Chapter 25

Spices

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1. Introduction

Conventional plant breeding has contributed significantly to crop improvement over past fifty years. However, there is intense pressure to produce further improvements in crop quality and quantity as a result of population growth, social demands, health requirements, environmental stress and ecological considerations. Traditional plant breeding has not able to withstand this increasing demand due to the limited gene pool and restricted range of organism between which genes can be transferred due to the species barriers. Genetic transformation holds great promise for overcoming these major constraints to crop productivity as it involves recombination of an efficient cell or tissue culture regeneration system with recombinant DNA technology, which would transfer specific genes from other taxa, unrelated plants, microbes, and animals. Because of these possibilities, it is now feasible to introduce genes that have previously been inaccessible to the conventional plant breeder or which did not exist in the crop of interest to impart desired traits, such as high yield, insect resistance, disease resistance and herbicide resistance, without disrupting their fundamental genetic background within a short period of time. The first stably transformed plants were reported in early 1980s (Herrera-Estrella *et al.,* 1983). The production of novel chimeric genes in 1980s resulted in the expansion of plant transgenic technologies, transformation vectors, DNA delivery systems, combined with plant regeneration systems.

The major components for the development of transgenic plants are: (1) the development of reliable tissue culture regeneration systems; (2) efficient transformation techniques for the introduction of genes into the crop plants; (3) preparation of gene constructs in suitable vectors; (4) selection and multiplication of transgenic plants; (5) molecular and genetic characterization of transgenic plants

for stable and efficient gene expression; and (6) evaluation of transgenic plants. Genetic transformation methods in plants are mainly classified into direct and indirect transformation systems. The majority of gene transfer experiments have focused on maximizing the efficiencies for the recovery of stably transformed plants, and also extending the range of genotypes that could be engineered using a specific procedure. Plant transformation is performed using a wide range of tools such as *A. tumefaciens* Ti plasmid vectors, biolistic bombardment, micro injection, chemical (PEG) treatment of protoplasts and electroporation of protoplasts. Though all methods have advantages that are unique to each of them, transformation using *Agrobacterium* and biolistic bombardment are the most extensively used methods. In general, *Agrobacterium* has been used as the vector for genetic transformation of diverse dicotyledonous species, but biolistic bombardment has been a very useful technique to introduce foreign DNA into plant cells of monocotyledons and dicotyledonous plants.

India is known as "The Home of Spices". Spices have been considered important in the culinary art from time immemorial. They are used for flavouring, seasoning and imparting aroma in foods. Besides, some of them are known to be fungistatic, antimicrobial or antibiotic. Their antioxidative activity helps to preserve foods from oxidative deterioration, increasing their shelf life. They are also processed into numerous 'value added' attractive spice products of importance such as spice oleoresins, essential oils, curry powder *etc.* Spices may comprise different plant components or parts such as floral parts or fruits or berries or seeds or rhizomes or roots or leaves or kernel or aril or bark or bulbs *etc.* (Pruthi, 1992). There is a constant mention of the important role spices have played in the life of the ancient people and also in changing the course of the world history. It was the lure of the exotic spices that inspired the European navigators and explorers that brought them to the shores of India and South-East Asia. Even today, spices are of considerable economic importance for all spice producing, exporting and importing countries of the world. The global spice trade is expected to increase with the growing consumer demand in importing countries for more exotic, ethnic tastes in food (Peter, 2001).

International Organization for Standardization (ISO) lists 109 herb and spice plant species useful as ingredients in food. India is known as one of the largest producers, consumers and exporters of spices and spice products. India grows over 50 spices in different parts. However, the country has one of the lowest productivity in many of the spice crops. Abiotic and biotic factors are among the important factors that contribute for the lower productivity of spices in India. Conventional breeding programmes are cumbersome and time consuming. Besides, in many spice crops source of resistance to biotic and abiotic stresses are not available or not characterized in the available germplasm collections. Hence transgenic approaches are one of the alternatives to impart abiotic and biotic stresses and to enhance quality and yield of spice crop. In this chapter, developments in the transgenics of different spice crops are discussed.

2. Black Pepper (*Piper nigrum*) (Family: Piperaceae)

Black pepper (*Piper nigrum*), known as the 'King of Spices', originated in the

tropical evergreen forests of Western Ghats of India (Ravindran, 2000). It is one of the most ancient crops cultivated in India. It is used for a variety of purposes including in medicine. It constitutes an important component of culinary seasoning and an essential ingredient of numerous commercial foodstuffs. India has the largest area and production of black pepper in the world. India is a leading exporter of black pepper that accounts for about 40 per cent of export earnings through spices (Ravindran, 2000).

2.1. Development of Transformation System

There are two preliminary reports on *Agrobacterium* mediated transformation of black pepper. Sasikumar and Veluthambi (1996) reported black pepper transformation using cotyledon and primary leaves as explants and *Agrobacterium* strain LBA4404 harbouring binary vector PGA 472 with β-glucuronidase (*GUS*) and neomycin phosphotransferase (*npt*II) as the selectable markers. They could get callusing (20 per cent) in infected cotyledon explants where as there was no appreciable callusing in infected primary leaves under kanamycin selection. Transformed calli remained fresh and proliferated in medium containing up to 150 µg/ml of kanamycin but in the case of control calli, no callus proliferation was observed above 50 µg/ml of kanamycin. Sim *et al.* (1998) cultured leaf, petiole and stem explants from axenic seedlings of black pepper and inoculated with *Agrobacterium* strain LBA4404 containing plasmids pMOG23 and pTOK47 containing *GUS* and *npt*I genes in callus inducing medium in the dark at 28º C. After co cultivation for two days, the explants were transferred to Murashige and Skoog (MS) based medium supplemented with carbenicillin and cefotaxime to kill *Agrobacterium*, and kanamycin at 75 mg/l for selection of transformed tissue. After 10 days, the leaf explants tested showed expression *GUS* gene but PCR analysis using *npt*II specific primers failed to detect the presence of transgene. Both these reports on black pepper transformation did not report regeneration of transformed tissue into plantlets.

Nair and Gupta (2006) reported a very efficient micropropagation strategy through cyclic secondary somatic embryos obtained from secondary embryos that were borne from the root pole region of primary somatic embryos derived from micropylar region of germinating mature seeds of black pepper. Using these somatic embryos as explants, Jiby and Bhat (2011) developed an efficient *Agrobacterium* mediated transformation protocol for black pepper. Cyclic secondary somatic embryos (embryogenic mass), co-cultivated with *Agrobacterium* carrying the *GUS* reporter gene, were cultured on plant growth regulator free Schenk and Hildebrandt (SH) medium and transformants were selected in the medium containing cefotaxime and step wise increase in kanamycin concentration from 25 to 100 µg/ml (Fig. 1). The transient GUS gene expression was determined histochemically. Transformants that survived in the selection medium were hardened in the green house. An average of nine hardened putative plantlets was obtained per gram of embryogenic mass. Presence of transgene in these plantlets was assayed by PCR, dot blot and Southern blot hybridization. Maju and Sonia (2012) reported direct regeneration of shoots from the bulged portion of shoot tip and nodal explants cultured on SH and MS medium with various combinations of cytokinins and auxins. They also reported

