MONTANA STATE UNIVERSITY

Safflower (Carthamus tinctorius L.) tissue culture and transformation using Agrobacterium tumefaciens by Miaocheng Ying

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
Montana State University
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Abstract:
Safflower is an important oilseed crop in North America, India, and Mexico. Safflower oil is desirable for human nutrition due to its high degree of polyunsaturation and elevated levels of α-tocopherol. To facilitate in vitro manipulation of safflower germplasm, tissue culture conditions and an Agrobacterium tumefaciens-mediated transformation system were developed and optimized. About 90% of 'Centennial' cotyledon and leaf segments produced callus on MS basal salts medium containing 1 mg/L 6-benzylaminopurine and 1 mg/L 1-naphthaleneacetic acid. Multiple buds were regenerated from callus in the same medium. Safflower cotyledon, stem and leaf segments were transformed using A. tumefaciens strain LBA4404 carrying the Ti plasmid pBI121 which contains the β-glucuronidase (GUS) reporter gene and confers kanamycin resistance. Transformation frequency was about 13% based on assay of GUS activity in transgenic calli. Southern hybridization analysis confirmed the integration of NPT II and GUS genes. Transformation of germinating seeds with A. tumefaciens was evaluated as an alternative method. This method requires no tissue culture steps and manipulations are technically simple. However, seedling survival and transformation efficiency were very low based on assays of GUS activity in plantlets. Transformation of plant apical meristems was not observed.

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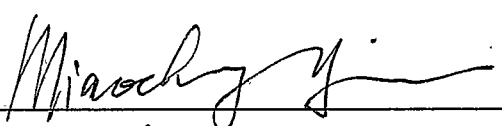
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ABSTRACT

Safflower is an important oilseed crop in North America, India, and Mexico. Safflower oil is desirable for human nutrition due to its high degree of polyunsaturation and elevated levels of α-tocopherol. To facilitate *in vitro* manipulation of safflower germplasm, tissue culture conditions and an *Agrobacterium tumefaciens*-mediated transformation system were developed and optimized. About 90% of 'Centennial' cotyledon and leaf segments produced callus on MS basal salts medium containing 1 mg/L 6-benzylaminopurine and 1 mg/L 1-naphthaleneacetic acid. Multiple buds were regenerated from callus in the same medium. Safflower cotyledon, stem and leaf segments were transformed using *A. tumefaciens* strain LBA4404 carrying the Ti plasmid pBI121 which contains the β-glucuronidase (GUS) reporter gene and confers kanamycin resistance. Transformation frequency was about 13% based on assay of GUS activity in transgenic calli. Southern hybridization analysis confirmed the integration of NPT II and GUS genes. Transformation of germinating seeds with *A. tumefaciens* was evaluated as an alternative method. This method requires no tissue culture steps and manipulations are technically simple. However, seedling survival and transformation efficiency were very low based on assays of GUS activity in plantlets. Transformation of plant apical meristems was not observed.

CHAPTER 1

LITERATURE REVIEW

Safflower

Safflower (*Carthamus tinctorius L.*) belongs to the tribe *Cynaceae* of the *Compositae*, which also includes the genera *Cynara* (artichoke), *Cirsium* (thistle), and *Centaurea* (star thistle). The genus *Carthamus* is represented by safflower, an annual species, but there are also perennial species in the genus. Safflower is a diploid (2n=2x=24) (Knowles, 1975). It is a coarse, erect herb which usually grows to 50 to 100 centimeters in height. Most safflower varieties are open pollinated, with 5 to 30 per cent outcrossing (Knowles, 1975). Flower buds contain from 20 to 100 individual florets, each of which usually bears one seed (Claassen, 1949).

Safflower has historically been grown from central India to eastern Europe (Shaw and Leonard, 1963). In the United States, most safflower is grown in California, while acreage in western Nebraska, eastern Colorado, Wyoming, and Montana fluctuates. Safflower is well-adapted to semi-arid regions of the western United States with at least 120 frost-free days (Claassen, 1949). Weed control is a major problem in safflower production since the crop does not compete well with weeds. Herbicides currently used in safflower production

include trifluralin, EPTC, and metolachlor for the control of wild oat (*Avena fatua*), kochia (*Kochia scoparia*), Russian thistle (*Salsola kali*), and Canada thistle (*Cirsium arvense*). These weeds are the principle weed problems in safflower production.

Safflower has been grown primarily for its flowers which are a source of carthamin, a red dye used to color cloth in Asia and Europe. It is still grown for this purpose in parts of northern India and adjacent countries. Only within the last century has safflower been grown extensively as an oil crop (Bergman and Flynn, 1987; Claassen, 1949; Shaw and Leonard, 1963).

The oil extracted from safflower seed is desirable in the paint and varnish industries because of its non-yellowing properties. Research conducted by the US Department of Energy has shown that industrial grade safflower oil has great promise as a viable alternative to the use of fossil fuels (Bergman and Flynn, 1987). Safflower oil has also recently become an important edible oil (Bergman and Flynn, 1987). Safflower seed contains about 40% oil, composed mainly of either linoleic or oleic fatty acids, depending upon genotype (Bergman and Flynn, 1987; Lyon et al., 1982;). The predominance of one fatty acid (usually about 80%) permits safflower oil composition to be genetically modified more rapidly than other vegetable oils. Fatty acid composition of safflower genotypes is very stable even when grown under

different environmental conditions - a desirable quality for marketing (Bergman and Flynn, 1987).

Safflower meal remaining after expression and extraction of the oil contains 25% to 30% protein and 30% to 35% crude fiber. Safflower meal is commonly separated by screening to yield a high protein fraction containing about 42% protein and 16% crude fiber, and a high fiber fraction containing about 20% protein and 38% fiber. The high protein fraction is unsuitable for human or animal use because of its high fiber content and the presence of two phenolic glucosides, 2-hydroxyarctiin and matairesinol monoglucoside, which make it bitter and mildly cathartic, respectively (Lyon et al., 1982; Palter et al., 1972). Safflower protein isolates from seed meal defatted with hexane are almost free of deleterious glucosides and can be used as a protein supplement in wheat bread (Lyon et al., 1982). The remaining safflower oil in the complex may be used to replace some of the shortening or dough conditioner in bread formulations. These complexes successfully replaced 10% of the flour in a standard wheat bread (Betschart et al., 1975; Lyon et al., 1982).

Correlations between oil content, protein content, and several morphological characters have been reported (Bergman and Flynn, 1987). There was a high negative correlation between oil and protein content with hull thickness. Decrease in hull percentage resulted in a corresponding increase in oil content of the seed up to 50% or higher and an increase in

meal protein content up to 35%. Positive correlations between spininess or seed size and oil content have been observed in many breeding programs (Claassen, 1949). Taste differences of meal from various safflower lines have been reported (Bergman and Flynn, 1987).

Tissue Culture

The concept of totipotentiality of plant cells was introduced by Schwann in 1839. About 70 years later, Gottlieb Haberlandt attempted the first plant cell culture in order to develop a more versatile tool to explore morphogenesis (Dodds and Roberts, 1985). A significant development in methods for plant tissue culture commenced with the work of Phillip White and R. Gautheret (Dodds and Roberts, 1985). They established the conditions under which cell division and growth would take place in explants, and explored nutritional and hormonal requirements of tissues.

In agriculture, the major contributions of plant tissue culture have been in the areas of haploid breeding, clonal propagation, the production of somaclonal mutants, pathogen-free plants, and secondary plant products (Bajaj and Reinert, 1975). In addition, the cyropreservation of plant tissues and the establishment of *in vitro* gene banks have attracted considerable interest (Bajaj and Reinert, 1975; Dixon, 1985; Dodds and Roberts, 1985; Wilkins and Dodds, 1983).

Tissues from various plant parts can be grown *in vitro*. Procambial stem tissues, tissues containing primary and secondary cambia, vascular parenchyma of roots and tubers, ovaries, and primary root and stem meristems are the most frequently used tissues (Dixon, 1985). Flowers, placenta, anthers, nucella, and pollen may also be cultivated. Factors which affect the response to culturing include the physiological condition of the organ, season of the year in which the explant is obtained, size of the explant, and overall quality of the donor plant (Murashige, 1974). Among *N. tabacum* stem section explants, for example, those from nearer the apical region produce adventitious roots and shoots more readily than those from the basal region. A progressive decline in organogenic characteristics has been observed down the length of the tobacco stem. Among seedling parts, the cotyledon is usually the most regenerative (Jelaska, 1972). For safflower, George and Rao (1982) reported that the donor plant genotype had a large influence on the regenerative potential of cotyledons. Also, the degree of success of safflower tissue culture depended upon the genotype selected and the medium used for culture.

