

## Review paper

**Status of transgenics in Indian spices**

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**Abstract**

Biotechnology tools involving plant tissue culture and recombinant DNA technologies are powerful to complement conventional breeding and expedite spices improvement. The rate of progress in improvement of perennial spices is relatively slow due to their long pre-bearing period and other crop specific problems compared to other horticultural crops and studies are underway aimed at genetic improvement against pests, diseases and abiotic stresses. Hairy root transformation strategy is also being used for secondary metabolite production using seed spices, which are repertoire of medicinally important compounds. This review presents a consolidated account of contemporary information on biotechnological advances made in spice crops and future perspective in this direction.

**Key words:** *Agrobacterium* mediated transformation, *In planta* transformation, Plant regeneration, Spices improvement, Transgenics

**Introduction**

India has been recognized as the 'Land of Spices' from prehistoric times and western Ghats of India is believed to be the centre of origin of many spices particularly, black pepper, cardamom and other zingiberaceous spices. Among the 109 spices listed by International Organization for Standardization, India grows about 60 and Indian spices flavor foods in over 130 countries. Indian spices have obtained geographical indicators such as Malabar pepper, Alleppey Green Cardamom, Coorg Green Cardamom and Naga chilli etc. due to their intrinsic values in terms of taste, colour and fragrance. India possesses many innate advantages over other spice producing countries - its large genetic base, varied soil and climatic conditions. Indian spices export is remarkable but not spectacular considering the

historical importance. Crop loss due to diseases, lack of resistant varieties and post harvest losses are the major reasons for low productivity of spices. In perennial spices like black pepper, cardamom etc., conventional breeding programmes are time consuming and cumbersome. Sources of resistance to biotic and abiotic stresses are absent in the cultivated germplasm. In addition, crops like ginger and turmeric have no or very rare seed set, making conventional breeding programmes ineffective. Under such circumstances, developing high yielding and disease resistant varieties through biotechnological programmes has an important role to play for spices improvement and the future spice trade. Genetic improvement of plants for resistance to pathogens requires discovering resistance genes and understanding the inherent mechanisms involved. For this purpose, the development of a

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genetic transformation procedure would help to validate the role of discovered genes.

### ***Current status of transgenic spice crops***

#### ***Black pepper (*Piper nigrum* L.)***

Black pepper, the 'king of spices', is one of the major export earning crops. Preliminary attempts at transformation in black pepper were attempted by Sasikumar and Veluthambi (1994, 1996) and primary transformants were obtained for kanamycin resistance in the cotyledons using *Agrobacterium tumefaciens* binary vector strains LBA 4404 and EHA 105. The optimum concentration of kanamycin concentration (50 µg ml<sup>-1</sup>) to completely inhibit callus formation and growth was also standardised. Sim et al. (1998) reported *Agrobacterium* mediated transfer of GUS to black pepper. But regeneration of the transformed tissue is not reported.

Reports are available on optimization of *Agrobacterium* mediated genetic transformation system in black pepper and related *Piper* species aimed towards development of disease resistant varieties against the most dreaded disease, foot rot, caused by *Phytophthora capsici*. Babu et al. (2005) successfully transformed black pepper leaf tissues with osmotin gene, a Pathogenesis-related (PR) protein known for inducing *Phytophthora* resistance. *Agrobacterium* mediated transformation was attempted using osmotin gene construct in pGV2260. Among the 70 putative transgenics regenerated, five putative transgenics showed delayed response to infection and decreased spread of foot rot caused by *Phytophthora capsici*.

Asha and Rajendran (2009) reported *in planta* transformation in black pepper variety Panniyur 2 via pollen tube pathway using the total exogenous DNA of *Piper colubrinum*, a wild relative species of *Piper* resistant to *Phytophthora capsici*. The resulted putative transformant seeds were germinated *in vitro* by embryo rescue and the

germinated plantlets were screened *in vitro* by incorporating the toxic culture filtrate of the pathogen *P. capsici* in the rooting media. The surviving putative transformant plantlets were later screened artificially for disease tolerance under *ex vitro* conditions. 39.21% of the putative transformants survived the screening and RAPD analysis of these plantlets showed variation in banding pattern compared to the DNA recipient parent *P. nigrum* variety Panniyur-2.