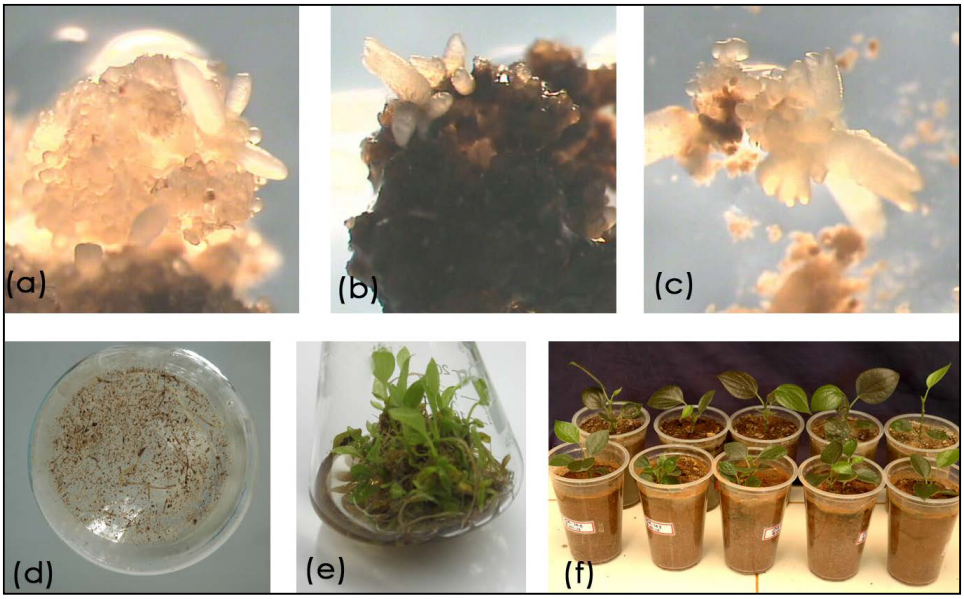

Figure 25.1: Different Stages of Transgenesis in Black Pepper.

(a) Co-cultivated embryogenic mass, (b) Growing points under kanamycin selection, (c) Embryo clusters formed under kanamycin selection, (d) Embryogenic mass in liquid SH medium, (e) Fully developed plantlets in liquid SH medium, (f) Hardened plants maintained in green house (Source: Jiby and Bhat, 2011).

genetic transformation of Panniyur 1 variety of black pepper by infecting seedling derived explants with *Agrobacterium* carrying pCAMBIA 1301 vector with GUS and HPT markers. Shoot regenerated from explants in the presence of hygromycin were analysed by PCR and GUS histochemical assay for confirmation of transgenic nature of plants.

Sasi *et al.* (2015) reported a loop-mediated isothermal amplification (LAMP) and real-time LAMP based assays as an alternative to PCR for quick and sensitive detection of transgenic black pepper plants. Primers (six each) were designed based on the nucleotide sequence of two target regions [kanamycin and *Cauliflower mosaic virus* (CaMV) 35 S promoter] integrated into the genome of transgenic black pepper. The assay successfully detected the transgenic plants whereas no cross-reaction was recorded with non-transgenic plants. The detection limit for LAMP was up to 10000 times that for conventional PCR and 1/1000 times that for real-time LAMP. The assays were validated by testing putative transformants of black pepper.

2.2. Development of Transgenic Black Pepper for Fungal and Viral Resistance

Babu *et al.* (2013) reported delayed response to infection by the fungus, *Phytophthora capsici* causing foot rot disease in black pepper plants transformed with *osmotin* gene. Bhat *et al.* (2014) attempted pathogen derived resistance approach to get virus resistant plants by transforming black pepper with sequences from

Cucumber mosaic virus (CMV) and *Piper yellow mottle virus* (PYMoV) sequences. Three constructs (CMV coat protein gene in sense orientation, reverse transcriptase region of PYMoV in sense and antisense orientations) were prepared in the binary vector, pBI121, mobilized into *A. tumefaciens* and were used for transforming embryogenic mass of four varieties of black pepper. After co-cultivation, explants were selected in a step wise increase in the concentration of kanamycin from 25 to 100 µg/ml in the SH medium. Of the 23 transgenic lines of PYMoV in the sense orientation and 40 transgenic lines in antisense orientation, 15 and 21 transgenic lines respectively showed presence of transgene when tested through PCR. The integration of the transgene was ascertained through Southern hybridization using transgene specific probe while the transcript production was confirmed though RT-PCR and northern hybridization. Out of 67 transgenic plants carrying the CMV coat protein gene when subjected to PCR, 12 plants showed the presence of transgene. Southern analysis of these plants using transgene specific probe confirmed the presence of transgene in nine plants with three insertions in all plants. All nine plants showed transcript and protein production when subjected to northern and western blotting. Short listed transgenic lines of PYMoV and CMV when subjected to challenge inoculation with respective viruses, four plants with PYMoV sequences showed symptom remission and two plants showed resistance to CMV.

Piper colubrinum, an exotic species distantly related to black pepper, shows high degree of resistance to the oomycete pathogen *Phytophthora capsici*, which causes the devastating 'foot rot' disease in black pepper. *In planta* transformation of black pepper *via* pollen tube pathway using total exogenous DNA of *P. colubrinum* was reported (Asha and Rajendran, 2010). When resultant seeds were germinated and tested *in vitro* and *ex vitro* against *P. capsici*, 39 per cent of plants did not take up the infection. Enhanced expression of serine/threonine protein kinase (*PcSTPK*) gene in *P. colubrinum* was noticed upon infection by *P. capsici* (Krishnan *et al.,* 2015). A *Tobacco Rattle Virus* (TRV)-based virus-induced gene silencing (VIGS) construct was established for functional validation of PcSTPK in *Piper colubrinum* by Krishnan *et al.* (2015). The construct TRV:PcSTPK VIGS vector was inltrated into young leaves of *Piper colubrinum* and the time course study revealed that STPK transcript levels was signicantly down regulated. Knock-down of *PcSTPK* by VIGS increased the susceptibility to *P. capsici* infection, as evidenced by the appearance of foliar necrotic lesions and increased proliferation and sporulation of *P. capsici* on the leaf surface indicating the possible role of PcSTPK in modulating antifungal defense response in the plant. The osmotin *PR 5* gene homologue from *Piper colubrinum* showed significant over expression in response to *P. capsici* infection. The functional validation of the same was reported by Anu *et al.* (2015) using a TRV based VIGS in *Piper colubrinum*. The TRV construct carrying *Piper colubrinum* osmotin (PCOSM) was infiltrated into *Piper colubrinum* plants. Three weeks post infiltration, significant down regulation of PCOSM was observed. The silenced plants when challenged with *P. capsici*, showed increased *P. capsici* growth accompanied by decreased accumulation of H_2O_2 indicating that osmotin gene is required for resisting *Piper colubrinium* infection and has possible role in hypersensitive cell death response and oxidative burst signaling during infection.

3. Caraway (*Carum carvi*) (Family: Umbelliferae)

Caraway or 'caraway seed' of commerce is the fruit of a biennial herb known botanically as *Carum carvi*. It is native to North and Central Europe, and is extensively cultivated in Holland, Russia, Poland, Bulgaria, Rumania, Syria and Morocco. The seeds, on steam distillation, yield an aromatic essential oil which finds greater use in medicine (Pruthi, 1992).