The requirements of plant tissues grown *in vitro* are similar to those of intact plants growing in nature. However, in the vast majority of cases only isolated plant tissues or small plant organs are cultured instead of whole plants. These isolated tissues and organs lack the capacity to

synthesize their own supply of carbohydrates, most vitamins, and plant growth substances. Accordingly, all substances needed by whole plants in nature must be provided artificially to cultured tissues (Dodds and Roberts, 1985).

Media components for plant tissue cultures can be classified into five groups: i) major inorganic salts such as nitrogen, phosphate, magnesium, and calcium; ii) minor trace elements such as manganese and cobalt; iii) iron source; iv) carbon source (usually sucrose); and v) organic supplement (vitamins and plant growth regulators) (Dixon, 1985; Gamborg et al., 1981). In some cases, to obtain primary proliferation of plant tissues in culture and to maintain a high rate of nondifferentiated tissue growth, it is necessary to add undefined components such as coconut milk (Dixon, 1985).

In almost all plant tissue culture systems, five or six standard formulations are used, with key differences in the type and quantity of hormones used. Auxins and cytokinins are the primary plant hormones used in tissue culture. Common auxins used are 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indolebutyric acid (IBA). The preferred auxin is IAA, but is perhaps the weakest auxin and is inactivated readily by fluorescent lights (Murashige, 1974). In contrast, 2,4-D is the most stable and widely used auxin, but is known to depress secondary product formation in some cases (Zenk et al., 1975). Cytokinins used are the naturally-occurring zeatin and

synthetics such as kinetin, 6-benzylaminopurine (BAP), and N-isopentenylaminopurine (2-ip). Auxins stimulate cell elongation while cytokinins promote cell division in plant tissues. These hormones are instrumental in the regulation of cell division, cell elongation, cell differentiation, and organ formation (Dodds and Roberts, 1985; Maltzahn, 1959; Mitra and Allsopp, 1959; Skoog and Miller, 1957).

The breakthrough which has made tissue culture possible for propagation of diverse plants was the discovery by Skoog and Miller (1957) that root and shoot initiation is basically regulated by interactions between auxin and cytokinin. Their work with tobacco callus cultures showed that whereas both substances are necessary for tissue growth, the pattern of organogenesis is determined by their relative concentrations in the nutrient medium. A relatively high ratio of auxin to cytokinin favors root initiation and represses shoot formation. In contrast, a relatively high concentration of cytokinin induces shoot initiation and suppresses rooting. Control of root and shoot initiation by the auxin to cytokinin ratio appears to be a general phenomenon among plants (Maltzahn, 1959; Mitra and Allsopp, 1959).

In safflower tissue culture, cotyledons produced shoots on media containing 0.5 to 2.0 mg/L BA, and on media containing 0.5 to 2.0 mg/L BA plus 0.1 or 0.5 mg/L NAA (George and Rao, 1982; Tejovathi and Anwar, 1984). Media containing BA and NAA encouraged shoot formation from safflower

cotyledons, but other cytokinins (kinetin, 2-ip, and zeatin) in combination with NAA did not promote shoot formation (George and Rao, 1982). Jin et al. (1989) reported that safflower callus was initiated from excised roots, hypocotyls, and cotyledons of four safflower varieties on 0.05 mg/L NAA plus 1.2 to 2 mg/L BA, and 0.25 mg/L 2,4-D plus 0.5 mg/L BA. The optimum hormone concentration for root induction was 10 mg/L IAA plus 0.1 mg/L kinetin. Rajendra et al. (1991) reported that Murashige and Skoog (MS) medium (1962) was very effective in inducing safflower callus. Shoot regeneration was observed on MS medium containing 2.0 mg/L BAP and 0.5 mg/L NAA. Rooting was observed on one-half strength MS containing 0.1 mg/L NAA and 1% sucrose in the Indian cultivar 'Mangira.'

The most important factors of the external tissue culture environment are light and temperature (Murashige, 1974). Illumination of plant cultures must be considered in terms of intensity, length of daily exposure period, and quality. Light is needed to regulate certain morphogenetic processes, including shoot formation (Nebel and Naylor, 1968), root initiation (Gautheret, 1969), cladophyll differentiation (Murashige, 1974), and in asexual embryogenesis (Haccius and Lakshmanan, 1965). Cultures are usually maintained at a constant temperature around 25^0C (Dixon, 1985; Dodds and Roberts, 1985; Murashige, 1974). In studies using *Helianthus tuberosus* tuber sections, Gautheret (1969) reported that rooting occurred best under alternating temperatures of 26^0C

(day) and 15^{0}C (night). He suggested that the higher temperature was essential for the formation of cambia, which differentiated into root primordia at low night temperatures.

Embryo culture, somatic embryogenesis, and organogenesis are three commonly used methods for plant regeneration through tissue culture (Dixon, 1985). Embryo culture involves the *in vitro* development or maintenance of isolated mature or immature embryos. Embryos are excised from either seeds or ovules and cultured in a substitute endosperm environment (i.e., nutrient medium). Subsequent embryo development and germination occurs as it would from the seed. Somatic or asexual embryogenesis involves the induction of embryo-like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin. Such embryos can further develop and germinate into plantlets through events corresponding to zygotic development (Dodds and Roberts, 1985). This phenomenon occurs in several plant species *in vitro* during culture of various cell types, tissues and organs; in nature such events are usually intra-ovular occurrences (Dodds and Roberts, 1985). Plant regeneration through organogenesis is the differentiation by which plant organs are formed *de novo* or from preexisting structures. Plant shoots are unipolar structures physically connected to the tissue of origin. Occasionally, roots may give rise to shoots. Changes in plant

growth regulator concentration and/or ratio are usually needed for shoot and root formation (Dixon, 1985).

Somatic hybridization using protoplasts offers a potential means of producing hybrids between unrelated plants when conventional breeding is not possible (Gamborg et al., 1981). The production of protoplasts (cells without cell walls), their hybridization and subsequent culture is a recent application of plant regeneration that provides the starting point for many techniques of genetic modification of plant cells and whole plants (Dixon, 1985).

Since all plants arising from tissue culture should be exact copies of the parental plant, plant tissue culture was originally viewed as a method of cloning a particular genotype (Larkin and Scowcroft, 1981). However, somaclonal variation, or phenotypic variation occurring during tissue culture, is frequently observed. Somaclonal variation can therefore be applied as a method to create novel genetic variants. It has been reported that somaclonal variation occurs in different explants from all species tested and has been observed for a wide variety of characteristics (Larkin and Scowcroft, 1981; Nagarajan and Walton, 1989). Selection at the cellular level and screening of regenerants for a desired characteristic could provide a powerful option for plant improvement (Larkin and Scowcroft, 1981). However, somaclonal variation has also been shown to result in detrimental genetic changes in chromosome number and structure, ploidy levels, gene position

and expression, chromatin amount, and activation of transposable elements (Nagarajan and Walton, 1989).

Plant Transformation Using *Agrobacterium tumefaciens*

There are several methods for introducing DNA into plant cells and obtaining stably transformed plants: transformation using bacterial- or viral-derived vectors (French et al., 1986; Takamatsu et al., 1987), transformation with naked DNA using $CaCl_2$ or PEG (Davey et al., 1980; Krens et al., 1982), fusion of liposome-encapsidated DNA with protoplasts (Walden, 1989), microinjection (Crossway et al., 1986), and most recently microprojectiles (Mendel et al., 1989). Progress in vector development and the availability of genetic markers now allow the conclusive identification of a transformed cell or whole plant. The presence of foreign genes in regenerated plants can be demonstrated by Southern hybridization analysis (Komari, 1989; Moloney et al., 1989; Southern, 1975).

Genetic transformation may be used to produce new hybrid plants with resistance to herbicides, pathogens, or pests (DeBlock et al., 1987; Gabard et al., 1989; Lyon et al, 1989). Other potential applications include manipulating the quality of seed proteins, improving photosynthesis, introducing the ability to fix nitrogen, and engineering tolerance to environmental stress. Transformation technology also provides the tools to understand basic plant molecular biology, gene expression, and viral genetics (Walden, 1989).