Genetic transformation is an excellent tool to study gene function; vacuum infiltration method is used to transform oomycete- resistant wild *Piper* sp., *Piper colubrinum*, as a rapid transient method for expression of GUS (β-Glucuronidase) reporter gene and introduce hairpin vector for endogenous gene silencing (Mani and Manjula, 2011). *Agrobacterium* (EHA 105) harbouring GUS binary vector were vacuum-infiltrated into young detached *in vitro* leaf explants, which showed detectable GUS gene activity within 4 days of infiltration. They also reported the application of transient gene silencing in *P. colubrinum* by the delivery of *in vitro* synthesized hairpin vector construct (pHELLSGATE) containing endogenous serine threonine protein kinase (STPK) gene homologue into *in vitro* shoots which resulted in significant reduction in transcript accumulation of the endogenous gene.

Genetic transformation in black pepper showed retarded regeneration potential from mature tissues due to associated problems like high phenolic exudation and presence of endophytic fungi. A very efficient micropropagation strategy through somatic embryogenesis developed by Nair and Gupta (2003) is promising for rapid regeneration of transformed tissues which can ease genetic manipulations of black pepper.

Embryogenic mass derived from primary somatic embryos that were obtained from the micropylar region of mature germinating seeds of black pepper were used by Jiby and Bhat (2011) for efficient *Agrobacterium*-mediated transformation of black

pepper plants. Embryogenic mass co-cultivated with *Agrobacterium* carrying the  $\beta$ -glucuronidase (GUS) reporter gene was cultured on plant growth regulator-free Schenk and Hildebrandt (SH) medium and transformants were selected in medium containing cefotaxime and step-wise increase in kanamycin concentration.

The utility of Coat protein of Cucumber mosaic virus (CMV) gene (Bhat et al., 2005) in inducing virus resistance in black pepper is being studied using transgenic pathway. Gene constructs were prepared in plant transformation vector (pBI 121) and mobilized into *Agrobacterium*: (i) *Cucumber mosaic virus* coat protein (CMV-CP) in sense and antisense orientation, (ii) Portion of open reading frame (ORF) III of *Piper yellow mottle virus* (PYMoV) in sense and antisense orientation. Putative transgenics developed in black pepper (IISR Subhakara and Panniyur 1) are under evaluation (IISR, 2011-2012).

Maju and Soniya (2012) established genetic transformation system for *Piper nigrum* L. var. Panniyur-1 plants by infecting seedling derived explants with *Agrobacterium tumefaciens* strain EHA105 carrying binary plasmid pCambia 1301, which contains scorable marker,  $\beta$ -1,3-glucuronidase and selectable marker hygromycin phospho-transferase gene (hpt) under the control of CaMV 35S promoter.

#### *Cardamom (Elettaria cardamomum* Maton.)

Cardamom, (Zingiberaceae), the 'Queen of Spices' is also native to India and the productivity of cardamom is hampered by various diseases of viral etiology.

A preliminary study on transformation of cardamom was attempted using biolistic process to study the optimum conditions for gene delivery and the efficiency of the plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) for transformation and gene expression in cardamom embryogenic

callus. Transient expression of GUS gene was noticed in the bombarded callus tissue (Babu, 1998).

In order to develop pathogen derived resistance in cardamom, Backiyarani et al. (2005) reported cloning of coat protein gene of Kursuppara isolate, a highly variable isolate of *Cardamom mosaic virus* (CdMV) infecting cardamom. The PCR amplified coat protein and coat protein + 3' UTR region were separately cloned in pXcmkn 12 vector and subcloned into a plant expression vector (pAHC17) at the *Bam* H1 site, under the ubiquitin promoter. Developing virus resistant lines using coat protein genes through transgenic pathway may help in mitigating the viral problems.

#### *Ginger (Zingiber officinale* Rosc.)

Ginger, (Zingiberaceae) is the third most important spice originated in South Asia. Since ginger is vegetatively propagated, the genetic variability is very much limited which is a hurdle in crop improvement programmes to develop varieties resistant to rhizome rot caused by *Pythium aphanidermatum* and bacterial wilt caused by *Ralstonia solanacearum*.