Krens *et al.* (1997) reported a high-frequency direct regeneration when cotyledonary node explants were used. Transient expression of *GUS* was obtained when cotyledonary node explants were used. This explant type proved to be the best for stable transformation resulting in transgenic plants. Parameters determining regeneration and transformation efficiency were optimized. The percentage of explants giving transgenic plants was as high as 13 per cent. This system for the rapid production of transgenic caraway plants opens up possibilities for studying metabolic engineering with this crop.

4. Cardamom (*Elettaria cardamomum*) (Family: Zingiberaceae)

Cardamom is the second most important national spice of India and hence known as "Queen of Spices". Cardamoms of commerce are the dried capsules of these plants, which contain seeds possessing a pleasant characteristic aroma. The plant is indigenous to India and Sri Lanka and more than 70 per cent of the world production is from India. It is cultivated in evergreen rain forests at 760-1500 m above sea level (Pruthi, 1992). Low production of cardamom is mainly due to the onslaught of pests and diseases caused by viruses and fungi.

In order to produce transgenic cardamom resistant to *Cardamom Mosaic Virus* (CdMV), Backiyarani *et al.* (2005) cloned coat protein gene of CdMV in plant expression vector, pAHC 17 under the ubiquitin promoter. Based on the *in vitro* studies, Josephrajkumar *et al.* (2006) suggested production of transgenic cardamom expressing protease inhibitor, such as aprotinin, for the management of cardamom shoot and capsule borer, *Conogethes punctiferalis*. A preliminary standardization of biolistic based transformation of cardamom embryogenic callus using GUS reporter gene was reported (Babu *et al.,* 2013).

5. Celery (*Apium graveolens*) (Family: Umbelliferae)

Celery seed is the dried ripe fruit of the umbelliferous herb. The native habitat of celery extends from Sweden to Egypt, Algeria and Ethiopia, and in Asia. It is one of the important minor spices of India. The dried ripe fruits are used as spice. Leaves and stalks are used as salads in soups. It is rich in beta- carotene, folic acid, vitamin C, calcium, magnesium, potassium, and fiber. The crop is susceptible to abiotic and biotic stresses. Thus, genetic engineering strategies could be a powerful means for introducing agriculturally valuable traits such as herbicide, disease or pest resistance.

Only a few reports to date are available concerned transformation efforts of celery. Catlin *et al.* (1988) obtained a total of 20 kanamycin resistant plants using *A. tumefaciens* mediated transformation of petiole explants of cv. PI257228. Celery

callus and seedlings were also used as models for transient transformation by Liu *et al.* (1992). Glufosinate herbicide resistant celery plants were obtained from cvs. XP85 and XP166 using *A. tumefaciens* with the bar transgene (Loskutov *et al.*, 2008). Morphologically typical and atypical transgenic plants were regenerated from both leaf and seedling explants using a selection system of 0.25 mg/ammonium glufosinate.

Song *et al.* (2007) reported a protocol for rapid and efficient production of transgenic celery plants *via* somatic embryo regeneration from *A. tumefaciens* inoculated leaf sections, cotyledons and hypocotyls. Co-cultivation was carried out for four days in the dark on callus induction medium supplemented with acetosyringone followed by selection in the medium containing kanamycin and timentin. Explants that survived for 12 weeks in the selection were regenerated *via* somatic embryogenesis on Gamborg B5 + 4.92 µM 6 (γ-γ-dimethyl allyl amino) purine (2iP) + 1.93µM a-naphthaleneacetic acid (NAA) + 25 mg/l kanamycin + 250 mg/l timentin after 8 weeks. Using this protocol, the transformation frequency of 5 per cent for leaf sections, 18 per cent for cotyledons, and 16 per cent for hypocotyl explants were obtained. Stable integration of the model transgenes with 1–3 copy numbers was conrmed in selected transgenic events by Southern blot analysis of *gusA*. Progeny analysis by histochemical GUS assay showed stable Mendelian inheritance of the transgenes.

Loskutov *et al.* (2008) evaluated methods for celery transformation using *A. tumefaciens* and the *bar* gene as selectable marker. Callus selection (CS) and the flamingo-bill explant (FB) methods were evaluated for efficacy in transformation. *A. tumefaciens* strains EHA105 and GV3101, each with the *bar* gene under the promoters NOS (pGPTV-BAR) or 35S (pDHB321.1), were used. Leaf explants were inoculated and co-cultivated for two days in the dark. Calluses emerged on the explants after 4-6 weeks were selected for glufosinate (GS) resistance by a two step method with increasing concentration of glufosinate. The explants that survived the selection were allowed to shoot on Gamborg B5 medium + 2iP (4.9 µM) + NAA (1.6 µM) and rooted on MS in 5–6 months time. Conversely, using FB with inoculation by GV3101/pDHB321.1, putative transgenic celery plants were obtained in just six weeks. Southern blot analyses indicated 1–2 copies in CS lines and 1 copy in FB lines. Herbicide assays on whole plants with 100 and 300 mg/l glufosinate indicated a range of low to high tolerance for lines derived by both methods. The *bar* gene was found to be Mendelian inherited in one self-fertile CS derived line.

6. Coriander (*Coriandrum sativum*) (Family: Umbellifereae)

The green leaves and seeds of coriander are used as spice. Mexico and USA are the major growers of coriander. It is a native of Mediterranean region and India is one of the major producers of coriander in the world. Stem, leaves and fruits of coriander have a pleasant aromatic odour. The entire plant is used in preparing chutneys and sauces, and leaves are used for flavouring curries and soups. The fruits are used as condiment in the preparation of several items (Pruthi, 1992).

The phytohormone ethylene is involved in many developmental processes, including leaf and flower senescence. Ethylene is perceived by plants through

receptors that trigger the downstream signal transduction pathway. The mutated ethylene receptor *ERS1* (ethylene response sensor) from *Arabidopsis* is of a dominant negative nature and confers ethylene insensitivity in Arabidopsis. To investigate if the altered *ERS1* gene can affect the tissue senescence in heterologous plants, *ERS1*was introduced into coriander by *Agrobacterium*-mediated transformation (Wang and Kumar, 2004). Transgenic plants were regenerated by co-cultivating hypocotyl segments with *A. tumefaciens* harboring binary vector pCGN1547 that carried the *ERS1* gene. The presence and expression of the transgene was confirmed by genomic Southern blot and reverse transcriptase-PCR analyses. Leaf and flower senescence were delayed significantly in the transgenic plants. The ability of the mutated ERS1 gene to confer the ethylene- insensitive phenotype can be exploited for extending the shelf-life of leafy vegetables.

7. Cumin (*Cuminum cyminum*) (Fam: Apiaceae)

Cumin is an annual herbaceous plant cultivated in tropical regions of the world including Egypt, India, China, Pakistan, Spain and the Eastern Mediterranean. India is the largest producer of cumin, contributing about 70 per cent of the total world production. Its root, stem, leaf and flower are a rich source of essential oils and polyphenols with antioxidant properties. However, cumin production has declined in recent years and the area of cumin cultivation is becoming depleted due to various factors that include abiotic stress such as salinity. The development of an abiotic stress-tolerant plant that can cope with adverse conditions might be an alternative so that unsuitable land is used for sustainable development. Conventional breeding methods have provided limited scopes to improve traits in cumin due to its low-genetic diversity and also due to inefficient and time-consuming approaches. Recent advances in gene manipulation, DNA technology, and genetic transformation provide a potential approach for the development of transgenic cumin.

