous tissues of central pith and plumular trace regions [33]. Hypocotyls of seedlings have seldom been used as explants, even though the shoot regeneration frequency from hypocotyl segments was found to be higher than from cotyledons [22]. Franklin et al. [31] investigated the factors affecting adventitious shoot regeneration from the proximal end of mature and immature cotyledons. The presence of BAP and TDZ in the medium exerted a synergistic effect, in that regeneration efficiency was higher than for either cytokinin alone.

Indirect organogenesis is important as an alternative source of genetic variation in order to recover somaclones with interesting agronomic traits. Callus regeneration is advantageous over direct regeneration for transformation since effective selection of transgenic cells can be achieved [1]. However, the efforts made to regenerate plants from callus have yielded poor

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results since plants could not be regenerated from any type of soybean callus [42]. Yang et al. [32] compared different explants excised from immature and germinated seeds for callus mediated organogenic regeneration, although induction of organogenic callus was easily achieved by culture of immature cotyledons, development of adventitious buds from these calluses and the subsequent growth of these buds to shoots were inefficient, suggesting that only part of the callus was competent for regeneration. Sairam et al. [1] developed a rapid and efficient protocol for regeneration of genotype-independent cotyledonary nodal callus for cultivars Williams 82, Loda and Newton through manipulation of plant growth regulators and carbohydrates in the medium. Hong et al. [43] reported organogenic callus induction from cotyledonary node and leaf node explants in media supplemented with TDZ and BA, the system has been successfully utilized for *Agrobacterium*-mediated transformation

2.2. Genotype

Among the different factors affecting soybean regeneration, the genotypic dependence is ranked quite high. Since there is strong genotype specificity for regeneration of different soybean genotypes, a major limiting factor, it is pivotal to formulate genotype specific regeneration protocols. Genotype specificity for regeneration in soybean is well documented, although organogenesis is less genotype dependent and has become routine in several laboratories [18,20,28,29&33]. Reichert et al. [41] tested organogenic adventitious regeneration from hypocotyl explants excised from 18 genotypes. Plant formation from hypocotyl explants showed that all genotypes were capable of producing elongated shoots that could be successfully rooted. This study confirmed the genotype independent nature of this organogenic regeneration from the hypocotyl explant. Sairam et al. [1] developed an efficient genotype independent cotyledonary nodal callus mediated regeneration protocol for soybean cultivars Williams 82, Loda and Newton developed through manipulation of plant growth regulators and carbon source. Callus induction and subsequent shoot bud differentiation were achieved from the proximal end of cotyledonary explants on modified MS [37] media containing 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA), respectively. Sorbitol was found to be the best for callus induction and maltose for plant regeneration. The genotypic dependence of regeneration from cotyledon explants could be reduced by the use of combinations of cytokinins (Franklin et al. [31]). Though there was no significant difference in shoot bud formation among different genotypes, but there was significant difference in conversion of the number of regenerated plants in each cultivar (Delzer et al. [44]).

2.3. *Agrobacterium* mediated transformation

Agrobacterium-mediated transformation of soybean was first demonstrated by Hinchee et al. [10] through delivering, T-DNA into cells in the axillary meristems of the cotyledonary-node. After that scientists have attempted to introduce a lot of genes using *Agrobacterium* [25, 45-47]. The cotyledonary-node method is a frequently used soybean transformation system based on *Agrobacterium*-mediated T-DNA delivery into regenerable cells in the axillary meristems of the cotyledonary-node [16]. The efficiency of this transformation system remains low, apparently because of infrequent T-DNA delivery to cells in the cotyledonary-node axillary meristem, inefficient selection of transgenic cells that give rise to shoot