Transient expression of GUS was successfully induced in ginger embryogenic callus bombarded with plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) callus tissue. (Babu, 1997; Babu et al., 1998). Helium bombarded ginger embryogenic calli with microprojectiles (1.6  $\mu$ m gold particles) using 'BioRad' PDS-1000/He gene gun at 900 and 1100 psi helium pressure with the target distance of either 6 or 9 cm. The pAHC 25 vector used contained GUS ( $\beta$ -glucuronidase) and BAR (phosphinothricin - acetyl transferase) as reporter and selectable marker genes respectively. The best GUS score was obtained when the target distance was 9 cm with 900 psi helium pressure. The GUS score of 133 blue spots per cm<sup>2</sup> indicated not only the optimisation and efficiency of the biolistic process, but also the ability of the ubiquitin promoter to drive the expression of the reporter gene.

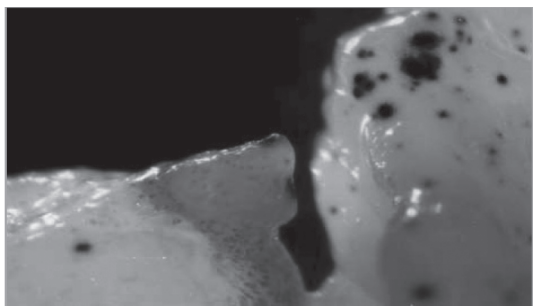


Figure 1. Transient expression of GUS in ginger embryogenic callus using p AHC25 vector

Suma et al. (2008) reported the genetic transformation of ginger buds through somatic embryogenesis. They found that young buds had very good embryogenic potential and were superior to other explants. The transformation protocol included three day pre-culture of explants on callus induction medium, bacterial (*Agrobacterium tumefaciens* strain EHA105/p35SG) dilution of 1:20 (v/v) as the initial inoculum, an infection time of 5 minutes, two day co-cultivation with *Agrobacterium* and post cultivation on callus induction medium with 100 mg L<sup>-1</sup> kanamycin and 300 mg L<sup>-1</sup> cefotaxime under darkness for two weeks, followed by a 16/8 h photoperiod regime. Acetosyringone was effective at a concentration of 200 µm for *vir* induction. With young bud as explant, a transformation frequency of 1.1 to 2.2% was noticed. The callus growth was very slow in the presence of antibiotics.

#### *Turmeric (Curcuma longa L.)*

Turmeric is conventionally propagated vegetatively through mother or finger rhizomes with one or two buds. It is reported to set seeds rarely in some locations and scarcity of seed set hampers recombination breeding. Good amount of morphological variability is observed in many cultivated types of turmeric, which is mostly due to vegetative mutations or due to environmental effects. In such circumstances biotechnological tools gain relevance in solving many crop specific problems and for crop improvement.

An efficient protocol for genetic transformation for turmeric through particle bombardment was reported by Shirgurkar et al. (2006). 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 and transgenic lines were established on selection medium from 50% of the bombarded calluses. Transgenic shoots regenerated from these were multiplied and stably transformed plantlets were produced. PCR and histochemical GUS assay confirmed the stable transformation. A protocol for regeneration and genetic transformation was established in *C. alismatifollian* using retarded shoots as explants, and employing using *A. tumefaciens* strain AGLO harbouring binary vector pBI121 or pBI121-Ca-ACSI. Transformation events were confirmed by PCR, GUS assay and southern blotting of regenerated plants (Mahadatanapuk et al., 2006).

#### *Chilli (Capsicum annuum L.)*

*Capsicum spp.*, consumed both as vegetable and spice is of high economic importance due to distinctive sensory attributes of aroma, pigments and taste. Cultivated peppers are affected by biotic and abiotic stresses, prominent being viral, bacterial and fungal diseases. Fruit colour, pungency and male sterility are interesting genetic characters of capsicum with high economic importance.