A simple and efficient method was developed for multiple shoot regeneration of cumin from imbibed embryo cultures (Ebrahimie *et al.,* 2003). The method yielded a large number of shoots within short period of time (30–50 days) without any subculturing. The effects of different media, different embryo explants and various combinations of plant growth regulators on callus formation and shoot regeneration were also studied. Simultaneous callus formation and shoot regeneration was obtained. The best response for multiple shooter generation was observed on B5 medium containing 1.0 mg/l BAP, 0.2 mg/l NAA and 0.4 mg/l IAA, with an average of 140 shoots per explants. A microprojectile bombardment-mediated genetic transformation of embryo axes and plant regeneration in cumin was reported by Singh *et al.* (2010). Pre-cultured cumin embryos were bombarded under 27 inches Hg vacuum, 25 mm distance from rupture disc to macrocarrier, 10 mm macrocarrier ight distance using 1100 psi rupture disc and 9 cm microprojectile travel distance. About 91 per cent embryos showed transient GUS expression after 24 hours. Shoot tips and roots of T0 plantlets exhibited GUS expression done after three months of bombardment. Transformation was conrmed by performing PCR detection of *nptII* and *GUS* genes respectively from T0 transgenics and Southern blot analysis using PCR amplied DIG labeled *nptII* gene as probe.

Pandey *et al.* (2013) reported an efficient and reproducible method of *Agrobacterium*-mediated genetic transformation for this crop. A direct regeneration method without callus induction was optimized using embryos as explants material in Gamborg's B5 medium supplemented with 0.5 µM 6 BA and 2.0 µM NAA. Pre-cultured elongated embryos wounded with razor blade were co-cultivated with *Agrobacterium* carrying the binary construct for 72 hours in the medium containing 300 µM acetosyringone. About 95 per cent embryos showed transient *GUS* expression after co-cultivation. Putative transformed embryos were cultured on B5 medium for shoot proliferation and regenerated plants were allowed to root. T0 plantlets showed *GUS* expression and gene integration was confirmed *via* PCR amplification. A transformation efficiency of 1.5 per cent was obtained and gene copy number analysed by Southern blot analysis indicated single-copy gene integration.

8. Fenugreek (*Trigonella foenum-graecum*) (Family: Papilionaceae)

Fenugreek is an annual plant extensively cultivated in India and northern Africa, the seeds and leaves of which are used as food and also as traditional medicines. Two biologically active alkaloid metabolites, galactomannan and diosgenin, are found in the seed of fenugreek plants. Diosgenin is found in roots, stems, and young leaves and used for the synthesis of oral contraceptives, hormones, and other steroids. Most of the studies concerning the production of diosgenin have focused on root and also hairy root cultures. Genetically transformed hairy roots are highly differentiated and cause stable and extensive production of secondary metabolites.

A. rhizogenes induced hairy roots is ideal for production of secondary metabolites such as diosgenin in fenugreek. In order to evaluate the transformation frequency and the efficiency of transgenic hairy root induction, leaf and stem explants from two fenugreek ecotypes, Karaj and Bushehr, were infected with *A. rhizogenes* strain K599 harboring a *GFP* (green fluorescent protein) gene (Shahabzadeh *et al.,* 2013). Regardless of ecotype, the ability of stem explants for the induction of hairy roots (8.09) and the transformation frequency (81.3 per cent) was higher compared with leaf explants with the values of 5.97 and 71.88 per cent, respectively. The number of transgenic GFP-positive hairy roots ranged from 4.2 to 13.5 in the Karaj ecotype and 3.8 to 9.9 in Bushehr. The effect of genotype, type of explants and bacterial concentration on the hairy root production, transformation frequency and rate of growth of transgenic roots were also studied (Shahabzadeh *et al.,* 2013).

9. Garlic (*Allium sativum*) (Family: Lilliaceae)

Garlic is an important minor spice or condiment known all over the world as a valuable condiment for foods, and a popular remedy or medicine for various ailments and physiological disorders. It is a hardy bulbous perennial with narrow flat leaves and bears small white flowers and bulbils. Garlic does not readily produce seeds and is propagated vegetatively thus preventing improvement of garlic by conventional breeding methods.

9.1. Development of Transformation System

The temperature and number of days of co-cultivation with *Agrobacterium* was

shown to be an important factor in transient expression of the *uidA* in garlic (Kondo *et al.,* 2000). After a culture period of five months in selection medium containing hygromycin, 20 shoots were induced from ca. 1000 calluses among which 15 plants expressed *GUS* gene and shoots developed into transgenic garlic plants. Integration of the *uidA* was confirmed by Southern blot analyses. Robledo-Paz *et al.* (2004) used microprojectile bombardment to introduce DNA into embryogenic callus of garlic and produce stably transformed garlic plants. Embryogenic calluses were bombarded with plasmid DNA containing genes coding for *hpt* and *gus*. Putative transformed calluses were indentured in the bombarded tissue after four months of selection on 20 mg/l hygromycin B. The transgenic nature of the selected material was demonstrated by *GUS* histochemical assay and Southern blot hybridization analysis.

Eady *et al.* (2005) reported *A. tumefaciens*-mediated transformation of leek (*Allium porrum*) and garlic using immature leek and garlic embryos as explants. The method involved the use of a binary vector containing the m-GFP- ER reporter gene and *npt*II selectable marker. The presence of transgenes in the genome of the plants was conrmed using TAIL-PCR and Southern analysis. Kenel *et al.* (2010) reported efficient *A. tumefaciens*-mediated transformation and regeneration of garlic immature leaf tissue. The method involved the use of immature embryos and a binary vector containing the m-GFP-ER reporter gene and *hpt* selectable marker. The presence of transgenes in the genome of the plants was conrmed using Southern analysis.

9.2. Development of Transgenic Garlic Resistant to Herbicide and Insects

Park *et al.* (2002) reported generation of chlorsulfuron (a sulfonylurea herbicide) resistant transgenic garlic plants by particle bombardment. The callus tissue from the apical meristem of garlic cloves were subcultured and repeatedly selected calli with brittle, non-mucilaginous surfaces were selected for over six months was used as explants for transformation. Recombinant DNA that contained the acetolactate synthase (*ALS*) gene from a chlorsulfuron-resistant *Arabidopsis* mutant, the cauliower mosaic virus 35S promoter, the *GUS* reporter gene, and the hygromycin phosphotransferase (*hpt*) selectable marker gene was used for transformation. The explants were bombarded twice with tungsten particles coated with the DNA constructs. Transformed calli were selected in hygromycin B and regenerated into plants were confirmed using PCR, Southern and northern blot analyses. The regenerated plants survived in the presence of 3 mg/l chlorsulfuron, demonstrating that their *ALS* was insensitive to the herbicide.

Zheng *et al.* (2004) reported the development of a reliable *Agrobacterium* mediated transformation system for garlic and its application in producing insect resistant transgenic garlic lines. Callus induced from apical and non-apical root segments of *in vitro* plantlets, true garlic seeds and bulbils were used as explants, *gusA* and the *GFP* gene coding for green uorescent protein were used as reporter genes. The protocol required short time period of about six months. The highest transformation frequency was 1.47 per cent in the cv. 'Printanor'. PCR and Southern hybridization showed that the reporter gene *gusA* and the selectable marker gene

hpt were stably integrated into the garlic genome. Using this protocol transgenic garlic resistant to beet armyworm using the cry1Ca and H04 resistance genes from *Bacillus thuringiensis* was developed. After transfer of the transgenic *in vitro* garlic plants to the green house, the cry1Ca plants developed normally and grew well to maturity with normal bulbs. However, all transgenic *in vitro* H04 garlic plants did not survive after transfer to the greenhouse. Transgenic cry1Ca garlic plants proved completely resistant to beet armyworm in a number of *in vitro* bio-assays.