meristems, and low rates of transgenic shoot regeneration and plant establishment. The development of an effective Agrobacterium transformation method for soybean depends on several factors including plant genotype, explant vigor, Agrobacterium strain, vector, selection system, and culture conditions [48, 49]. Increased soybean transformation efficiency, may be achieved by further optimizing the selection system, enhancing explant-pathogen interaction and improving culture conditions to promote regeneration and recovery of transformed plants. It has been reported that soybean genotype contributed to variation in susceptibility to *Agrobacterium* and regenerability in tissue culture [50, 51]. In addition, surface sterilization of plant tissue material for *in vitro* tissue culture and transformation is one of the critical steps in carrying out transformation experiments. While a short time of sterilization cannot completely decontaminate explants, prolonged sterilization may cause damage to explants and consequently affect their regenerability [52]. Antioxidant reagents such as cysteine, dithiothreitol, ascorbic acid and polyvinyl pyrrolidone have been used in plant transformation optimization to enhance either tissue culture response or transformation efficiency [53-55]. Recently, high transformation efficiency has also been reported in soybean by adding cysteine and thiol compounds to the cocultivation media [16, 56,57]. Liu et al. [35] established *Agrobacterium* mediated transformation using shoot tip explants of Chinese soybean cultivars. It had the advantage over the cotyledonary node by having no necrosis after infection, and showed more transient *gus* expression as embryonic tips are more sensitive to *Agrobacterium* because they contain promeristems and procambium. Yun, [58] established liquid medium to select transformed plants from the cotyledonary node. Liquid selection has proven to be more efficient than solid selection due to the direct contact of the explants with the medium and the selection agent in the medium. Olhoft et al. [59] transformed soybean cotyledonary nodes using *Agrobacterium rhizogens* strain SHA17 for the first time. The transformation efficiency was as high as 3.5 fold when compared with *Agrobacterium tumefaciens* strain AGL1. Clemente et al. [60] successfully used and evaluated the effect of glyphosate as a selective agent within the *Agrobacterium mediated* cotyledonary transformation system. Imazapyr is a herbicidal molecule that inhibits the enzymatic activity of acetohydroxyacid synthase, which catalyses the initial step in the biosynthesis of isoleucine, leucine and valine. Aragao et al. [47] used Imazapyr as a selection agent for selection of meristematic soybean cells transformed with the *ahas* gene from Arabidopsis. The *bar* gene encodes for phosphinothricin acetyltransferase (PAT) which detoxifies glufosinate, the active ingredient in the herbicide. Zhang et al. [61] successfully used glyphosate to select transformed cells after *Agrobacterium* transformation of cotyledonary node cells.

2.4. Particle bombardment

Even though particle bombardment is a widely used technique for transforming soybean embryogenic cultures, it was rarely explored for shoot morphogenesis. McCabe et al. [25] was the first to report particle bombardment mediated transformation in soybean. Transforming meristems of soybean bu DNA coated gold particles followed by shoot regeneration in the presence of cytokinin, resulting in the development of chimeras. In subsequent studies, non-chimeric plants were obtained through the use of screening methods for the selection of plants that contained transgenic germ-line cells [32,62&63]. Shoot apex transfor-

mation is labour intensive because the meristematic tissue is diffcult to target and, without selection, a large number of plants must be regenerated and analysed [64].

2.5. Genes for trait improvement

Soybean has been improved by *Agrobacterium* mediated transformation followed by shoot regeneration. Wheat germin gene (gf-2.8) encoding an oligomeric protein and oxalate oxidase (*oxo*) genes were introduced into soybean to improve resistance to the oxalate-secreting pathogen *Sclerotina sclerotiorum* [65]. Li et al.[6] successfully utilized *Agrobacterium*-mediated transformation to transfer chitinase gene (*chi*) and the barley ribosome-inactivating protein gene (*rip*) into soybean cotyledonary node cells. Piller et al. [66] investigated the feasibility of expressing the major Enterotoxigenic Escherichia coli K99 fimbrial subunit, FanC, in soybean for use as an edible subunit vaccine. Xue et al. [67] successfully expressed jasmonic acid carboxyl methyltransferase (NTR1) gene from *Brassica campestris* into soybean cv.Jungery that produces methyl jasmonate and showed tolerance to water stress. Soybean oil contains very low level of α-tocopherol which is the most active form of tocopherol. The tocopherols present in the seed are converted into α- and β-tocopherols by overexpressing γ-tocopherol methyltransferase from *Brassica napus* (BnTMT) [68]. Jiang et al. [69] transferred isoflavone synthase (IFS) gene into soybean callus using *Agrobacterium*-mediated transformation and the transgenic plants produced increased levels of the secondary metabolite, isoflavone. Transgenic soybean plant containing PhyA gene of *Aspergillus ficuum* exhibited a lower amount of phytate in different soybean tissues including the leaf, stem and root. This indicated that engineering crop plants with a higher expression level of heterologous phytase could improve the degradation of phytate and potentially in turn mobilize more inorganic phosphate from phytate and thus reduce phosphate load on agricultural ecosystems [70].