Genetic transformation of peppers poses constraints such as low transformation frequency, problems associated with shoot elongation, genotype dependency, inconsistency and non-repeatability of protocol (Brummell and Pathirana, 2007). Virus resistance in pepper has been achieved through satellite RNA genes or coat protein mediated approach. Dong et al. (1995) generated two transgenic pepper lines by transforming cotyledonary petioles of pepper cv 89-1 with *Agrobacterium* containing plasmid with an unspecified CMV satellite RNA gene under the

control of CaMV 35 S promoter. The transgenic lines showed delayed symptoms (from approximate 13 to 24 days) upon virus inoculation. Prior to that, Lee et al. (1993) used a cDNA transgene of CMV<sub>17N</sub> satellite RNA under the control of 35 S promoter for transforming cotyledonary explants of hot pepper var. Golden tower which resulted in four independent transformants. Zhu et al. (1996) and Lee et al. (2004) transformed capsicum peppers with CMV and TMV coat protein genes. Cai et al. (2003) also generated transgenic chilli pepper plants with combined coat protein genes from both TMV and CMV by transforming the hypocotyls explants.

Over expression of transcription factor genes have been used to transform pepper plants as a means for imparting broad spectrum resistance. Shin et al. (2002) over expressed tobacco Tsi1 gene in transgenic hot pepper plants with a 35S::Tsi1::nos construct and 15 primary transformants were generated. Pathogen challenge of plants of the T1 generation showed reduced multiplication of TMV and CMV virus, reduced incidence and severity of infection by the oomycete pathogen *Phytophthora capsici* (late blight) and slightly reduced accumulation of the bacterial pathogen *Xanthomonas campestris* (bacterial spot disease). Similarly PPI1 (pepper- PMMV interaction1, a bZIP transcription factor gene isolated from *Capsicum chinense* was over expressed in hot pepper under the control of 35S promoter (Lee et al., 2004). Use of MADS box genes such as Os MADS1 (MADS box gene isolated from rice) for transformation of chilli pepper by Kim et al. (2001) have shown promise in modifying plant growth habit.

There is only a single report of transgenic hot pepper plants developed for insect resistance from Korea against the major insect pest, the oriental tobacco bud worm prevalent there (Kim et al., 2002). The pepper plants were transformed with *cryIAc* gene under the control of 35S promoter or the ribulose 1, 5- biphosphate carboxylase small-subunit (*rbcS*) promoter from rice to give tissue specific expression in green organs.

The function of ripening related endo-1,4- $\beta$ -glucanase (EGase) gene *CaCell* in fruit softening was investigated by suppression of *CaCell* gene in transgenic pepper plants using constitutive expression of truncated sense *CaCell* gene (Harpster et al., 2002). They used the protocol of Engler et al. (1993) and produced the largest number of pepper primary transformants to date. Out of these 57 transformants, only two lines exhibited strong post-transcriptional gene silencing of *Ca Cell* and undetectable accumulation of *Ca Cell* protein and endo-1,4- $\beta$ -glucanase activity. This work confirmed that *CaCell* alone is responsible for ripening related EGase activity in pepper.

Transgenic expression has been used to study carotenoid biosynthesis in pepper. Deruere et al. (1994 a,b) cloned capsanthin/capsoburin synthase (CCS) involved in the synthesis of red carotenoid pigment not found in tomato and a fibrillin (fib), a structural protein involved in carotenoid deposition in chromoplasts. Capsinoid biosynthesis in transgenic *C. annuum* plants upon *Agrobacterium* transformation using different gene silencing techniques has been reported by Kisaka et al. (2011).

Virus induced gene-silencing approach has been used to study the functional role of resistance gene (CaRGA2) expression against *P.capsici* in *Capsicum annuum*. The suppression of the *CaRGA2* gene renders the pepper plant unable to transduce a signal downstream of the broad-spectrum resistance response, thereby allowing enhanced susceptibility to pepper pathogens (Zhang et al., 2013).

### Garlic (*Allium sativum* L.)

A stable transformation system was developed by *Agrobacterium*-mediated gene transfer using highly regenerative calluses. The temperature and number of days of co-cultivation was found to be an important factor in transient expression of *uid* A gene and its presence was confirmed by southern blot analysis (Kondo et al., 2000). Twenty transgenic plants were regenerated following



introduction of DNA into embryogenic calli of garlic by microprojectile bombardment (Robledo-Paz et al., 2004). *Agrobacterium*-mediated transformation of garlic embryogenic calli was done with tobacco chitinase and glucanase genes for tolerance to the fungus *Sclerotium cepivorum*. Thirty putative transgenic clones were obtained from inoculated calli after six months. Transformed plants were not completely resistant but showed a delayed infection (Lagunes-Fortis et al., 2013).