10. Ginger (*Zingiber officinale*) (Family: Zingiberaceae)

Ginger is an herbaceous perennial, the rhizome of which is used as spice. India is a leading producer of ginger in the world. The aroma of ginger is pleasant and spicy, and flavours penetrating. Hence it is used in the manufacture of a number of food products and in medicine. Ginger oil is a food flavorant in soft drinks. The absence of seed set in ginger makes conventional breeding methods inapplicable warranting genetic modification through biotechnological means. Suma *et al.* (2008) reported development of *A. tumefaciens* based genetic transformation and regeneration of ginger using *GUS* reporter gene. Optimum concentration of bacteria, co-cultivation period, concentration of acetosyringone and kanamaycin required were standardized. Transformants were recovered on selection media containing 100 mg/l kanamycin and a combination of 2,4-D 1.0 mg/l and BA 0.5 mg/1, and regenerated in half strength MS media of BA 3.0 mg/1 and 2,4-D 0.5 mg/l. Successful transformation was confirmed by histochemical GUS assay and PCR analysis.

11. Onion (*Allium cepa*) and Shallot (*Allium cepa* var. *aggregatum*) (Family: Lilliaceae)

Onion and shallot are used both for cooking and as a condiment for flavouring or for pickling. The flavour of shallot is somewhat milder than that of onions and is used for flavouring curries. Mild onions are used for cooking or as salad. Pungent varieties are used as condiment for flavouring a number of foods. Dehydrated onions, onion flakes, kibbled onions and onion powder are used for flavouring ketchups, sauces *etc.* There is a need to improve the production and nutritive value of these economically important crops and genetic engineering techniques can be exploited as an additional method for introduction of useful traits into established cultivars (Pruthi, 1992).

11.1. Development of Transformation System

Zheng *et al.* (2001) described a reliable transformation protocol which could be used year-round for onion and shallot. It was based on *Agrobacterium tumefaciens* as a vector, with three-week old callus, induced from mature zygotic embryos, as target tissue. Successful transformation was Subspecies (onion and shallot) and cultivar specific as shallot was responsive to transformation than onion and shallot cv. Kuning gave the best results. Also, it was found that constant reduction of the size of the calli during subculturing and selection by chopping, enhances exposure to the selective agent hygromycin, thereby improved the selection efficiency signicantly. Callus induction medium and co-cultivation period showed a signicant effect on successful stable transformation. The usage of different *Agrobacterium* strains, callus

ages, callus sources and osmotic treatments during co-cultivation did not inuence transformation efficiency. The highest transformation frequency (1.95 per cent) was obtained with shallot cv. Kuning. PCR and Southern hybridization were used to confirm transgene integration and its copy number. FISH performed on 12 plants from two different lines representing two integration events showed that original T-DNA integration had taken place on the distal end of chromosomes 1 or 5. A total of 83 transgenic plants were transferred to the greenhouse and these plants appeared to be diploid and normal in morphology.

A new selection system for onion transformation by *Agrobacterium* and biolistic, that does not require the use of antibiotics or herbicides, was developed by Aswath *et al.* (2006). The selection system used the *Escherichia coli* gene that encodes phosphomannose isomerase (*pmi*). Transgenic plants carrying the *manA* gene that codes for *pmi* could detoxify mannose-6-phosphate by conversion to fructose-6-phosphate, an intermediate of glycolysis. Six week old embryogenic callus initiated from seedling radicle was used for transformation. Transgenic plants were produced efciently with transformation rates of 27 and 23 per cent using *Agrobacterium* and biolistic system, respectively. Untransformed shoots were eliminated by a stepwise increase from 10 g/l sucrose with 10 g/l mannose in the rst selection to only 10 g/l mannose in the second selection. Integrative transformation was conrmed by PCR, RT-PCR and Southern hybridization. Cheng *et al.* (2009) reported transient expression of linear gene cassettes containing a *GUS* reporter gene in onion epidermal cells *via* direct transformation. The basic transformation solution used was MS liquid medium. Hypertonic pretreatment of explants and transformation cofactors, including Ca2+, surfactant assistants, *Agrobacterium* LBA4404 cell culture on transformation efficiency were evaluated. Prior to the incubation of the explants and target linear cassette in transformation solution for 3 hours, the onion low epidermal explants were pre-cultured in darkness for 48 hours and then transferred to MS solid media for 72 h. FITC-labeled linear DNA was used to trace the delivery of DNA entry into the cell and the nuclei. By GUS staining and flow-cytometry- mediated fluorescent detection, a significant increase of the ratios of fluorescent nuclei as well as expression of the GUS reporter gene was observed by each designed transformation solution.

Sandhu and Gosal (2009) reported transient GUS expression in onion epidermal layer cells through particle bombardment. The onion epidermal layer(s) were bombarded with tungsten coated plasmid pWRG2426 containing *GUS* gene under the control of CaMV 35S promoter. The *GUS* gene expression was assessed within 24 hours following particle bombardment by counting the number of islands of cells showing indigo coloration and the measure of intensity was based on relative indigo color development among the bombarded cells. Mythili *et al.* (2012) studied factors influencing *in vitro* regeneration such as the age of the embryogenic callus, the salt content in the basal medium (MS or BDS), gelling agent strength, plant growth regulators (picloram, Kinetin, 2,4-D) in the regeneration medium and aeration of the culture vessels. They also studied factors such as method of inoculation, influence of acetosyringone; and selection agents (kanamycin or geneticin) influencing *Agrobacterium m*ediated transformation of onion var. 'Arka Niketan'. Inoculation

of the explants with *Agrobacterium* under vacuum filtration was suitable only for immature embryos while inoculating with bacterial suspension was suitable for the embryogenic callus. The effect of acetosyringone was influenced by basal medium and plant growth regulators. Geneticin was found to be a better selectable agent.

Callus obtained from basal meristem plate and twin scale leaves of two cultivars (Bellary and CO3) were used by Malla *et al.* (2015) for *Agrobacterium* mediated transformation using binary vector pCAMBIA 1301 containing the *GUS* gene with different concentrations of acetosyringone. The study revealed that the frequency of callus induction was the maximum in MS fortified with B5 vitamins supplemented with 0.5 and 1 mg/l picloram. Regeneration of plantlets from the callus was observed on MS supplemented with 0.5 mg/1 each of BAP and KIN and 0.1 mg/1 NAA. *In vitro* bulb response was observed on MS with B5 vitamins supplemented with 2.0 mg/l BAP. *GUS* gene integration in the transgenic plants was confirmed by PCR. The maximum *GUS* gene expression was observed at a concentration of 150 µM acetosyringone in the transformed plants by histochemical assay.