3. Somatic embryogenesis and transformation

Somatic embryogenesis is a process by which a plant somatic cell develops into a whole plant without gametic fusion but undergoes developmental changes as that of zygotic embryogenesis [71, 72]. The first demonstration of *in vitro* somatic embryogenesis was reported in *Daucus carota* by Reinert [73]. The concept of embryogenesis has drawn a lot of attention because of its significance in theory and practice. Primarily, somatic embryos can be produced easily and quickly, so that it provides an economical and easy way to study plant development. Secondly, synthetic seeds developed from somatic embryos open the possibility of developing high quality seeds and may allow us to produce seeds from those plants that require a long period for seed production. Somatic embryogenesis is also useful in plant genetic engineering since regeneration *via* somatic embryogenesis is frequently single of cell origin, resulting in a low response of chimeras and high a number of true transgenic regenerants [74, 75].

3.1. Somatic embryogenesis

The first record of soybean somatic embryogenesis was reported by Beversdorf & Bingham [76], followed by Christianson et al. [77] who regenerated plants through the method. The immature cotyledon is the preferred explant for soybean somatic embryogenesis as it has pre-determined embryogenic cells. Somatic embryogenesis is a multi-step regeneration process starting with the formation of proembryogenic cell mass, followed by somatic embryo induction, their maturation, desiccation and finally plant regeneration [78].

Soybean somatic embryos were induced from immature cotyledon explants cultured on medium containing high levels of 2,4-D [79]. Even though NAA induced somatic embryogenesis from immature cotyledons, the mean number of embryos produced on 2,4-D was significantly higher [80]. Explant orientation, pH, solidifying agent, and 2,4-D concentration have a synergic effect on somatic embryo induction [81]. The early-staged somatic embryos can be maintained and proliferated by subculturing the tissue on either semi-solid medium [79] or liquid suspension culture medium [82]. Somatic embryos incubated in a medium containing NAA do not proliferate so well as those produced on a medium containing 2,4-D [83]. Somatic embryos initiated on NAA are more advanced in embryo morphology than those induced on 2,4-D and the efficiency of somatic embryo induction was highest with a medium containing 2-3% sucrose. Cultures initiated on lower sucrose concentrations tended to produce a higher amount of friable embryos, while increased concentrations of this sugar impaired embryo induction [80,84-86]. Histodifferentiation and maturation of somatic embryos doesn't need exogenous auxin or cytokinins [87]. Indeed, poorly developed meristem or swollen hypocotyls may be an undesired outcome of the application of exogenous auxins and cytokinins, respectively. Moon and Hildebrand, [88] investigated the effects of proliferation, maturation, and desiccation methods on conversion of soybean somatic embryos to plants. Somatic embryos proliferated on solid medium showed a higher regeneration rate when compared with the embryos proliferated in liquid medium. The growth period of somatic embryo development can be reduced one month by culturing in a medium devoid of 2,4-D and B_5 vitamins. Carbon source is critical for embryo nutritional health and improves somatic embryo maturation. The effects of carbohydrates on embryo histodifferentiation and maturation on liquid medium were analyzed by Samoylov et al. [89]. FNL medium supplemented with 3% sucrose (FNL0S3) or 3% maltose (FNL0M3) were compared. Data indicated that sucrose promotes embryo growth and significantly increases the number of cotyledon-stage embryos recovered during histodifferentiation and maturation. However, the percentages of plants recovered from embryos differentiated and matured in FNL0S3 was lower than those grown in FNL0M3 (Samoylov et al. 1998b). The quality of somatic embryos can be positively influenced by a low osmotic potential in maturation medium [90, 91]. Carbohydrates can act as an osmotic agent. Polyethylene glycol 4000, mannitol and sorbitol were tested as supplements to a liquid Finer and Nagasawa medium-based histodifferentiation/maturation medium FNL0S3, for soybean (*Glycine max* L. Merrill) somatic embryos of 'Jack' and F138 or 'Fayette'[90]. Overall, 3% sorbitol was found to be the best of the osmotic supplements tested. The ability of histodifferentiation and conversion of somatic embryo have been improved by the use of ethylene inhibitor aminoethoxyvinylglycine [92].

http://dx.doi.org/10.5772/54268

The effects of ethylene on embryo histodifferentiation and conversion were genotype-specific. The germination frequency of soybean embryos is very low [93], and therefore, partial desiccation of somatic embryos was emphasised with a view to improving the germination frequency in soybean [87,94&95]. Desiccation induced a physiological state there by increase the germination ability of somatic embryos [87].