#### *Vanilla (Vanilla planifolia)*

Genetic transformation in *Vanilla planifolia* using thin shoot-tip-sections under the influence of external incorporation of putrescine was reported by Malabadi and Nataraja (2007) using an expression vector containing nptII and GUS genes driven by CaMv 35s promoter. An efficient transformation protocol using protocorm-like bodies (PLBs) derived from shoot tips was developed for vanilla (*Vanilla planifolia*) by Retheesh et al. (2011). PLBs were co-cultured with *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector pBI121 containing the  $\beta$ -glucuronidase (*gusA*) and neomycin phospho-transferase II (npt II) genes.

#### *Seed Spices*

##### *Cumin (Cuminum cyminum L.)*

India contributes 70% of the total world production of cumin and abiotic stress such as salinity is the major constraint in cumin production. Low genetic diversity attributes limited scope to improve traits in cumin via conventional breeding. Microprojectile bombardment-mediated genetic transformation has been established using precultured cumin embryos by Singh et al. (2010) and 91% of the embryos showed transient GUS expression after 24 hrs. Shoot tips and roots of  $T_0$  plantlets also showed GUS expression after 3 months of bombardment exhibiting possibility of stable transformation in cumin. An efficient method for *Agrobacterium* mediated genetic transformation and plant

regeneration using embryos as explants was reported by Pandey et al. (2013) with transformation efficiency of 1.5% at the hardening stage.

##### *Coriander (Coriandrum sativum L.)*

Wang and Kumar (2004) have developed transgenic coriander plants in an attempt to investigate the role of mutated ethylene receptor ERS1 from *Arabidopsis thaliana* in tissue senescence of heterologous plants. Transgenic coriander was regenerated by co-cultivating hypocotyl segments with *A. tumefaciens* harbouring binary vector pCGN1547 that carried the ERS1 gene. The *Arabidopsis* ERS1 mutant effectively conferred ethylene insensitive phenotype to coriander plants with a transformation efficiency of 6.6%.

##### *Fennel (Foeniculum vulgare L.)*

Mugnier (1988) reported hairy root induction of fennel by inoculating with *Agrobacterium rhizogenes* strain A4.

##### *Fenugreek (Trigonella foenum-graecum L.)*

Hairy root cultures of *T. foenum* have been widely used in production of secondary metabolites. Diosgenin production was established in *T. foenum* by hairy root induction by *Agrobacterium rhizogenes* strain A4 (Merkli et al., 1997). The highest diosgenin production was observed in half-strength woody plant medium (0.040% dry weight) which represents almost twice the amount detected in non-transformed roots (0.024% dry weight). Stolon and its postulated precursors were detected in hairy root cultures of *T. foenum* (Paraza-luna et al., 2001).

*In vitro* crown galls were induced in fenugreek by *Agrobacterium tumefaciens* strain A281 using root, cotyledon and hypocotyl explants and the presence of *uidA* (*gus*) gene was confirmed by polymerase chain reaction (Khawar et al., 2004). The production of the trigonellin by hairy root cultures of *Trigonella*

*foenum* was described in two Iranian masses -Zanjan and Borazjan (Raheleh et al., 2011).

### *Advent of spice genomics*

Initial genomic efforts in black pepper was focused on identification of candidate genes responsible for pathogenesis in black pepper and related *Piper* species. Nazeem et al. (2008) also reported the role of  $\beta$ -1, 3 glucanase and related enzymes in the defense mechanism in foot rot tolerant variety of black pepper 'Kalluvally' and found that the resistant genotype *P. colubrinum*, possessed higher enzyme activities than *P. nigrum* varieties. An attempt to isolate and clone cDNA fragments encoding the defense related protein,  $\beta$ -1,3-glucanase in black pepper (*P. nigrum* L.) and *Piper* spp. was initiated by George et al. (2006), and methyl glutaryl CoA reductase in *Piper colubrinum* by Giriya et al. (2005 a, 2005b).