11.2. Development of Transgenic Onion and Shallot Resistant to Herbicide and Insects

Eady *et al.* (2003a) produced transgenic onion plants tolerant to herbicides containing active ingredients *viz.*, glyphosate and phosphinothricin from immature embryos of open pollinated and hybrid parent onion lines were used as explants and a maximum transformation frequency of 0.9 per cent was obtained. Transformants of different onion cultivars, grown on different selective agents and confirmed by Southern analysis, thrived with no apparent ill effects when sprayed with the respective herbicides at double the recommended field dosage for weed eradication. Eady *et al.* (2003b) studied inheritance and expression of introduced DNA in transgenic onion plants. Transgenic onion plants containing the *Cauliflower Mosaic Virus* 35S promoter (CaMV35S) and *GFP* gene construct encoding the visual green fluorescent reporter protein from pBin m GFP ER and the CaMV35S-bar gene construct encoding resistance to the herbicide phosphinothricin from pCAMBlA3301 were produced by *Agrobacterium*-mediated transformation were used in the study. These plants were grown to maturity and selfed in order to determine the expression and inheritance of the transgenes. Both the expression of GFP and tolerance to phosphinothricin inherited in a Mendelian fashion. Levels of expression in F1 offspring varied due to environmental and genetic factors and copy number did strongly influence GFP protein production and expression. In the majority of plants there were no obvious detrimental phenotypic effects caused by the transgene, the integration event, or somaclonal variation.

Agrobacterium-mediated genetic transformation was applied to produce beet armyworm (*Spodoptera exigua*) resistant tropical shallots. A cry1Ca or a H04 hybrid gene from *Bacillus thuringiensis*, driven by the chrysanthemum ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SSU) promoter, along with the HPT driven by the CaMV 35S promoter, was employed for genetic transformation. An average transformation frequency of 3.68 per cent was obtained from two shallot cultivars, Tropix and Kuning. After transfer of the *in vitro* plants

to the greenhouse 69 per cent of the cry1Ca and 39 per cent of the H04 transgenic shallots survived the rst half year. After one year of cultivation in the greenhouse, the remaining cry1Ca and H04 transgenic plants grew vigorously and had a normal bulb formation, although the cry1Ca transgenic plants (and controls) had darker green leaves compared to their H04 counterparts. PCR and Southern analysis conrmed integration of T-DNA into the shallot genome. Northern blot and ELISA analyses revealed expression of the *cry1Ca* or *H04* gene in the transgenic plants. The amount of Cry1Ca expressed in transgenic plants was higher than the expression levels of H04 (0.39 vs. 0.16 per cent of the total soluble leaf proteins, respectively). There was a good correlation between protein expression and beet armyworm resistance. Cry1Ca or H04 gene expression of at least 0.22 or 0.08 per cent of the total soluble protein in shallot leaves was sucient to confer complete resistance against beet armyworm. The study also conrmed earlier findings that H04 toxin was more effective in controlling *S. exigua* than Cry1Ca toxin.

12. Paprika/Chilli (*Capsicum annuum*) (Family: Solanaceae)

Chilli or Paprika or Hungarian paprika, also called sweet pepper or Spanish pimento, is the mild or non-pungent variety of chilli capsicum. The dried ripe red paprikas are valued chiefly for their brilliant red colour and mild flavour. The crop is susceptible for attack by several fungi and viruses. The most transformation studies in chilli refer to the use of marker (*npt*II) or reporter gene (*GUS*) in order to establish adequate protocols; however some genes have also been utilized to generate transgenic plants with tolerance to viruses and fungi.

12.1. Development of Transformation System

The first report of *Agrobacterium* mediated genetic transformation of chilli using explants such as hypocotyls, cotyledons and leaves was reported by Liu *et al.* (1990). Although kanamycin resistant shoot buds were obtained, no further elongation and plant formation occurred. Manoharan *et al.* (1998) reported transformation using *A. tumefaciens* carrying a binary vector plasmid pBI 121and cotyledonary explants. Shoot buds produced were elongated and rooted in the presence of kanamycin (25 mg/l). The transgenic nature of regenerated plants was conûrmed histochemical staining of *GUS*, PCR and Southern hybridization analyses of *NPT*II gene.

Kim *et al.* (2002) developed a transformation system for paprika based on mannose as selection agent. A dosage curve for optimizing the selection conditions was established by mixing mannose and sucrose. They found that mannose selection has an increased transformation frequency compared to kanamycin selection. Nianiou *et al.* (2002) established a regeneration and transformation protocol for the sweet red pepper type 'Florinis' and for two pepper hybrids PO1 and C using hypocotyl explants. The rate of plant regeneration was found to depend on the types of explants cultured and the media used. Shoot bud initiation is more effective on MS media supplemented with IAA and BAP and shoot bud development with addition of GA3. Rooted shoots are successfully established in soil. *Agrobacterium* and the particle gun were used for transformation. *A. tumefaciens* strain LBA4404 harboring a plasmid containing GUS reporter gene and the NPT II selection gene or a plasmid with chloroplastic Cu/Zn SOD gene of tomato. Pepper hypocotyls

were bombarded with plasmid that contained the GUS reporter gene driven by the CaMV-35S promoter employing particle gun mediated transformation. Of the two methods, more transgenic plants were obtained in the case of particle gun.

A highly efficient transformation system using cotyledons as explants was reported by Li *et al.* (2003). Cotyledon explants were preconditioned for transformation for two days in a medium supplemented with sucrose. After two days of co-cultivation, explants were selected on 500 mg/l carbenicillin for two days. Explants were then placed on medium containing $AgNO_3$, kanamycin sulfate and carbenicillin. After 4-5 weeks, the explants with buds were transferred to medium supplemented with sucrose, $AgNO_3$ and hormones such as IAA, BA, gibberellic acid along with kanamycin and carbenicillin for elongation of buds. After 1-6 weeks, 1-2 cm long elongated shoots were excised and placed in MS medium containing NAA, IAA, kanamycin and carbenicillin for rooting. All four tested genotypes showed a high differentiation efficiency (81 per cent), elongation rate (61 per cent) and rooting efficiency (90 per cent). PCR results showed that 41 per cent of the plants were transgenic. High frequency shoot regeneration and *Agrobacterium* mediated transformation using shoot tips, cotyledons and hypocotyls explants was reported by Sobhakumari and Lalithakumari (2005).

A tissue culture independent *Agrobacterium* mediated *in planta* transformation was attempted in two varieties of chilli (Kumar *et al.*, 2009). In order to establish a reliable and highly efficient method for genetic transformation of pepper, a monitoring system featuring *GFP* as a marker was applied to *Agrobacterium*-mediated transformation by Jung *et al.* (2011). A callus-induced transformation (CIT) system was used to express *GFP* gene. Expression of GFP was observed in all tissues of T0, T 1 and T2 peppers in which the whole pepper plant exhibited GFP uorescence. The transformation rate ranged from 0.47 to 1.83 per cent depending on the genotype. This technique could enhance selection power by monitoring GFP expression at the early stage of callus *in vitro*. The detection of GFP expression in the callus led to successful identication of shoots that contained the transgene.

Kumar *et al.* (2012) reported an effective and reproducible auxin free regeneration method for six different red pepper cultivars (ACA-10, Kashi Anmol, LCA-235, PBC-535, Pusa Jwala and Supper) using hypocotyl explants and an efficient *Agrobacterium*-mediated transformation protocol. The explants (hypocotyls, cotyledonary leaves and leaf discs) collected from axenic seedlings were cultured on either hormone free MS medium or MS medium supplemented with BAP alone or in combination with IAA. Inclusion of IAA in the regeneration medium resulted in callus formation at the cut ends of explants, formation of rosette leaves and ill defined shoot buds. Regeneration of shoot buds was achieved from hypocotyls grown in MS medium supplemented with different concentrations of BAP unlike other explants which failed to respond. Incorporation of GA3 in shoot elongation medium at 0.5 mg/l concentration enhanced the elongation in two cultivars, LCA-235 and Supper, while other cultivars showed no significant response. Chilli cultivar, Pusa Jwala was transformed with βC1 ORF of satellite DNA β molecule associated with Chilli leaf curl Joydebpur virus through *A. tumefaciens*. Transgene integration in putative transformants was confirmed by PCR and Southern hybridization analysis.