Most success in plant transformation has been achieved by using DNA delivery vectors derived from plant pathogens. One pathogen, *Agrobacterium tumefaciens*, has been used extensively to transfer a wide variety of foreign DNAs into the genome of dicotyledonous plants (An, 1985; DeBlock et al., 1989; Moloney et al., 1989). The soil phytopathogen *Agrobacterium tumefaciens* is a sophisticated parasite that uses genetic engineering processes to induce infected plant cells to divert some of their carbon and nitrogen supplies for the synthesis of nutrients (called opines), which *Agrobacterium* can specifically catabolize (Lemmers et al., 1980). The genetically engineered plant cells are also stimulated to proliferate and thus form tumor tissues called crown galls (Schell, 1987). The ability of *Agrobacterium tumefaciens* to transform plant cells is correlated with the presence of a tumor-inducing (Ti) plasmid. Genetic studies indicate that two regions in the Ti plasmid and two chromosomal genes are essential for transformation. These are the transfer DNA (T-DNA) and the virulence (*vir*) region on the Ti plasmid and the chromosomal virulence genes *Chv*A and *Chv*B. While the internal sequence information of the T-DNA is not required for transfer and integration, one of the regions at the ends of the T-DNA, which are 25 bp direct repeats, is directly responsible for T-DNA transfer (Wang et al., 1984). These direct repeat sequences are the recognition sequences for a site-specific endonuclease encoded by *vir*D (Yanofsky et al.,

1986). Cleavage by the *vir*D product results in a linear, single-stranded DNA molecule, designated the T-strand, which is presumed to be an intermediate in the T-DNA transfer process. Any DNA sequence less than 24 kb placed between the border regions will be excised and transferred to the plant cell. The *vir* region mapping outside the T-DNA is required for transformation but is not itself transferred to the plant cell (Lemmers et al., 1980). The *Chv* loci mediate attachment of the bacterium to the plant cell (Schell, 1987).

Although multiple tandem repeats of T-DNA may occur in transformed plants, they may also be separated in different regions of the plant genome. Generally, major rearrangements of DNA sequences do not take place during the transformation process. The site of T-DNA integration into the plant genome is apparently random (Mayerhofer et al., 1991; Walden, 1989). Within tumor cells the T-DNA is transcribed to produce a variety of polyadenylated mRNAs. However, the levels of T-DNA transcripts are relatively low compared with other plant mRNAs and the relative abundance of each can differ, apparently due to the insertion location within the plant genome (Bevan et al., 1985).

Agrobacterium tumefaciens vir gene expression is activated specifically by the plant molecules acetosyringone (AS) and α-hydroxyacetosyringone (OH-AS) (Stachel et al., 1985). These molecules induce the entire *vir* operon in the Ti plasmid as well as stimulating the formation of T-strands. AS

and OH-AS are synthesized specifically in wounded but metabolically active plant cells and probably allow *Agrobacterium* to recognize susceptible cells under natural conditions.

Plant transformation vectors based on *Agrobacterium* can generally be divided into two categories: those that cointegrate into a resident Ti plasmid and those that replicate autonomously (binary vectors) (Klee et al., 1987). Cointegrating vectors include a region of homology between the vector plasmid and the Ti plasmid. The vector is usually designed to cointegrate into one or a few specific sites in the Ti plasmid by recombination. Once the cointegrate has been formed, the plasmid is very stable in *Agrobacterium*. In contrast, binary vectors are not stable in *Agrobacterium* in the absence of drug selection. Instead of a region of homology with the Ti plasmid, binary vectors contain origins of replication from a broad host range plasmid. These replication origins permit autonomous replication of the vector in *Agrobacterium*. Since binary vectors do not cointegrate, they must contain the T-DNA border sequences. A major advantage of binary vectors is their lack of dependence on a specific Ti plasmid. The vector may be introduced into virtually any *Agrobacterium* host containing any Ti plasmid, as long as the *vir* helper functions are provided (Walden, 1989).

One of the most important advances in plant vector construction has been the development of genetic markers

applicable in plant tissue. The use of a selective agent that is inhibitory to untransformed plant cells allows the direct selection of transgenic cells by their ability to grow and proliferate under selective conditions. The best selective agents are compounds that arrest growth of nontransformed cells or slowly kill them. The most widely used selectable marker is the gene encoding neomycin phosphotransferase II (NPT II) (Klee et al., 1987). The enzyme detoxifies aminoglycoside compounds such as kanamycin and G418 by phosphorylation. The NPT II gene has been used successfully to transform a large number of plant species. For convenient detection of transformation, reporter genes are used. Those frequently used in plant transformation include β-glucuronidase (GUS), chloramphenicol acetyl transferase (CAT), luciferase, opine synthase, streptomycin phosphotransferase (SPT), and dihydrofolate reductase (DHFR) (Walden, 1989).

For Ti plasmid-based vectors, four transformation methods have been developed: 1) co-cultivation of protoplasts with *Agrobacterium* followed by callus formation and plant regeneration (Davey et al., 1980; Krens et al., 1982), 2) leaf disk inoculation with *Agrobacterium* (Atkinson and Gardner, 1991; Daniell et al., 1991; Ledger et al., 1991), 3) *Agrobacterium*-mediated transformation of germinating seeds (Chee et al., 1989; Feldmann and Marks, 1987), and 4) microinjection into plant meristems (Schrammeijer et al., 1990).

Co-cultivation involves isolating protoplasts from plant tissue, incubating them with a fresh culture of *Agrobacterium* containing the desired Ti plasmid construction, and selecting for transformed tissue and whole plant regeneration. However, because of the difficulty in regenerating many species of plants from protoplasts, this procedure is not used widely.

Leaf disk transformation is probably the most convenient method of producing transgenic plant material. In this procedure, leaf disks are co-cultured with *Agrobacterium* on agar regeneration medium for several days. Besides leaves, a wide variety of tissue explants may be used including stems, hypocotyls, cotyledons, roots and tubers. The best choice of explant is usually one that regenerates well for the species of interest. Following co-culture, explants are transferred to regeneration/selection medium, where only transformed callus will grow and differentiate into shoots. Feeder cells are usually used during coculture to enhance transformation efficiency by increasing the frequency of regenerated shoots. Shoots are excised, rooted on an appropriate medium, and transferred to soil.

The above methods involve tissue culture techniques that can be time-consuming and require specialized laboratory facilities. Moreover, since tissue culture is involved, induced genetic and/or morphological changes (somaclonal variation) may be observed in regenerated plants. Such variation may interfere with assessing possible transgenic

plant tissue. A recent report suggests that these difficulties may be overcome by the transformation of germinating seeds (Feldmann and Marks, 1987). Chee et al. (1989) transformed soybean by infecting germinating seeds with *Agrobacterium tumefaciens*. The identification of neomycin phosphotransferase (NPT II) enzyme activity in the tissues of R_0 plants (plants regenerated from transformed explants) and presence of the transferred Nos-NPT II gene in R_1 plants (plants from seeds produced by R_0 plants) indicated that about 0.7% of the surviving inoculated seeds yielded transformed tissues in R_0 plants, and that about 10% of these plants yielded transformed R_1 plants. However, the mechanism by which transformation takes place is not known.

Schrammeijer et al. (1990) reported meristem transformation via *Agrobacterium tumefaciens* in sunflower. Their goal was to transform meristem cells that eventually give rise to embryos. Expression of GUS and NPT II genes was observed and confirmed using the polymerase chain reaction and Southern hybridization in transformed plants. Stable transformation of shoot meristem cells was demonstrated, but occurred at low frequencies.

Much attention in plant transformation research has been focused on potential applications in crop improvement including engineering specific traits into a wide variety of plants (Walden, 1989). Some of these traits include changes in seed oil content or quality, manipulation of seed proteins,

introduction of tolerance to environmental stress, pathogens, and herbicides. However, most characteristics of crop plants are determined by the interaction of many gene products involved in a variety of biochemical processes. Specific enzymes in these pathways need to be identified and characterized before they may be used for plant genetic engineering. Nevertheless, substantial advances have been made recently in transferring genes into plants, directing their correct expression, and targeting protein products into correct cellular compartments. Many engineered plant lines are already undergoing field trials (Walden, 1989). A recent example reported by DeBlock et al. (1987) demonstrated the introduction of commercial levels of herbicide resistance. The *bar* gene which confers resistance to bialaphos, a tripeptide herbicide that inhibits glutamine synthetase, was transferred to tobacco cells using *Agrobacterium*-mediated transformation. Regenerated transgenic plants showed complete resistance to commercial formulations of bialaphos.