One of the *Piper* species viz., *Piper colubrinum* is found to be highly resistant to all known strains of *Phytophthora capsici*. But due to sexual incompatibility, hybridisation to mobilise the resistance genes in *P. colubrinum* to cultivated black pepper is difficult. Hence isolation and incorporation of resistance related genes into black pepper genome through transgenic pathway is the alternate way. Sandeep et al. (2009) has reported differential induction of chitinase in *Piper colubrinum* in response to inoculation with *Phytophthora capsici*. An interspecific hybrid between *Piper colubrinum* and *Piper nigrum* was developed by Vanaja et al. (2008) having partial resistance to the dreaded disease *Phytophthora* foot rot. Tagging and isolation of gene imparting partial resistance to *P. capsici* from interspecific hybrid may be a promising alternative in developing foot rot resistant transgenics in black pepper.

The SSH approach has been chosen by two research groups to generate cDNA libraries aimed at identifying genes involved in defense mechanism involved in host-pathogen interaction. *Piper colubrinum* genes that are differentially expressed

in response to the signaling molecule, salicylic acid (SA) were identified by Dicto and Manjusha (2005). A leaf-specific subtracted cDNA library of *P. nigrum* containing 30% of the clones having homology to metallothionein type-2 homologues has been constructed (Susan et al., 2008). The high levels of expression of metallothionein type-2 in leaves, especially in trichomes is correlated to the secretory function of trichomes to exude excess heavy metals accumulating preferentially in leaves (Garcia-Hernandez et al., 1998) and moreover, leaf trichomes produce secretions that are thought to provide a first line of defense against pests and pathogens (Wang et al., 2002).

Cloning and sequence characterization of two isoforms of *osmotin*, an antifungal PR-5 gene homologue, was done from a salicylic acid-induced subtracted cDNA library earlier generated in *Piper colubrinum* (Mani and Manjula, 2010). The larger form of the gene is 693 bp long, encoding a 21.5 kDa protein. The smaller form comprises a 543 bp long coding sequence which codes for a protein of 16.4 kDa. A notable feature of the smaller form was a prominent internal deletion of 150 bp besides certain point mutations. Cloned isoforms of *osmotin* from resistant species could be candidates for molecular breeding for the improvement of black pepper as well as candidates for the study of structure based mechanism of antifungal activity attributed to PR-5 family (Mani et al., 2012).

Tiing et al. (2012) has reported cloning and characterization of 10 partial resistance gene analogues from *Piper nigrum* L. cv. Semongok Aman and *Piper colubrinum* Link. Root transcriptome (Gordo et al., 2012) and leaf transcriptome (Joy et al., 2013) will be 'resourceful' tools for biotechnological improvement of black pepper.

Nair and Thomas (2006) reported isolation, characterization, and expression of resistance gene candidates (RGCs) using degenerate primers based on conserved motifs from the NBS domains of plant

resistance (*R*) genes from cultivated and wild *Zingiber* species. Nair and Thomas (2007) have provided a base for future RGC mining in ginger and valuable insights into the characteristics and phylogenetic affinities of non-TIR NBS–LRR *R*-gene subclass in ginger genome.

Priya and Subramanian (2008) reported isolation and molecular analysis of *R*-gene against *Fusarium oxysporum* f.sp. *zingiberi* in resistant ginger cultivars. They observed that the CC–NBS–LRR class of plant resistance *R*-gene is present only in the resistant cultivars and these cloned *R*-genes provide a new resource for developing *Fusarium* yellows resistant ginger cultivars.

Kavitha and Thomas (2008a, 2008b) employed AFLP markers and mRNA differential display to identify genes whose expression was altered in a soft rot-resistant accession of *Zingiber zerumbet* before and after inoculating it with *Pythium aphanidermatum*. A few differentially expressed transcript-derived fragments (TDFs) containing defense/stress/signalling group which are homologous to genes known to be actively involved in various pathogenesis-related functions in other plant species were identified. They found that *Z. zerumbet* shows adequate variability both at DNA level and in response to *Pythium*. Nair et al. (2010) identified a member of the pathogenesis-related protein group 5 (PR5) gene family in *Z. zerumbet* that is expressed constitutively but upregulated in response to infection by *P. aphanidermatum*. Isolation of resistance genes from such related species will help in ginger improvement via transgenic approaches.