3.2. Genotype

Soybean somatic embryogenesis is highly genotypic when compared to organogenesis. The existence of strong genotype specificity in the regeneration capacity of the different cultivars represents a major limiting factor for the advancement of soybean biotechnology. The embryogenic efficiency of soybean was shown to be different among cultivars at each stage (induction, proliferation, maturation, germination) of somatic embryogenesis [92,96] and it is very challenging to identify genotypes highly responsive to all stages. Simmonds and Donaldson, [97] screened 18 short season soybean genotypes for proliferative embryogenesis. Five genotypes produced embryogenic cultures which were proliferative for at least 6 months. Yang et al. [98] screened 98 Chinese soybean varieties for somatic embryogenesis and selected 12 varieties based on their embryogenic capacity. The greatest average number of plantlets regenerated per explant (1.35) was observed in N25281. Bonacin et al. [99] demonstrated the influence of genotype on somatic embryogenic capability of five Brazilian cultivars. Droste et al. [100] reported somatic embryo induction, proliferation and transformation of commercially grown Brazilian soybean cultivars for the first time. Soybean somatic embryo conversion is genotype dependent; germination frequency of H7190 was approximately three fold lower than that of PI 417138 [101]. Hiraga et al. [102] examined the capacity for plant regeneration through somatic embryogenesis in Japanese soybean cultivars and identified Yuuzuru and Yumeyutaka as having high potential for somatic embryogenesis. Several cultivars were identified as uniformly embryogenic at the primary induction phase at all locations, among which Jack was the best [103]. Kita et al. [104] evaluated somatic embryogenesis, proliferation of embryogenic tissue, and regeneration of plantlets in backcrossed breeding lines derived from cultivar Jack and a breeding line, QF2. The backcrossed breeding lines exhibited an increased capacity for induction and proliferation of somatic embryos and were used successfully to generate transgenic plants.

3.3. *Agrobacterium* mediated transformation

Recovery of the first transgenic plant *via* somatic embryogenesis in soybean was reported by Parrott et al. [105]. Immature cotyledon tissues were inoculated with *Agrobacterium* strain which contained 15 kD zein gene and the neomycin phosphotransferase gene. The explants were placed on medium containing high auxin for somatic embryo induction. Three transgenic plants containing the introduced 15 kD zein gene were regenerated. Unfortunately, these plants were chimeric and the 15 kD zein gene was not transmitted to the progeny. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) of immature cotyledons tremendously improves the efficiency of *Agrobacterium* infection by introducing large numbers of micro wounds into the target plant tissue [48]. The highest GUS

expression was obtained when immature cotyledons were sonicated for 2s in the presence of *Agrobacterium* followed by co-cultivation for 3 days. Trick and Finer, [108] successfully employed Sonication-assisted *Agrobacterium*-mediated transformation of embryogenic suspension culture tissue and when SAAT was not used, no transgenic clones were obtained. Yan et al. [109] demonstrated the feasibility of *Agrobacterium* mediated transformation of cotyledon tissue for the production of fertile transgenic plants by optimising the *Agrobacterium* concentration, using co-cultivation time and selecting proper explant. Ko and Korban, [110] investigated optimal conditions for induction of transgenic embryos followed by *Agrobacterium* mediated transformation. Using cotyledon explants from immature embryos of 5-8mm length, a 1:1 (v/v) concentration of bacterial suspension and 4-day co-cultivation period significantly increased the frequency of transgenic somatic embryos. The *Agrobacterium* tumefaciens strain KYRT1 harboring the virulence helper plasmid pKYRT1 induces transgenic somatic embryos at a high frequency from infected immature soybean cotyledons [111]. Recently, the successful recovery of a high number of soybean transgenic fertile plants was obtained from the combination of DNA- free particle bombardment and *Agrobacterium*-mediated transformation using proliferating soybean somatic embryos as targets [112].