Several reports of safflower tissue culture have been published (George and Rao, 1982; Jin et al., 1989; Rajendra et al., 1991; Tejovathi and Anwar, 1984). However, regeneration has only been achieved from a few Indian genotypes. Optimization of regeneration conditions and genotype characterization are needed for domestic cultivars. Since safflower production is limited by effective registered herbicides and the potential for additional herbicide

registrations is low, the introduction of herbicide resistance into commercial safflower varieties would be highly desirable.

In addition to a high content of quality oil, safflower seed meal contains a high percentage of protein. However, it is not used for human consumption since it contains phenolic glucosides which make it bitter and mildly cathartic. *Agrobacterium*-mediated safflower transformation could be a promising approach to improve seed meal quality as well as seed oil composition and amount. The gene encoding β-glucosidase which hydrolyses the monoglucoside and may results in a loss of bitterness has been cloned and expressed in *Saccharomyces cerevisiae* (Kohchi and Toh-e, 1986).

The objectives of the following studies are to 1) optimize safflower tissue culture conditions, 2) develop an efficient regeneration system, and 3) demonstrate transformation of safflower tissues and stable integration of test genes.

CHAPTER 2

SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) TISSUE CULTURE AND REGENERATION

Abstract

In vitro callus production and plant regeneration from cotyledon-, stem-, and leaf-derived safflower explants were studied under defined nutritional, hormonal and environmental conditions. Twenty safflower varieties and breeding lines were evaluated for their response to tissue culture conditions. Calli were formed from about 90% of 'Centennial' cotyledon and leaf segments on MS basal salts medium containing 1 mg/L 6-benzylaminopurine (BAP) and 1 mg/L 1-naphthaleneacetic acid (NAA) solidified with 0.7% agar. Regeneration of multiple buds from primary callus occurred within 3 weeks of culture in the same medium.

Introduction

Several reports have described safflower tissue culture conditions (George and Rao, 1982; Jin et al., 1989; Tejovathi and Anwar, 1984). Safflower regeneration was also reported for a few Indian genotypes (Rajendra et al., 1991). However, there are no reports that safflower regeneration has been

achieved for domestic cultivars. The objectives of this study were to 1) determine optimal culture conditions for domestic safflower varieties, and 2) develop an efficient regeneration system for safflower.

Materials and Methods

Safflower Genotypes

Twenty safflower varieties and breeding lines were screened for their response to tissue culture conditions. Safflower seeds of American varieties and breeding lines were obtained from Dr. Jerald W. Bergman of the Eastern Agricultural Research Center, Sidney, Montana. Indian safflower varieties were obtained from the Western Regional Plant Introduction Station, USDA ARS, Pullman, Washington (Table 1).

Seed Sterilization, Germination, and Seedling Growth

In initial experiments, explants were excised from nonsterile seedlings and the tissue surface sterilized with 5% commercial chlorine bleach plus 0.05% sodium dodecyl sulfate (SDS) followed by thorough washing with sterile water. In all subsequent experiments, safflower seeds were surface sterilized in 15% (v/v) commercial chlorine bleach containing 0.05% (v/v) Triton X-100 with shaking for 15 minutes followed by thorough rinsing with three to four changes of 300 ml sterile distilled water for 2 hours. Seeds were germinated

aseptically on wet filter paper in 6 cm x 6 cm x 10 cm square Magenta GA7-3 vessels at 26±2^{0}C under a 16 hour photoperiod with cool-white fluorescent lights (80-100 $\mu E \cdot m^{-2} \cdot sec^{-1}$) for 4 days. Sterile seedlings were transferred to Medium A (described below) and grown under the conditions described above for 2 weeks.

Table 1. List of Safflower Cultivars and Lines Tested.

Accession	Genotype (Cultivar)	Origin
Centennial	Centennial	USA
S-208	S-208	USA
PI 254364	V. Garhia-pusa	India
PI 254365	V. Guara-pusa	India
88C 700	88C 700	USA
88C 719	88C 719	USA
88C 739	88C 739	USA
88C 762	88C 762	USA
88C 763	88C 763	USA
88C 764	88C 764	USA
88C 765	88C 765	USA
88C 766	88C 766	USA
88C 791	88C 791	USA
88C 806	88C 806	USA
88C 813	88C 813	USA
88C 814	88C 814	USA
88C 822	Girard	USA
88C 838	88C 838	USA
88C 854	88C 854	USA
Montola 2000	Montola 2000	USA

Media Preparation

Safflower Medium A consisted of MS basal salts (MS basal salt mixture, Sigma catalog number M5524), vitamins (Vitamin mixtures, Sigma catalog number M7150), 3% sucrose and 0.7% Bacto agar (Difco Laboratories, 0140-01). Other gelling agents tested were 0.7% Noble agar (Difco Laboratories, 0142-01) and 0.2% Phytagel (Sigma catalog number P-8169). Growth regulators tested were 2,4-D (Sigma catalog number D-2128), NAA (Sigma catalog number N0640), and BAP (Sigma catalog number B-9395) at concentrations ranging from 0.1 mg/L to 2.0 mg/L. Growth regulators were dissolved in a small volume of 1N NaOH, diluted to 1 mg/ml with sterile distilled water and stored at -20^0C. All media were adjusted to pH 5.80±0.01 with KOH or HCl prior to adding the gelling agent. Media were autoclaved at 1.4 kg/cm^2 and 121^0C for 20 min, cooled to 50-55^0C, and dispensed into 170 cm^3 culture jars (30 ml medium per jar).

Tissue Culture and Maintenance

Cotyledons, stems, and leaves from 2 to 4 week old sterile seedlings were cut transversely into 0.5 x 1.0 cm^2 segments and placed on solid medium so that the cut ends contacted the medium. Explants from the basipetal, acropetal, and central sections cotyledons were evaluated for their ability to produce callus. Calli were maintained under the conditions described above and subcultured onto fresh medium

every 3 to 4 weeks. For shoot induction, calli with dark green spots (green islands) were selected, divided into 0.5 x 1.0 cm^2 pieces, and transferred to fresh medium containing various hormone concentrations. Visible buds (about 5 mm long with two to four well formed true leaves) present on calli after about 3 weeks were counted as regenerated buds to calculate efficiency of regeneration.

Regeneration of Shoots and Whole Plants

Calli with green islands were transferred to media with various hormone concentrations as shown in Table 2.

To induce rooting, 0.5 to 1.5 cm buds were cut from calli and transferred to rooting media as shown in Table 3. Shoots or buds were cultured for 3 to 5 days on media supplemented with IAA, and then transferred to the same medium without IAA.

The effect of light quality was examined on safflower callus and bud production and whole plant regeneration. Light from cool white fluorescent lights was compared with yellow (440 to 720 nM) light created by wrapping cool white bulbs with Roscolux Theatrical Gel (#10 medium yellow, Rosco).

Table 2. Hormone Composition of Media Used for Bud Induction also Containing 1X MS Basal Salts, 3% Sucrose, and 0.7% Noble Agar.

NAA (mg/L)	BAP (mg/L)	GA_3 (mg/L)
		0.01
		0.1
0.1	0.1	
0.1	0.3	
0.1	1.0	
0.1	3.0	
0.3	0.1	
0.3	0.3	
0.3	1.0	
0.3	3.0	
1.0	0.1	
1.0	0.3	
1.0	1.0	
1.0	3.0	
0.1	0.5	
0.5	2.0	

Table 3. Media Components Used for Root Induction also Containing 0.7% Noble Agar.

MS Salts Concentration	IAA (mg/L)	% Sucrose
1X		3
1X		6
1X		9
1/2X		3
1/2X		6
1/2X		9
1X	0.01	
1X	0.03	
1X	0.1	
1/2X	0.01	
1/2X	0.03	
1/2X	0.1	

Results and Discussion

Callus Production

In initial experiments, surface-sterilized explants cultured on Medium A plus 1 mg/L BAP and 1 mg/L NAA (Medium B) rapidly turned brown starting at the cut surfaces, and only a few explants survived. Large quantities of brown exudates (presumably phenolics) were released into the medium. Browning of *Eucalyptus tereticornis* explants was prevented by adding 500 mg/L polyvinylpyrrolidone (PVP) in the culture medium (Subbaiah and Minocha, 1990). However, the addition of charcoal (0.1%) or $AgNO_3$ (1 or 10 mg/L) to Medium B was not

successful in preventing browning of safflower explants. Therefore, surface sterilization of explants was avoided by using aseptically grown seedlings for all subsequent experiments.