Indian mango ginger, *C. amada* Roxb., exhibited significant resistance to both *Ralstonia solanacearum* and *Pythium aphanidermatum* proving some promise for developing bacterial wilt resistant ginger (Kumar et al., 2006). Prasath et al. (2011) amplified two putative PR5 like protein genes, *CaPR5* and *ZoPR5* from *Curcuma amada* and *Zingiber officinale*, which encodes precursor proteins of 227 and 224

amino acid residues. *CaPR5* is readily induced by the bacterium in *C. amada*, while *ZoPR5* induction was very weak and slow in *Z. officinale*. Promoter analysis indicates the presence of a silencing element binding factor in *ZoPR5*-promoter, but not in *CaPR5*. Prospective promoter elements, such as GT-1 box and TGTCa, implicated as being positive regulatory elements for expression of PR proteins, occur in the 5'-flanking sequences of the *CaPR5*. A subtractive approach was used to analyse differentially expressed genes in *Curcuma amada* compared to *Zingiber officinale* upon inoculation with *Ralstonia solanacearum* (Prasath et al., 2013).

Nair and Thomas (2013) have isolated full length sequence of *ZzR1* resistance gene from *Zingiber zerumbet* with potential for imparting resistance to soft rot in ginger. Real-time PCR analysis of *ZzR1* transcription in *Z. zerumbet* following pathogen infection demonstrated activation at 3 hpi thus suggesting an involvement of *ZzR1* in *Z. zerumbet* defense mechanism. *ZzR1* gene, showing high homology with other CC-NBS-LRR class of *R* genes, represents a valuable genomic resource in designing strategies for engineering resistance in ginger.

Joshi et al. (2010) reported isolation and characterization of NBS-LRR-resistance gene candidates in *Curcuma longa* cv. *surama*. *R* gene conferring resistance to *Pythium aphanidermatum* was characterized in *Curcuma zedoaria* by Basudeba et al. (2013). Transforming *Curcuma longa* with potential *R* genes is one of the solutions to obtain disease-resistant cultivars in 'golden spice'. Annadurai et al. (2013) reports the presence of novel transcripts related to anticancer and antimalarial terpenoids in the transcriptome of *Curcuma longa*.

### Regulation of GMO's in India

No food products derived from genetically modified spices are currently available in the market. Under current Indian law, GM crops including spices, before commercialization, requires legal approval



from the Genetic Engineering Approval Committee (GEAC), the highest body for GM regulation in India, under the Ministry of Environment and Forest. Efforts to regulate biosafety measures are vigorously made in India. As directed by Govt. of India, Dept. of Biotechnology (DBT), has been entrusted with the responsibility of setting up of National Biotechnology Regulatory Authority (NBRA). Setting of NBRA will require the promulgation of new legislation, namely 'National Biotechnology Regulatory Act' or NBR Act which is now under the consideration of Indian Parliament.

GM crops must go through a risk assessment procedure where they are evaluated in laboratory tests and field trials and must undergo safety analysis. The necessary tests include molecular characterization, compositional assessment and 90-day rat toxicity assays. Agronomic, phenotypic, environmental and allergenicity testing may also be required.

Only in chilli peppers various assessment systems have been developed in terms of food safety. Chen et al. (2003) proved that the fruits from the CMV-resistant genetically modified sweet pepper *Capsicum frutescens* cv. Zhongjiao plants are comparable to those from the non-transgenic plants. Coat (CP) gene of CMV derived from a Chinese CMV under the control of CaMV promoter and NOS terminator was used to transform sweet pepper. When assessed *in vitro* and *in vivo*, no genotoxicity could be detected nor significant differences observed in growth, body weight gain, food consumption, haematology, blood biochemical indices, organ weights and histopathology between rats or mice either sex.

Assays developed by Shim et al. (2007); Song et al. (2007) and Chaouachi (2008) are useful to detect presence of transgenic content in chilli peppers. An immunoassay (ELISA) for the quantitative detection of phosphinothricin-N-acetyltransferase enzyme encoded by the bar gene and qualitative and quantitative PCR analysis based on the detection of the

bar gene using the Ccs (capsanthin–capsorubin synthase) as the endogenous reference gene in genetically modified chilli pepper tolerant to BASTA™ herbicide was developed by Shim et al. (2007). Real-time PCR analysis for the differential detection and quantification of genetically modified chilli peppers using a  $\beta$ -fructosidase gene as the endogenous reference along with other solanaceous species (tomato, potato, eggplant) will be useful in terms of food safety (Chaouachi et al., 2008).