12.2. Development of Transgenic Paprika Resistant to Herbicide, Fungi and Viruses

Yamakawa *et al.* (1998) produced transgenic chilli expressing phenylalanine ammonia lyase (*PAL*) gene from parsley using A. rhizogenes strain A13 harbouring recombinant binary vector pBI121 and hypocotyls as explants. After four weeks, the hairy roots produced were transferred onto medium supplemented with cefataxime and subsequently selected in kanamycin. The integration of the transgene was confirmed through Southern hybridization. Hairy roots containing *PAL* showed different *PAL* activity, slow growth and altered morphology.

In another study, *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* (TMV) coat protein (*CP*) genes have been transferred to chili pepper cultivar 8212 by a modied procedure of *A. tumefaciens*-mediated transformation using hypocotyl as the explants (Cai *et al.*, 2003). PCR analysis revealed the presence of both CMV and TMV CP genes in at least 11 primary transformants out of 49 kanamycin-resistant chili pepper plants. Ten T1 lines, from ve independent transformation events, were identied as putative homozygous transgenic lines based on the rooting assay of their T2 seedlings on the kanamycin-containing media. Integration and expression of CMV CP and TMV CP transgenes in one of the homozygous line, 16-13, were conrmed by Southern blot, RT-PCR and western blot analyses. Line 16-13 was highly resistant to infection of homologous CMV and TMV strains in greenhouse conditions when successively challenged with CMV and TMV or challenged with TMV alone. Furthermore, eld trials on T2, T3 and T4 progenies of Line 16-13 were performed on scales of 123, 300 and 10,000 plants, respectively, in consecutive years 1996, 1997 and 1998 with the permission of the Chinese government authority. The transgenic plants displayed delayed symptom development and signicantly milder disease severity in eld conditions when compared to untransformed chili pepper plants, resulting in 47 and 110 per cent increase in pepper fruit yield in surveys conducted in 1997 and 1998 trials, respectively. Finally, quality analysis and biosafety assessment were performed on transgenic chili pepper fruit concurrently with the control fruit, and demonstrated that the transgenic chili pepper fruit is substantially equivalent to the non-transgenic pepper in terms of the quality and biosafety when consumed as a food additive.

Lee *et al.* (2004) used two genes, *TMV-CP* and *PPI1* (pepper-PMMV interaction 1 transcription factor), to transform commercially important chili pepper inbred lines (P915, P409) by means of *Agrobacterium* co-culture and obtained eighteen independently transformed T0 plants. They also reported that use of correct type of callus and selection of callus-mediated shoot formation are important in the transformation of chilli. Lee *et al.* (2009) produced transgenic peppers resistant to a new CMV pathotype, CMVP1 using coat protein gene from CMV pathotype, CMV P0 as transgene using *Agrobacterium* mediated transformation. Transgenic peppers tolerant to CMVP1 were selected in a plastic house as well as in the eld. Three independent T3 pepper lines highly tolerant to the CMVP1 pathogen were found to also be tolerant to the CMVP0 pathogen. These selected T3 pepper lines were phenotypically identical or close to the non-transformed lines. However, after CMVP1 infection, the height and fruit size of the non-transformed lines became

shorter and smaller, respectively, while the T3 pepper lines maintained a normal phenotype

Juan-Xu *et al.* (2009) reported an *Agrobacterium*-mediated transformation system with *Cre* and *Barnase* genes designed to control plant fertility by cell lethal gene Barnase expressing at specific developmental stage and in specific tissue of male organ under the control of Cre/lox system. PCR and Southern blotting analysis of kanamycin plantlet indicated that the foreign genes had been integrated into the genome of pepper. The transgenic plants with *Cre* gene developed well, blossomed out, and set fruit normally. The transgenic plants with *Barnase* gene grew well with normal appearance of flower, but they showed different fertility from complete sterility, partial sterility to complete fertility.

Aguilar-Barragán and Ochoa-Alejo (2014) used *Tobacco rattle virus* (TRV) based virus induced gene silencing system (VIGS) system to determine the role of MYB, MYC, and WD40 transcription factors (TFs) that regulate the expression of structural biosynthetic genes at different steps on chilli. The accumulation of anthocyanins in chilli pepper fruits of plants transformed with TRV2-MYB and TRV2-WD40 constructs was significantly reduced compared to the control or empty TRV2-transformed plants. A significant reduction in gene expression of both TFs was also detected. The expressions of the chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavonoid 3′,5′-hydroxylase (*F3´5´H*), dihydroflavonol 4-reductase (*DFR*), and UDP-glucose:flavonoid 3-O-glucosyltransferase (*3GT*) genes were decreased in the plants transformed with the TRV2-MYB construct but not the transcription of flavanone 3-hydroxylase (*F3H*). When chilli pepper plants were infected with the TRV2-WD40 construct, a significant reduction in *CHS*, *F3H*, *F3´5´H*, *DFR* and *3GT* expression, but not in *CHI* in the fruits was observed. Mythili *et al.* (2015) produced transgenic chili expressing baculovirus chitinase gene which showed *in vitro* inhibition against fungal pathogens such as *Colletotrichum capsici* and *Alternaria alternata* causing anthracnose disease.

13. Turmeric (*Curcuma longa*) (Family: Zingiberaceae)

Turmeric is a rhizomatous species known both for its culinary and medicinal uses. It is mainly cultivated in India, Pakistan, Sri Lanka, Bangladesh, and China. India is the largest producer and a major exporter of this spice. Turmeric powder is obtained from its boiled, dried and polished underground rhizomes. Cultivated turmeric is a sterile polyploid species that is propagated only clonally *via* rhizomes. Due to the vegetative propagation, the rhizome is susceptible to accumulation and transmittance of pathogens and soil-borne diseases. It is essential to develop new strategies that combine tissue culture and genetic engineering techniques to complement breeding programs, and effective transformation approaches for identication of gene function and improvement of physiological traits for this species. Preliminary studies in this direction included i*n vitro* micropropagation and plant regeneration from callus culture. An efficient method for stable transformation for turmeric was developed by Shirgurkar *et al.* (2006) using particle bombardment. Callus cultures initiated from shoots were bombarded with gold particles coated with plasmid pAHC25 containing the *bar* and *gusA* genes each driven by the maize

ubiquitin promoter. Transformants were selected on medium containing glufosinate. Transgenic shoots regenerated from these were multiplied and stably transformed plantlets were identified by PCR and histochemical *GUS* assay. Transformed plantlets were resistant to glufosinate. An efficient somatic embryogenesis system and a reliable *Agrobacterium*-mediated transformation protocol were developed for turmeric (He and Gang, 2014). Calli derived from turmeric inorescences were used as source tissues for transformation. Factors affecting transformation and regeneration efficiency were evaluated, including callus induction and culture conditions, *Agrobacterium* strains, co-cultivation conditions, selection agent sensitivity and bacterial elimination, and transformant selection. Optimized transformation conditions were identied, including use of *Agrobacterium* strain EHA105 with plasmid pBISN1 for infection; a modied B5 medium system for callus induction, subculture, co-culture and selection; and MS media for transformant regeneration. Transgenic plants and their vegetative (clonal) progeny stably expressed the transgene as indicated by *GUS* assay, PCR and Southern blot analysis. In addition, a transient gene expression system was also developed that involved *Agrobacterium* inltration of young turmeric leaves followed by *in vitro* regeneration of plantlets.