Genotype effects on response to tissue culture conditions have been well established (Espinasse and Lay, 1989). The present study confirms the importance of safflower genotype in callus production. Among twenty safflower cultivars and breeding lines examined, only six lines produced callus (Table 4). Percent callus formation of the six lines ranged from 16.7% to 90.2%. All other lines tested failed to form callus under these experimental conditions. The commercial variety 'Centennial' provided the highest percentage of callus production and therefore was used for all subsequent experiments.

In an attempt to further identify the best explant source for callus production and regeneration, cotyledons were cut transversely into three sections and cultured on Medium B. In general, all cotyledon sections produced callus, although the central section appeared to produce callus at a slightly higher frequency (Table 5).

Explant characteristics other than genotype of the source plant influenced the frequency of callus induction. Explants from young, healthy seedlings were more likely to produce callus than older tissues or tissues from stressed seedlings (data not shown).

Table 4. Frequency of Callus Induction in Cotyledon and Leaf Explants of Twenty Safflower Cultivars and Breeding Lines after Three Weeks Culture on Medium B.

Variety	No. of Explants Tested	No. of Explants Forming callus	Induction Frequency (%)
Centennial	143	129	90.2
830	27	19	70.0
254365	54	23	42.6
254364	69	26	37.7
S 208	171	59	34.5
700	18	3	16.7
739	16	0	0
762	20	0	0
763	17	0	0
764	18	0	0
765	18	0	0
766	19	0	0
791	21	0	0
806	17	0	0
813	18	0	0
814	17	0	0
822	16	0	0
719	18	0	0
854	19	0	0
Montola 2000	32	0	0

The effect of selected plant growth regulators on safflower callus induction is shown in Table 6. In general, MS basal medium containing 1 mg/L BAP and 1 mg/L NAA (Medium B) provided the most consistent incidence of callus production. When the BAP concentration was increased to 1.5

mg/L, no callus was produced regardless of NAA concentration. Tejovathi and Anwar (1984) reported that calli and shoots were initiated from two Indian safflower varieties on MS medium containing 0.5 mg/L BAP or kinetin plus 0.1 mg/L NAA. Also, Rajendra Prasad et al. (1991) observed shoot regeneration on MS medium supplemented with 2.0 mg/L BAP plus 0.5 mg/L NAA. These media were tested using 'Centennial' explants, but no calli or shoots were obtained. The lack of callus and shoot production may be due to differential response of genotypes to hormone levels. The synthetic auxin 2,4-D was also tested for callus induction (Table 6). In combination with 0.5 mg/L BAP, callus induction frequency increased up to 1.5 mg/L 2,4-D. However, callus was not formed on higher concentrations of 2,4-D and all explants turned brown and died.

Table 5. Effect of Explant Position on Callus Induction Frequency from 'Centennial' Cotyledons Cultured on Medium B.

Cotyledon Position	No. of Explants	No. of Explants Forming Callus	Induction Frequency (%)
Acropetal Section	55	13	23.6
Central Section	59	25	42.4
Basipetal Section	57	21	28.0

Table 6. Effect of Plant Growth Regulators on Frequency of Callus Induction from Leaf, Stem and Cotyledon Segments of 'Centennial' Safflower.

Plant Growth Regulator Composition (mg/L)	No. of Explants	No. of Explants Forming Callus	Induction Frequency (%)
0.5 BAP + 0.1 NAA	21	5	23.8
0.5 BAP + 0.5 NAA	21	6	28.6
0.5 BAP + 1.0 NAA	21	0	0
1.0 BAP + 0.1 NAA	21	2	10.0
1.0 BAP + 0.5 NAA	21	7	33.3
1.0 BAP + 1.0 NAA	21	17	81.0
1.5 BAP + 0.1 NAA	21	0	0
1.5 BAP + 0.5 NAA	21	0	0
1.5 BAP + 1.0 NAA	21	0	0
0.5 BAP + .25 2,4-D	36	3	8.3
0.5 BAP + 0.5 2,4-D	18	2	11.1
0.5 BAP + 1.0 2,4-D	18	6	33.3
0.5 BAP + 1.5 2,4-D	18	10	55.6
0.5 BAP + 2.0 2,4-D	18	0	0

Bud Induction and Regeneration

Calli and multiple buds were produced from cut surfaces of cotyledon, stem, and leaf segments of 2 to 4 week old seedlings (Figure 1). Calli were initiated from explants after an average of 10 to 15 days on Medium B.

When calli were transferred to fresh media with different growth regulator combinations, only calli on Medium B formed buds. On all other media, calli grew slowly for a short time and then gradually turned brown.

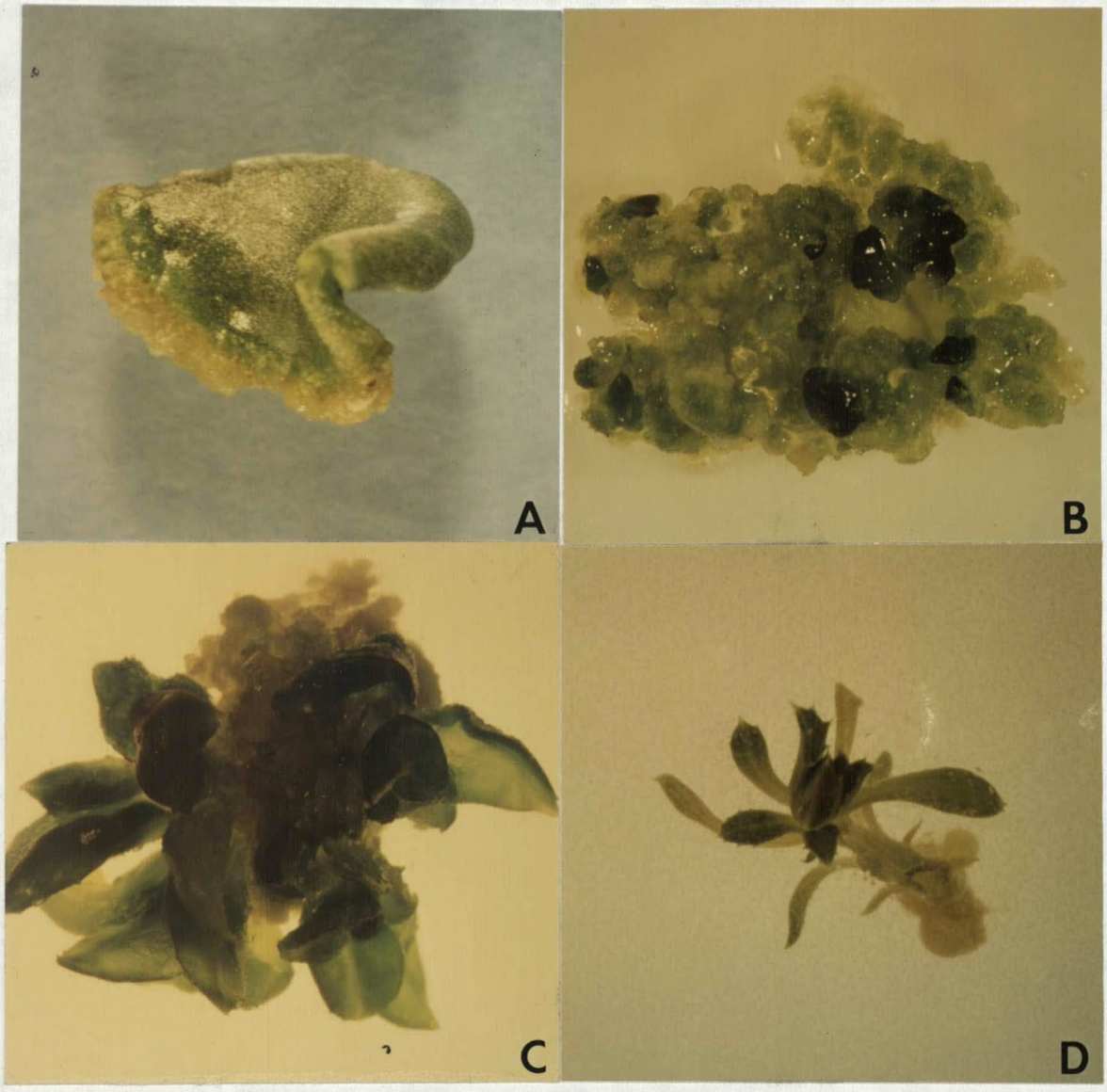


Figure 1. Shoot regeneration in callus derived from leaf explants of *Carthamus tinctorius* var. 'Centennial'. A: Callus initiated from cut edge of explant. B: 'Green island' formed in callus. C: Buds induced from callus. D: Regenerated shoot.

