

I.1 Soybean

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1 Introduction

Soybean [*Glycine max* (L.) Merr.] is grown in more than 50 countries and is the leading oilseed crop produced and consumed worldwide (Wilcox 2004). In addition to oil, soybeans are a rich and efficiently produced source of protein. They are consumed directly and, following fermentation or processing, are consumed as food, food ingredients, food additives and dietary supplements, and they are also used in animal feeds and for the production of a range of industrial compounds and materials. World soybean production has nearly doubled since 1985, by increasing yields on a per area basis and by increasing the overall area planted with the crop (Wilcox 2004). Substantial quantities of soybeans are traded on world markets and, therefore, the crop contributes significantly to the global economy (Wilcox 2004).

The worldwide scale of soybean production and the economic importance of the crop dictate that substantial private industry and public research efforts are directed towards crop improvement, management and production. Conventional plant breeding has been and will continue to be the mainstay of soybean improvement (Sleper and Shannon 2003). Over the past 30 years, major advances in molecular genetics and, more recently, genomics have been applied to crop improvement, resulting in molecular approaches that augment conventional breeding efforts. In soybean, a number of molecular marker maps have been constructed and applied to marker-assisted breeding. For example, mapping the *rhg-1* locus conferring resistance to soybean cyst nematode is used routinely for introgressing that region into new soybean varieties. The next step on this path of genome characterization is the completion of the alignment of genome sequences to genetic and physical maps and the overwhelming task of providing this information to the scientific community (Jackson et al. 2006). The majority of expressed genes in soybean have been at least partially sequenced, providing an extensive library of expressed sequenced tags (ESTs) for gene identification. Taken together, the molecular and genomic resources now available in soybean provide and will continue to provide a range of tools for investigating gene function, the genetics and regulation

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of important traits, and for crop improvement through both conventional and marker-assisted breeding, and genetic engineering approaches (Stacey et al. 2004).

Introgression of desired traits into crops using transformation approaches augments traditional breeding and add value. Genetic transformation of soybean has been and will likely continue to be an important component in crop improvement. For example, Roundup Ready soybeans are the most extensively planted transgenic crop worldwide occupying 109 million acres (ca. 44×10^6 ha) in 2004. Roundup Ready soybean was produced from some of the first successful soybean transformation experiments conducted in 1988, as described by Padgett et al. (1995). Since that time, other approaches have emerged as the predominant transformation methods (Trick et al. 1997; Parrot and Clemente 2004). Transformation of soybean remains to be highly challenging with relatively few laboratories routinely able to transform soybean. The methods commonly used for soybean transformation require personnel skilled in the art to produce healthy, fertile transgenic plants that transmit the transgene to the next generation. Currently, many soybean transformation systems are dependent on the production of tissue cultures as sources of regenerable target cells for DNA delivery. Regeneration of plants from cell cultures in soybeans occurs through either somatic embryogenesis or shoot organogenesis from apical and axillary meristematic cells. For somatic embryogenesis, embryo development is mainly induced from explants derived from immature zygotic embryos that were initially exposed to high concentrations of auxin in the medium, especially 2,4-dichlorophenoxyacetic acid (2,4-D). In contrast, shoots that regenerate via organogenesis are derived from explants exposed to low levels of cytokinins like 6-benzylaminopurine (BAP). In this review, various methods are discussed that successfully and repeatedly regenerate whole fertile plants from tissue culture, with a focus on those methods predominately used for transformation.

2 Somatic Embryogenesis

2.1 Background

Somatic embryo development progresses through the distinct phases of histodifferentiation, maturation, desiccation, germination and conversion, as reviewed by Parrott et al. (1995). Histodifferentiation begins with the induction of the embryogenic stage and lasts until auxin concentration is lowered and somatic embryo development commences. Embryos during this stage are actively undergoing cell division and differentiate through the globular, heart and torpedo stages, ending with the cotyledonary stage. At somatic embryo maturation, mitosis ceases and cell expansion occurs, with the accumulation of storage reserves and acquisition of desiccation tolerance. Physiological matu-

urity is followed by a period of desiccation that is associated with the synthesis of proteins that enables germination of the cotyledon-stage embryo. Conversion is completed with whole plant regeneration of the germinated embryo.