14. Vanilla (*Vanilla planifolia*) (Family: Orchidaceae)

Vanilla is the second most expensive spice on the world market. Natural vanillin obtained from the cured pods (fruits) of this plant is used for spicing a variety of food and confectionaries. There are three important cultivated species namely *V. planifolia* (Mexican vanilla), *V. pompona* (West Indian vanilla), and *V. tahitensis* (Tahitian vanilla). The important vanilla growing countries are Madagascar, Indonesia, Mexico, Comoro and Reunion. Vanilla is a native of Mexico and was introduced to India as early as 1835. Like other orchids, vanilla is also affected by fungal and viral diseases.

Wang *et al.* (1997) produced transgenic *Nicotiana benthamiana* plants using the coat protein gene of Vanilla necrosis potyvirus (a virus known to infect vanilla) via *Agrobacterium tumefaciens*-mediated transformation. Four constructs contained either: sense (+) CP sequence, antisense (-) CP sequence, sense CP sequence with a Kozak's consensus ATG resulting in a change in the first amino acid, or antisense CP sequence with the Kozak's modification. When transgenic *N. benthamiana* plants were mechanically inoculated with a high concentration of VNV, one of the plant lines containing the full-length sense CP gene was highly resistant to virus infection. Plants from the resistant lines expressed the CP at a relatively low level compared to susceptible lines containing the same construct. Plants containing the other three constructs were either susceptible or showed delayed symptom expression in tobacco. However transgenic vanilla harboring this sequence could not be developed.

Malabadi and Nataraja (2007) reported production of protocorm-like bodies (PLBs) in presence of putrescine from thin section culture of shoot tips of vanilla. Using PLBs as explants they established an *Agrobacterium* mediated genetic engineering of vanilla using *nptII* and *GUS* genes. The presence of transgene was confirmed by PCR followed by Southern and northern hybridization of PCR

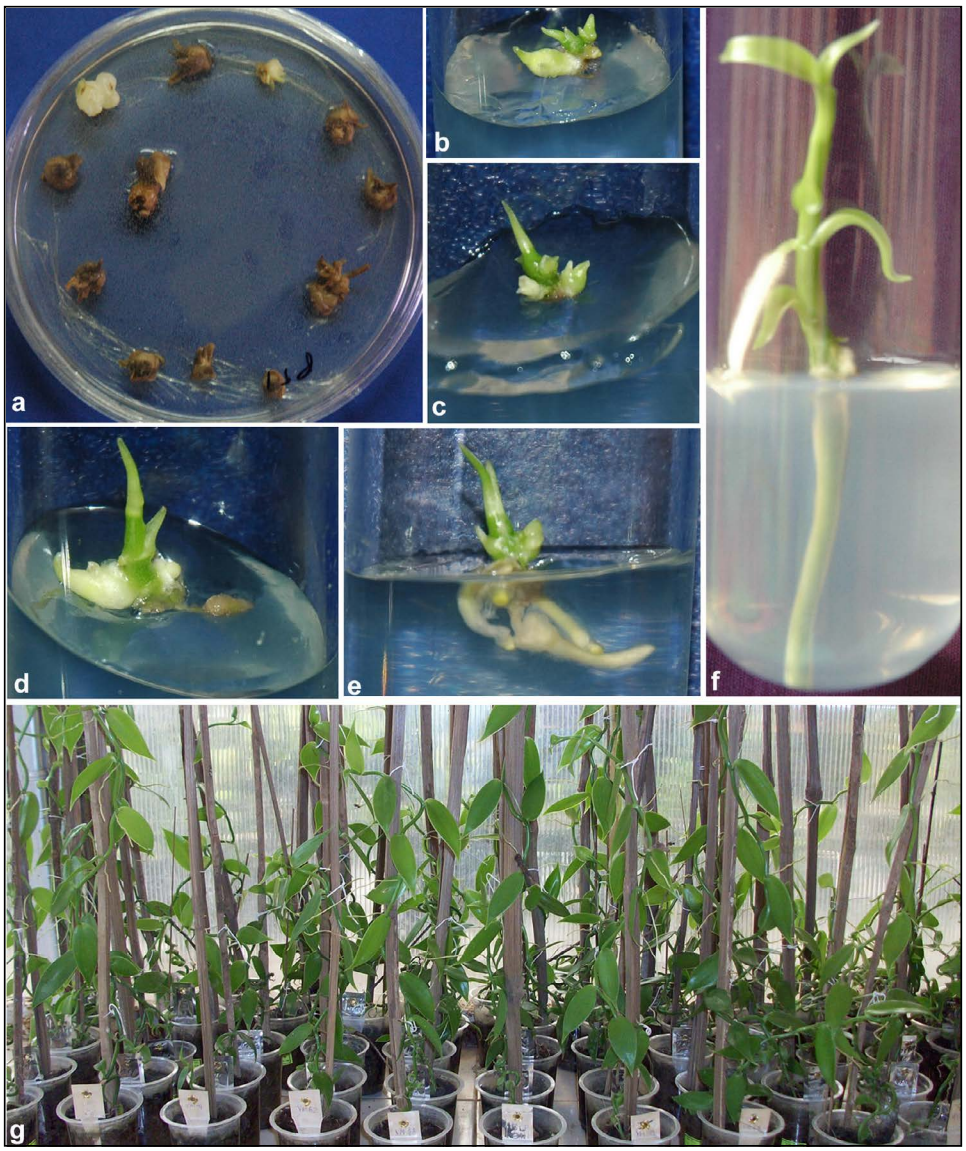

Figure 25.2: *Agrobacterium* Mediated Genetic Transformation in Vanilla.

(a) Protocorm like bodies (PLBs) in selection medium after 30 days of co-cultivation (b, c, d, e and f) after 40, 50, 60, 90 and 120 days of culture in regeneration medium (g) hardened transgenic plants in green house.

products. Retheesh and Bhat (2011) established an efficient transformation protocol for vanilla using PLBs derived from shoot tips as explants (Figure 25.2). Of the ten media tested, MS medium containing 0.45 µM thidiazuron (TDZ) produced maximum PLBs per shoot tip. PLB's were co-cultured with *A. tumefaciens* strain

EHA105 harbouring the binary vector pBI121 containing the *GUS* and *NPTII* genes for three days in MS medium supplemented with acetosyringone and transferred to selective regeneration medium containing 4.43 µM BA, 2.68 µM NAA supplemented with 50 mg/l kanamycin and 250 mg/l cefotaxime. After 15 days of culture, the surviving explants were transferred to the same regeneration medium but with a higher concentration of kanamycin (75 mg/l). Finally, explants that survived after 30 days were subjected to more stringent selection in the regeneration medium supplemented with 100 mg/l of kanamycin (Fig. 2). Integration of T-DNA into nuclear genome of transgenic plant was confirmed by PCR and Southern hybridization while expression of transgene was confirmed by Northern hybridization.

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