It is well established that the auxin:cytokinin ratio is the major determinant for callus and shoot induction (Dixon, 1985). In this study I tested the same ratio (1:1) but different concentrations of auxin and cytokinin. Only 1 mg/L NAA with 1 mg/L BAP (1:1) allowed callus and bud production. Explants cultured on other 1:1 combinations of auxin:cytokinin did not produce calli or buds. Therefore, I think that the absolute concentrations of auxin and cytokinin are more important for safflower tissue culture.

Several gelling agents were compared for callus production and bud initiation (Table 7). In general, all three gelling agents performed similarly well for callus production, with possibly slightly better results using Noble agar.

Table 7. Effect of Gelling Agents on Callus Production and Bud Regeneration from 'Centennial' Leaf Explants Cultured on Medium B.

Gelling Agent	No. of Explants	Callus Forming Frequency (%)	No. of Calli Forming Buds	Bud Forming Frequency (%)
Noble Agar	29	82.8	13	44.8
Bacto agar	29	93.1	10	34.5
Phytagel	29	75.9	11	37.9

Cotyledon tissue was compared with leaf and stem tissues for the frequency of callus and bud induction. The results showed that all three tissues performed equally well for bud

induction, although callus from leaf explants may produce buds at a slightly higher frequency (Table 8).

Table 8. Frequency of Callus and Bud Induction from 'Centennial' Explants Cultured on Medium B.

Explant Source	No. of Explants	No. of Explants Forming Callus	No. of Calli Forming Buds	Induction Frequency (%)
Cotyledon	90	74	15	16.7
Stem	63	54	12	19
Leaf	276	236	71	25.7

After small shoots were established, they were cut from calli and transferred to rooting media. Roots were not obtained on any media tested. Transferred shoots grew for only a short time and then died gradually. One shoot grew for about 3 weeks on Medium B and formed a flower bud before dying.

CHAPTER 3

SAFFLOWER (*CARTHMAS TINCTORIUS* L.) TRANSFORMATION

Abstract

An efficient protocol for *Agrobacterium*-mediated transformation of safflower was developed. Safflower explants from leaf disks, cotyledons and stems were co-cultivated with the avirulent *Agrobacterium tumefaciens* strain LBA4404 carrying the Ti plasmid pBI121 which contains the β-glucuronidase (GUS) reporter gene and confers kanamycin resistance (NPT II). About 25% of the resulting calli tested positive for GUS activity, demonstrating the first report of successful safflower transformation. Integration of cloned genes (NPT II and GUS) was confirmed by Southern DNA hybridization. Transformation was also conducted by inoculating apical meristem regions of germinating seeds with *Agrobacterium tumefaciens*, a method which requires no tissue culture steps. However, transformation frequency was very low as determined by assaying resulting plantlets for GUS activity.

Introduction

Weed control is the most limiting factor in safflower

production. Also, the quality of safflower seed meal needs improvement for human consumption. Genetic engineering is a promising approach to introduce herbicide resistance into commercial safflower varieties, to improve seed meal quality, and to change oil composition and amount. Therefore, an efficient transformation system for safflower is highly desirable. The objectives of this research were to: 1) develop an efficient, reproducible transformation system for safflower using tissue culture techniques, and 2) evaluate alternative transformation methods that do not require tissue culture steps.

Materials and Methods

Plant Materials and Tissue Culture

Plant tissue culture procedures were carried out as described in Chapter 2. For inoculation of germinating seeds, 'Centennial' seeds were surface sterilized and germinated on moistened filter paper in tissue culture vessels for 24 hours.

Agrobacterium Strain and Ti Plasmid

The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) containing the binary plasmid pBI121 was used for all transformation experiments. pBI121 contains two genes expressed in plants: a β-glucuronidase (GUS) reporter gene driven by the CaMV 35S promoter, and a neomycin phosphotransferase II (NPT II) gene, which confers kanamycin

resistance, driven by the NOS promoter. A map of the T-DNA region of pBI121 is shown in Figure 2.

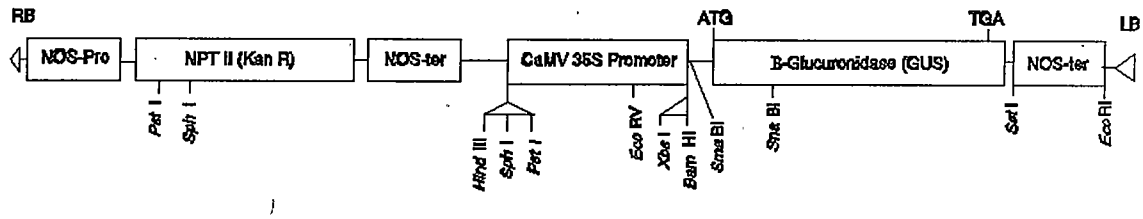


Figure 2. The T-DNA region of pBI121.

Kanamycin Sensitivity of Safflower Explants

Sterile 'Centennial' leaf segments were cultured on Medium B plus 12.5, 25, 50, 100, or 200 mg/L of kanamycin sulfate to determine the proper concentration of kanamycin for selecting transformants.

Co-cultivation and Selection

Agrobacterium strain LBA4404/pBI121 was grown 40 hours in TY liquid medium (0.5% tryptone, 0.3% yeast extract, 6mM $CaCl_2 \cdot 2H_2O$) containing 25 µg/mL streptomycin and 50 µg/mL kanamycin at room temperature with shaking at 150 rpm. Sterile 3 week old safflower cotyledon, stem, and leaf explants were cut into 0.4 by 0.5 cm pieces, submerged in the *Agrobacterium* culture (about 10^8 cells/ml) for 30 minutes, blotted dry between two sterile filter papers, and placed onto Medium B. After 2 days cocultivation, explants were transferred to selective medium containing 500 mg/L carbenicillin and 50 mg/L kanamycin. Callus induction and shoot regeneration conditions were as described in Chapter 2.

Calli and buds were subcultured onto fresh medium every 3 weeks.

One month old friable callus from cultivar 'S-208' was cut into 1 mm^2 pieces and cultured in MS basal salts liquid medium (pH 5.5) plus 1 mg/L BAP and 1 mg/L NAA at room temperature with shaking at 120 rpm. Cells were subcultured every three weeks. Homogeneous cell suspension cultures were obtained after 3 subcultures. To test the effect of feeder cells on growth of transformed callus, 1 ml of cells was spread on the surface of agar-solidified Medium B and covered with sterile filter paper. Inoculated explants were placed on the moistened filter paper and cocultivated for 2 days followed by the selection procedures described above.

Inoculation of Germinating Safflower Seeds

Seeds were surface sterilized and germinated for about 20 hours. Seed coats were removed, one cotyledon was excised, and the remaining plumule, cotyledonary node, and adjacent cotyledon tissues were inoculated with LBA4404/pBI121. Inoculation was performed by wounding and microinjection. For wounding, half seeds were wounded with a very fine glass needle at several places in the plumule region as shown in Figure 3. Wounded seeds were submerged in *Agrobacterium* culture for 30 minutes. For microinjection, about 3 μl *Agrobacterium* culture was injected with a glass needle around the plumule as shown in Figure 3. Following inoculation, half seeds were germinated on moistened filter paper for 3 days.

Surviving seedlings were transferred to flats containing peat:sand (1:1) and watered every other day with 1/2X Hoagland's Solution.

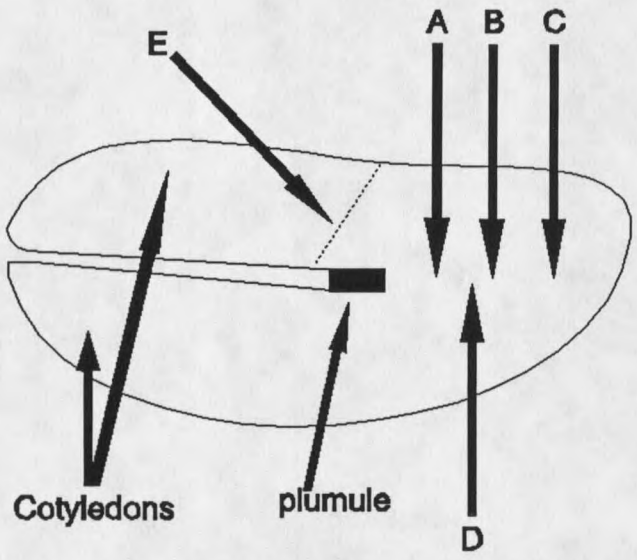


Figure 3. Germinating safflower seed with seed coat removed; A, B, and C are locations for wounding; D is injection position; One cotyledon was excised at E.