Kim et al. (2009) studied the gene flow from a genetically modified chilli pepper (*C. annum* L.) containing the CMVP0-CP (Cucumber mosaic virus pathotype 0-coat protein) gene to a non-transformed control variety 'P915' and two commercial F hybrids ('Manidda' and 'Taesdan') over two growing seasons in the field. Gene flow frequency of 17.89% between GM and 'Taesan' chilli pepper were observed at the closest distance (0.5 m) from the central GM plot.

### Future Perspectives

Preliminary work on isolation of genes responsible for biotic and abiotic stresses and agronomically important characters are available in the major spice crops. Candidate genes responsible for pathogenesis can also be identified from sequence information available in the databases, isolated and can be incorporated into promising varieties using transgenic pathway. Wild relatives of the crops within the family or species may be a repertoire of genes for various biotic and abiotic resistance, agronomically important traits etc. Even though breeding programmes involving hybridization to mobilize genes from wild relatives are cumbersome, incorporation of genes through transgenics is an alternative strategy. Due to restricted taxonomic functionality (RTF) of R genes, no R genes have been successfully expressed in a different family. So developing resistant genotypes within the family through transgenic pathway may be an alternative way.

Even though genetic transformation experiments are

now restricted to 'green house' level in the case of spices, it will be a powerful concept, to produce pesticide free spices, high yielding, drought and disease tolerant spice varieties, especially with changing climates of today. But the major concern will be about what effect genetically modified material could have on human health. As of now, research groups dealing with various spice crops have used antibiotic resistant markers to select transformants. The impact of such antibiotic resistant marker genes in altering nutritional values (Phillips, 1994), allergic reactions (Nordlee et al., 1996) are either unknown or untested. Plants engineered to contain virus particles as part of a strategy to enhance resistance could facilitate the creation of new viruses in the environment. The possibility of cross-pollination of genetically modified crops having herbicide and insect resistance with wild species (Hileman, 1999), evolution of superweeds, impact on genetic diversity especially in the native land of spices should be subjected to critical studies before introducing genetically modified spice crops to address fear among public.

Alternatives like cisgenics, intragenics and marker-less transgenics (Afolabi et al., 2005) could be also attempted in spice crops. Cisgenesis brings new possibilities for resistance strategy as stacking of R genes is more easy to handle, avoids linkage drag and moreover cisgenic resistance breeding using wild species is more safe. There are a repertoire of potential candidate genes identified in wild relatives of various spice crops (Dicto and Manjula, 2005; Sandeep et al., 2009; Mani and Manjula, 2012; Nair and Thomas, 2008) and research groups under spices improvement can focus on developing cisgenics in spices. Even though less regulatory measures are expected regarding intragenesis and cisgenesis as it shares the same gene pool as that of conventional breeding, now intragenic/cisgenic crops are regulated as only transgenic plants.

To conclude, *Agrobacterium* mediated genetic transformation and biolistics are dominating among

the various transformation strategies employed in the context of spices transgenics. Plant transformation technologies have been used to study the functional genomics in wild relatives of spices *via* gene silencing with a promise to extend genetic modification in spice crops. Hairy root transformation of various seed spices is promising for secondary metabolite production in large scale. Adoption rate of genetic transformation in spices is at a slow pace due to high regulatory burden of GE technology, market barriers and fear among general public. Development of food safety assessment protocols for detection of transgenic content in the spices and studies on the possibility of gene flow from genetically modified (GM) to conventional plants by the concerned research groups can alleviate the problems. Perfection of current transformation methods and application of new plant breeding technologies, like site-specific mutagenesis, cisgenics and intragenics, breeding with transgenic inducible lines, grafting techniques on GM rootstock and agro-infiltration methods will be of enormous value as tools in genetic improvement of spices against various diseases caused by phytopathogenic fungi, bacteria and viruses to make a commercial impact on the spice industry.

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