The primary method for producing somatic embryogenic cultures is through direct induction from immature zygotic cotyledons, meaning that embryos develop with few mitotic cell cycles before becoming embryogenetically determined. Thus, there is little intervening callus phase before induction (Parrott et al. 1995). Somatic embryo development is usually stimulated by culturing the immature cotyledons with high concentrations of auxin such as 2,4-D. However, α -naphthaleneacetic acid (NAA) is also used to induce embryogenesis. Exposure of the soybean cotyledon explants with 2,4-D results in the development of somatic embryos from most of the epidermal surface (Hartweck et al. 1988); and cotyledon explants exposed to NAA develop fewer normal embryos that are limited to the crescent-shaped distal periphery of explants (Lazzeri et al. 1987; Hartweck et al. 1988).

Indirect somatic embryogenesis from callus cultures derived from immature zygotic embryos has been reported (Christianson 1983; Barwale et al. 1986; Ghazi et al. 1986). A smooth, green, shiny embryogenic callus was induced from cotyledon tissue that was capable of regeneration when cultured in the presence of 10 mg l^{-1} 2,4-D ($45 \text{ } \mu\text{M}$; Ghazi et al. 1986). By changing the hormones in the callus induction medium, Barwale et al. (1986) reported plant regeneration from callus cultures derived from immature embryos from 54 genotypes through either embryogenesis or organogenesis. Embryogenic cultures capable of plant regeneration were best obtained by adding $43 \text{ } \mu\text{M}$ NAA to an MS-based medium (Murashige and Skoog 1962) after 4 weeks growth. When the MS media was amended with $13.3 \text{ } \mu\text{M}$ BAP, $0.2 \text{ } \mu\text{M}$ NAA and four times the normal concentration of MS salts, regenerable organogenic callus cultures from the immature embryos resulted (see Section 3: Organogenesis). Although these studies indicate callus cultures capable of regeneration were established, it is much more common to directly induce somatic embryogenesis without going through the callus phase.

Genotype is a critical factor in determining the somatic embryo quality and quantity and in regeneration competency and, therefore, affects all stages of somatic embryogenesis from induction, histodifferentiation, maturation and conversion (Delzer et al. 1990; Simmonds and Donaldson 2000; Meurer et al. 2001; Tomlin et al. 2002). Soybean genotypes are grouped according to maturity zones in North America from 000 to 10, with lower maturity groups belonging to Northern genotypes and higher maturity groups to the Southern genotypes. No relationship between maturity group and induction of embryogenesis was apparent in studies by Bailey et al. (1993). However, a few studies indicate that some of the most responsive genotypes are from lower maturity groups (Meurer et al. 2001; Tomlin et al. 2002). In particular, one of the most responsive cultivars used across many laboratories because of its consistent embryogenic response and reliable plant regeneration is the maturity group II cultivar 'Jack'. Because soybeans are photoperiod-sensitive, it is important to

factor in regeneration conditions with the maturity group of the cultivar being used for somatic embryogenesis. Problems associated with photoperiod sensitivity are partially overcome in somatic embryogenesis protocols by supplying the *in vitro* tissue cultures with a continuous light regime, thereby ensuring that plant development remains in the vegetative phase (Ghazi et al. 1986). After comparing both embryogenic induction and proliferation from immature cotyledons of nine cultivars, Meurer et al. (2001) concluded that, to successfully regenerate soybean plants via somatic embryogenesis, an established protocol should be used in conjunction with one of several embryogenic cultivars already identified, such as Jack.

2.2 Regeneration

Development of a successful regeneration protocol via somatic embryogenesis in soybean involved many years of optimization and experimentation in many laboratories. Some of the first successful efforts in establishing embryonic tissues were from suspension cultures of soybean initiated from hypocotyl or cotyledon callus tissue (Beversdorf and Bingham 1977). The hypocotyl or cotyledon callus was found to differentiate into embryos in liquid medium containing high sucrose and 2,4-D, but full plant regeneration was not achieved (Beversdorf and Bingham 1977). An early report of plant regeneration via somatic embryogenesis was by Christianson et al. (1983). In this study, a morphogenetically competent suspension culture was initiated from a single callus piece derived from the embryonic axes from which a single plant was regenerated. Ranch et al. (1985) reported plant regeneration using an intact zygotic embryo as an explant source and Lazzeri et al. (1985) reported regeneration using excised cotyledons. Currently, somatic embryogenesis methods in soybeans use the excised immature cotyledons to induce embryoids. A general outline for somatic embryogenesis is given below, from initiation of somatic embryos to plant regeneration.