3.4. Particle bombardment

Particle bombardment is a widely used technique for transformation of embryogenic cultures of soybean; the major advantage of this technique over *Agrobacterium* is the removal of biological incompatibilities. Particle bombardment in soybean was first reported by Finer and McMullen [64], in which embryogenic suspension culture tissue of soybean was bombarded with particles coated with plasmid DNAs encoding hygromycin resistance and β-glucuronidase. Analysis of DNA from progeny plants showed genetic linkage for multiple copies of introduced DNA. Using particle bombardment, fertile plants could be routinely produced from the proliferating transgenic embryogenic clones. Hazal et al. [113] studied growth characteristics and transformability of embryogenic cultures and found that cultures bombarded between 2-6 days after transfer to fresh medium showed more transient expression of the reporter gene. Histological analysis showed that the most transformable cultures had cytoplasmic-rich cells in the outermost layers of the tissue. Maughan et al. [114] bombarded embryogenic cultures with plasmid containing 630-bp DNA fragment encoding a bovine milk protein, β-casein. Hadi et al. [115] co-transformed 12 different plasmids into embryogenic suspension culture by particle bombardment. Hybridization analysis of hygromycin resistance clones verified the presence of introduced plasmid DNAs. Santarem and Finer [116] investigated the effect of desiccation of target tissue, period of subculture prior to bombardment and number of bombardments per target tissue for enhancement of transient expression of the reporter gene. Desiccation of proliferating tissue for 10 min, subculture on the same day prior to bombardment and three times bombardment on a single day enhanced the transient expression of β-glucuronidase [116]. Dufourmantel et al. [117] successfully transformed chloroplasts from embryogenic tissue of soybean using DNA carrying spectinomycin resistance gene (*aadA*) by bombardment. All transplastomic T0 plants were fertile and T1 progeny was uniformly

http://dx.doi.org/10.5772/54268

spectinomycin resistant, showing the stability of the plastid transgene. *Droste* et al. [100] successfully transformed embryogenic cultures of soybean cultivars recommended for commercial growing in South Brazil by bombardment, and this opened the field for the improvement of this crop in this country by genetic engineering.

3.5. Genes for trait improvement

Li et al. [6] attempted to transform two antifungal protein genes (*chitinase* and ribosome-inactivating protein) by co-transformation. Transgenic soybeans expressing the Yeast SLC1 Gene showed higher oil content [118]. They reported that, compared to controls, the average increase in triglyceride values went up by 1.5% in transgenic somatic embryos and also found that a maximum of 3.2% increase in seed oil content was observed in a T3 line. Transfer of Δ6 desaturase, fatty acid elongase and D5 desaturase into soybean under seed specific expression produced arachidonic acid (ARA) in seeds of soybean [119]. In an attempt to enhance soybean resistance to viral diseases, several groups successfully generated transgenic plants by expressing an inverted repeat of soybean dwarf virus SbDV coat protein (*CP*) genes [120], or soybean mosaic virus (SMV) coat protein gene [121]. The nutritional quality of soybean has been improved for enhanced amino acid, proteins and vitamin production by transgenic technology [114, 122, 123, 124, and 125]. The feasibility of genetically engineering soybean seed coats to divert metabolism towards the production of novel biochemicals was tested by transferring the genes phbA, phbB, phbC from *Ralstonia eutropha*. Each gene was under the control of the seed coat peroxidase gene promoter [126]. The analysis of seed coats demonstrated that polyhydroxybutyrate (PHB) was produced at an averge of 0.12% seed coat dry weight.

4. Conclusion and future prospects

As demands increase for soybean oil and protein, the improvement of soybean quality and production through genetic transformation and functional genomics becomes an important issue throughout the world. Modern genetic analysis and improvement of soybean heavily depend on an efficient regeneration and transformation process, especially commercially important genotypes. The transformation techniques developed until now till date do not allow high-throughput analyses in soybean functional genomics; though significant improvements have been made in the particle bombardment of embryogenic culture and *Agrobacetrium* mediated transformation of the cotyledonary node over the past three decades. However, routine recovery of transgenic soybean plants using either of these two transformation systems has been restricted to a few genotypes with no reports of transformation on other locally available commercial genotypes. Therefore, development of an efficient and consistent transformation protocol for other locally available commercial genotypes, will greatly aid soybean functional genomics and transgenic technology.

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