β-Glucuronidase (GUS) Assay

Histochemical localization of GUS activity in putative transgenic calli and plants was carried out essentially as described by Jefferson (1987). β-glucuronidase reduces X-gluc to produce an insoluble blue precipitate (Jefferson, 1987). Callus, bud, and leaf tissues were cut into 1 mm^2 sections and incubated overnight at 37^0C in wells of microtiter plates containing 60 μl of a solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) dissolved in 0.2 M $NaPO_4$ buffer, pH 7.0, containing 0.1 M $K_3[Fe(CN)_6]$, 0.1 M $K_4[Fe(CN)_6]\bullet 3H_2O$, and 1 M Na_2EDTA. Tissues were fixed in 50%

ethanol:glacial acetic acid:formaldehyde (17:1:2) for 24 hours to decolorize the tissue and dehydrated in a graded 50% to 95% alcohol series.

Genomic DNA Isolation and Probe Preparation

Safflower genomic DNA was isolated from kanamycin resistant calli and non-transformed calli as controls. About 0.5 g callus was frozen and ground in liquid nitrogen. The tissue was reground in 4 ml extraction buffer (50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA, 0.5% SDS, 0.75 M NaCl and 0.25% (v/v) 2-mercaptoethanol). After centrifugation to precipitate debris, the supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (PCI) followed by extraction with chloroform. DNA was precipitated by adding 1/3 sample volume of 7.5 M NH_4OAC and an equal volume of isopropanol. Following storage overnight at -20^0C, DNA was pelleted by centrifugation at 5000 g for 15 minutes, washed once with 75% ethanol, dried, and resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

For preparation of DNA hybridization probes, pBI121 was digested with *Pst*I to obtain a 1.9 kb NPT II fragment or *BamH*I and *EcoR*I for a 2.2 kb GUS fragment. NPT II and GUS fragments were separated from pBI121 by agarose gel electrophoresis, and the DNA recovered by electroelution followed by PCI extraction and ethanol precipitation.

Southern Analysis

Safflower genomic DNA was digested with *Pst*I, treated with DNase-free RNase and separated electrophoretically on 0.5% agarose gels (1.5 μg per lane). DNA was transferred by capillary blotting onto Magnagraph nylon membranes (0.45 μ) and fixed by baking at 80^{0}C for 2 hours. The membranes were prehybridized overnight at 42^{0}C in hybridization buffer [3X SSC (Standard Saline Citrate, 1X SSC contains 1.5 M sodium chloride and 15 mM sodium citrate), 5X Denhardt's (5X Denhardt's contains 0.1% Ficoll, 0.1% PVP, and 0.1% bovine serum albumin), 50% deionized formamide, 100 μg/mL sheared and denatured salmon sperm DNA, 1% sodium dodecyl sulfate (SDS)].

Probe DNA was labelled using the hexamer-random priming procedure described by Feinberg and Vogelstein (1983) with α^{32}P[dCTP]. After 30 hours hybridization at 42^{0}C, blots were washed once with 100 ml formamide wash buffer (50% formamide, 5X SSC, 0.2% SDS) for 30 minutes at 42^{0}C followed by three 30 minute washes in blot wash buffer (0.1X SSC, 0.1% SDS) at 62^{0}C. Blots were first hybridized with the NPT II probe, stripped with 1.5 liter blot wash buffer at 100^{0}C for 15 minutes, and rehybridized with the GUS probe. pBI121 DNA linearized with *Hind*III was included in each blot in amounts representing ten and twenty gene copies.

Results and Discussion

Factors Affecting Safflower Transformation

To obtain the highest transformation efficiency, the optimal kanamycin concentration to be used for selection was determined. Of 14 explants in each treatment, only two or one explant cultured on 12.5 mg/L or 25 mg/L kanamycin, respectively, formed callus (data not shown). All explants cultured on 50 mg/L or higher kanamycin became bleached and soon died. Initial transformation experiments were carried out using 25 mg/L kanamycin while 50 mg/L kanamycin was used for all subsequent experiments.

Newell et al. (1991) reported that the use of feeder cell layers during coculture enhanced transformation efficiency of *Solanum tuberosum*. However, feeder cells did not alter transformation efficiency of safflower in the present study (Table 9). Therefore, feeder cells were not used for subsequent experiments.

Table 9. Effect of Feeder Cells on Callus Induction from Transformed Cotyledon, Stem and Leaf Explants Cultured on 25 mg/L Kanamycin.

Variety	Feeder Cells (mL)	No. of Explants	No. of Explants Forming Callus	Frequency (%)
Centennial	1	36	34	94.4
Centennial	0	42	38	90.5
S-208	1	57	15	26.3
S-208	0	68	12	17.6

Inoculation of Germinating Safflower Seeds

Germinating safflower seeds were wounded at several locations as shown in Figure 3 and inoculated with *Agrobacterium tumefaciens*.

Transformation frequencies after wounding at several positions were compared by observing GUS activity in seedling tissues (Table 10). Frequency of transformation was directly associated with the position of wounding. No GUS expression was observed in leaf tissues wounded at position A or C. Transformation efficiency after wounding at position B was 2.7%; however, all GUS activity was localized at leaf tips. In cotyledon tissue, 37.5%, 47.5%, and 22.5% of cotyledon segments expressed GUS activity after wounding at positions A, B, and C, respectively. This high frequency is probably due to direct wounding of cotyledon tissues with the glass needle and forceps during the inoculation procedure. GUS activity was not observed either in stem tissue or tissues around shoot apical meristems.

Microinjection of *Agrobacterium* into germinating seeds was also attempted. Only about 12% of seedlings survived the injection procedure, and of those, transformation efficiency was very low. Similar to the wounding experiments, GUS activity in cotyledons was probably due to wounding with forceps.

Table 10. GUS Expression Frequencies in Leaf (L), Stem (S), and Cotyledon (C) Tissues after Different Inoculation Methods; Leaf Segments for GUS Assay Were Cut from Seedlings with Three True Leaves after Transformation.

Treatment	No. of Seeds Treated	No. of Seedlings	No. of Seedlings Expressing GUS	GUS Expression Frequency (%)		
				C	L	S
Wound A	150	94	0	37.5	0	0
B	150	119	4	47.5	2.7	0
C	150	56	0	22.5	0	0
Injection	129	16	1	7.2	0.78	0

Leaf Disk Transformation

After about 2 months growth on selective medium containing kanamycin and carbenicillin, putative transgenic calli were assayed for GUS activity. In preliminary transformation experiments, Medium A containing 25 mg/L kanamycin was used for selection. When portions of the resulting calli were assayed, 13.3% of calli expressed GUS activity. Since callus induction frequency under 25 mg/L kanamycin selection was about 90% (Table 9), the overall GUS transformation frequency was 13.3% x 90% = 12%. However, GUS staining in individual calli was only visible under the dissecting microscope (40X), since only small sections of calli stained blue. Apparently the kanamycin concentration was not high enough to kill nontransformed tissue, allowing chimeric calli to persist.

In subsequent transformation experiments, the kanamycin concentration was increased to 50 mg/L. GUS activity of calli selected on 50 mg/L kanamycin was easily visible without a microscope and most calli, sometimes the whole callus, were stained blue (Figure 4). Also, the frequency of GUS expression was increased to 25.6%.

Based on GUS activity, transformation efficiency using the leaf disk method was much higher than transforming germinating seeds, either by wounding or injection. However, the leaf disk method involves tissue culture procedures, including plant regeneration from single cells. Somaclonal variation associated with tissue culture remains a potential drawback of this technique.