2.2.1 Induction

The following protocol for induction of embryogenic tissues from immature cotyledons was described by Finer and Nagasawa (1988). Immature seeds from pods 7–14 days after flowering were collected and surface-sterilized before explant preparation. The optimal size of the cotyledons for induction of embryoids was 3–5 mm in length (Lazzeri et al. 1985; Finer 1988). The cotyledons from the immature seeds were separated from the embryo axes (Lazzeri et al. 1985) and cultured with the abaxial surface in contact with induction medium (MSD40) containing MS (Murashige and Skoog 1962) salts, B5 vitamins (Gamborg et al. 1968), 6% sucrose, 40 mg l⁻¹ 2,4-D (0.18 mM), and 0.8% agar (pH 5.7) to induce embryogenic callus growth. In some working protocols, lower concentrations of sucrose are used. Lippmann and Lippman (1984),

Komatsuda et al. (1991), and Hofmann et al. (2004) all reported that initiation of embryo structures was greater when 2–3% sucrose was used instead of 6%. After 1–2 months, globule-stage embryos and proliferative embryogenic tissue were formed on the immature cotyledon. These tissues grew from protrusions and swollen ridges on the entire surface of the immature cotyledon when 2,4-D was used as the auxin. In addition, the embryos were friable and translucent yellowish-green in colour. When NAA was used as the auxin, the embryos were compact, opaque, pale green and more advanced in stage, forming cotyledon-like structures and usually forming on the cut edges of cotyledons (Hofmann et al. 2004). Adventitious roots were also reported to form on embryos incubated using NAA (Lazzeri et al. 1987).

Embryogenic tissue can remain embryogenic indefinitely (Ranch et al. 1985; Finer 1988). Secondary somatic embryos arise from the apical or terminal portions of the older primary embryos, which may be highly responsive cotyledon tissue. In semi-solid medium, this is achieved using 20–40 mg l⁻¹ (0.090–0.18 mM) 2,4-D (Finer 1988; Wright et al. 1991) and in liquid medium 5 mg l⁻¹ (22.5 µM) 2,4-D (Finer and Nagasawa 1988). Secondary embryogenesis is inhibited and embryo differentiation and maturation are promoted when auxin is removed from the medium. Embryogenic suspension cultures are initiated by placing 20–50 mg of early-staged, highly embryogenic callus into a flask with 35 ml suspension culture medium 10A40N (also known as FN) consisting of MS salts (nitrogen replaced with 10 mM NH₄NO₃, 30 mM KNO₃), B5 vitamins, 6% sucrose, 5 mg l⁻¹ 2,4-D (22.5 µM) and 15 mM glutamine (eventually replaced with 5 mM asparagine to prevent tissue necrosis and reduce clump size; Finer and Nagasawa 1988). Amino acids are added to the medium to increase the frequency of somatic embryogenesis. An optimization of FN medium, called FN Lite, was reported to improve the proliferation of soybean suspension cultures by replacing the macro salts with 27.9 mM KNO₃ (NH₄NO₃ is removed), 3.5 mM (NH₄)₂SO₄, 1.4 mM KH₂PO₄, 2.0 mM CaCl₂ and 3% sucrose (Samoylov et al. 1998). High quality embryogenic tissues have fast growing, smooth, dense, nodular spheres that are green in colour. Eventually, the suspension cultures proliferate as clumps of globular embryos attached at their bases that range over 0.5–8.0 mm in diameter, which apparently arise from the apical surface of older embryos. Although it is possible to regenerate plants directly from these embryos (Finer and Nagasawa 1988), it is more common to undergo desiccation, germination and conversion.

2.2.2 Histodifferentiation

The induction of histodifferentiation given below was described by Bailey et al. (1993). Globular stage somatic embryos were transferred to semi-solid (MSM6AC) MS salts, B5 vitamins, 6% maltose, 0.5% activated charcoal, 0.2% Gelrite, pH 5.8, to differentiate the embryos into cotyledon-stage somatic embryos. Activated charcoal was added during maturation to aid in the bind-

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