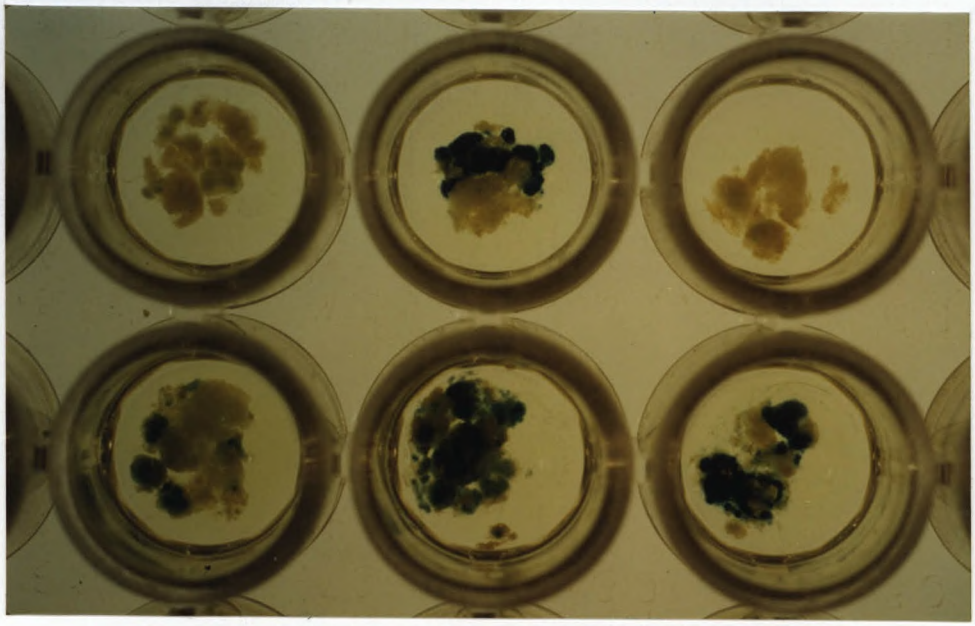


Figure 4. β-Glucuronidase assay of transgenic safflower calli after 2 months selection on 50 mg/L kanamycin.

As an alternative method, transformation of germinating seeds requires no tissue culture steps, and manipulations are technically simple. There are apparently some undifferentiated cells in the plumule or adjacent regions of germinating *Arabidopsis* seeds which later differentiate into ovules and/or pollen cells (Feldmann and Marks, 1987). These cells are the ideal target for *Agrobacterium*-mediated transformation. However, the overall transformation efficiency has so far been very low (Feldmann and Marks, 1987). A possible explanation for the low frequency is that the depth and position of wounding and injection with *Agrobacterium* was not precisely controlled. Chee et al. (1989) suggested that the use of high velocity microprojectiles might provide a viable option for increasing transformation frequency using this method.

Southern Analysis of Transformed Calli

Twelve independently transformed calli cultured on selective media for more than 2 months were analyzed for the presence of NPT II and GUS genes. Of those calli, calli 6, 8, 9, 10, 13, 14, and 15 stained blue in GUS assays and calli 4, 5, 7, 11, and 12 did not. To detect the presence of both genes, portions of the NPT II and GUS genes were labeled and used in Southern hybridization analysis. Results showed that all calli tested carried both NPT II and GUS genes (Figure 5). Although calli 4, 5, 7, 11, and 12 did not stain blue in GUS assays, the GUS gene was present in those calli as confirmed

by Southern hybridization. A possible explanation is that the GUS gene was integrated into those calli but not expressed.

Copy numbers of NPT II and GUS genes in transgenic calli ranged from low numbers up to about twenty copies. Chyi et al. (1986) reported that up to 50 copies of T-DNA can be integrated into the plant genome. An explanation for the presence of multiple transgene copies may be that transgenes are replicated during integration, or replicated transgenes are ligated prior to integration (Zambryski, 1988). The results also indicate that multiple gene copies were integrated at different chromosomal locations since several bands were observed in all blots probed with NPT II or GUS. Multiple gene copies may have also integrated as concatamers in some calli such as callus 4. A 2 kb DNA fragment was common to DNA from all transgenic calli probed with NPT II.

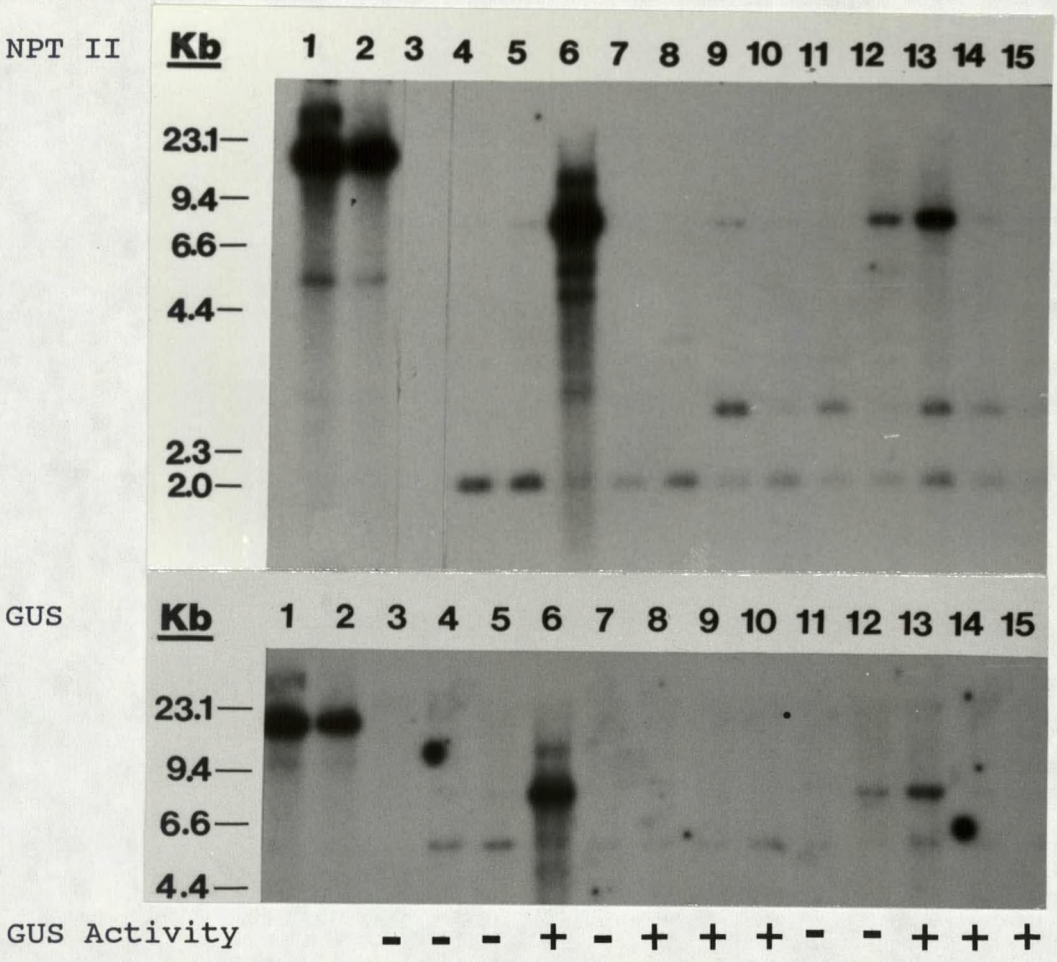


Figure 5. Southern hybridization analysis of safflower transgenic calli. 1.5 μg of genomic DNA digested with *Pst*I was loaded in each lane. Lanes 1 and 2 represent gene reconstructions containing 20 and 10 copies, respectively. Lane 3 contains DNA from nontransformed callus. Lane 4 through 15 contain DNA from transformed calli numbers 4 through 15, respectively.

CHAPTER 4

SUMMARY

Safflower tissue culture conditions were developed and optimized. Of twenty safflower varieties and breeding lines evaluated, 'Centennial' provided the highest incidence of callus production. High callus induction frequency (90%) was obtained on Medium B using 'Centennial' cotyledon, stem, and leaf explants. Regenerated multiple buds were induced from callus after 3 weeks culture on the same medium. One regenerated shoot formed a flower bud on Medium B. Rooting of regenerated shoots was not achieved.

Agrobacterium tumefaciens-mediated transformation of safflower was conducted using the disarmed *Agrobacterium* strain LBA4404 carrying the Ti plasmid pBI121 which confers kanamycin resistance and contains the GUS reporter gene. Transgenic calli assayed for GUS activity indicated about 25% transformation frequency. Integration of GUS and NPT II genes in transgenic calli was confirmed by Southern hybridization analysis.

Inoculation of germinating safflower seeds with *A. tumefaciens* was evaluated as an alternative method which does not involve tissue culture steps. Transformation of germinating seeds occurred at very low frequency as determined

by assay of GUS activity in surviving plantlets. Transformation of plant apical meristems was not observed.

The results provide a basis for the eventual incorporation of agronomically useful traits into commercial safflower varieties. Introduction of genes for herbicide resistance and improved nutritional quality will be possible using the method developed here.

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