Pathway Modulation of Medicinal and Aromatic Plants Through Metabolic Engineering Using *Agrobacterium tumefaciens*

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Abstract

Plants are the most appropriate source of variety of compounds that are useful to mankind as food, fiber, medicines, natural products, industrial raw material, etc. A large number of metabolites (primary and secondary) have been utilized by mankind since many years ago. However, there are certain limitations like overharvesting the natural plant, low amount of metabolite/compound, etc., which have been associated with the availability of natural products/compounds. This common problem has become major hurdles for researchers these days. To address such problems, scientists have moved toward more efficient technique like genetic transformation methods. In today's era, *Agrobacterium tumefaciens*-mediated transformation (ATMT) strategies have shown to be an efficient and most sophisticated technique to understand about modifications, cloning, and diversification in biosynthetic pathways *in planta.* The existing knowledge and many successful achievements in biotechnology sector have facilitated the development of new methods like metabolic engineering to divert the target metabolic flux in transgenic plants.

Keywords

ATMT • *A. tumefaciens* • Biosynthetic pathway • Genetic engineering • Metabolic engineering • MAPs • Metabolic flux

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Abbreviations

ATMT	*A. tumefaciens*-mediated genetic transformation
DW	Dry weight
MAPs	Medicinal and Aromatic Plants
ME	Metabolic engineering
PAL	Phenylalanine ammonia-lyase
T-DNA	Transfer DNA

Contents

1 Introduction

For millennia, significant progress has been made to use plant as an ultimate source of natural products. In highlighting strides to reengineer plant's genome over the past 20 years, genetic manipulation has shown much potential to benefit economies of scale in plant biotechnology sector. Metabolic/pathway engineering is basically an art to reengineer the already existing route in order to retrieve the target flux in enhanced concentration, and at times, accumulation of novel compounds has been reported so far. Plants in their native state comprise a phenomenal feat of metabolic cascades and innumerable diversion of biochemical pathways operated inside them. Although the conventional breeding programs have been proved fruitful that empower crop varieties to withstand biotic and abiotic stresses and to enhance the metabolite content, these breeding strategies are time consuming and laborious. However, metabolic engineering has significantly contributed many advances particularly in genetics/crop physiology and genomics by providing opportunities to alleviate the effect of these stresses and easily combat with factors involved in metabolism. Recent scientific discoveries in the area of ME have opened up new avenues which provide innumerable potential for plant improvement. Advancements in the ME leading to identification, annotation, and characterization of putative genes/enzymes in relation to particular aspect like primary or secondary metabolism have made this technology highly appreciable and feasible as well.

Strategies for diverting the metabolic flux include the engineering of an individual step in a particular pathway in order to enhance/reduce the content of target metabolite/product. In addition, to redirect the flux toward a more predictable aspect, sometimes suppression/arrest/blocking of competitive pathway has been also proved as an exciting tool. Moreover, reducing metabolic flux through competitive pathways overcome the rate-limiting steps in particular biosynthetic routes along with reduction in catabolic reactions, and the overexpression of regulatory genes also proved beneficial and key aspects of genetic engineering [1]. Recently, approaches have been applied to focus multiple genes/enzymatic steps in a particular pathway to control metabolite production. The parameters involved various strategies like the overexpression of enzymes and downregulation of consecutive genes/enzymes in a pathway while terminating those which are involved in competing pathways. This might also involve the various regulation transcription factors (TF) to recognize the multilocus genes which can control over a single and more consecutive pathways operating *in planta*. Some major technical limits, in context to how many numbers of genes can be transferred to a host plant, and new transformation techniques have been developed to transform a plant with multiple transgenes at a time and to express these transgenes in a coordinated manner [2]. The present scenario of paradigm in the biological science of complex cascades in plant system has diverted the current shift toward better, efficient transformation methods such as *Agrobacterium tumefaciens*-mediated genetic transformation systems (ATMT). The ATMT approach, an efficient method through which construct could be made per se, is used to investigate the type and the localization pattern (subcellular, intracellular) of gene of interest. Based on the studies, we here review the potential of metabolic engineering via *Agrobacterium tumefaciens* in the biosynthesis of novel compounds and discuss the prospects for establishing the technology to induce tailor-made synthesis of target metabolic flux. The above described aspects of genetic engineering have been made possible with approaches like biolistics, particle bombardment, but there are lacunas that still exist. The ATMT approach is highly feasible and efficient for these types of manipulation applied *in planta*.

2 Transformation Methods and Strategies

Recent progress has been made over last few years, which have led to the development of an efficient strategy that can be easily accessible with a broad range of agronomically important crops. There are approaches like biolistics (particle bombardment), electroporation, which considerably makes it easy to obtain transient expression at high levels (Table 1). The strategies have been used to check the efficiency of different gene constructs prior to transformation. While transient expression can be used to locate the presence of gene or study metabolic networks

Table 1 Common strategies used for transformation methods

Common methods used for introduction of foreign gene	Type of expression	Compatibility	Delivery system	Advantages	Disadvantages
Electroporation	Highly effective for transient expression	Compatible with every kind host range	High rate of DNA delivery	Very effective	Copy number is very high and therefore leads to gene co-suppression or silencing
Particle bombardment/ biolistic mechanism/gene gun	Highly effective for transient expression	Shows high compatibility with a range of host plant	High rate of DNA delivery	Highly effective	Copy number is very high and therefore leads to gene co-suppression or silencing
Agrobacterium-mediated transformation	Effective for both transient as well as stable expression but stable expression can be achieved in consecutive generations	Host range is limited due to hypersensitivity shown by plants	High rate of DNA delivery	Cheap, effective, and very simple. The technology may be useful in germ line transformation Copy number is often low and therefore high chances of getting transformants	Compatibility problem with a number of plant species especially leguminous species, monocot families, and tree species

in plant and cell tissue culture, it has been determined that the similar genes can also act differently in stable as well as in transiently transformed plants [3]. Transient expression is also used for analyzing the tissue-specific expression of target transgenes.

3 *Agrobacterium tumefaciens*: A Soil-Based Bioengineer in the Era of Advanced Biotechnology

Agrobacterium tumefaciens (scientifically named as *Rhizobium radiobacter*), a gram-negative, rod-shaped soil bacterium from family Rhizobiaceae, is responsible for crown gall disease in over 140 plus species of eudicots, dicotyledons, gymnosperms, and some monocots [4]. During the last years, the crown gall disease was acting as one of the major hurdle in horticultural biosector, as this disease leads to havoc loss in majority of economically important horticultural crops [5] like in grape [6], apple [7], cherry [8], and many more. However, problems like the mode of propagation through grafting of stems and the woody nature of these crops have been acted to be a common issue. The wounds on grafted tissue may serve as an infection channel and the crown gall disease takes place. In 1941 White and Braun observed that only a minute exposure of *A. tumefaciens* to wounded tissue may transform it permanently [9]. Soon after, tumorigenicity was proposed to be transferred through *A. tumefaciens* to host tissue [10, 11]. Further, during 1974, it was identified that the capacity to induce crown gall disease is inherently carried by Ti plasmid of *Agrobacterium* [12, 13]. However, in particular, the molecular characterization techniques like southern hybridization has led to the discovery that Ti plasmid transferred its DNA into the host plant cell and resulted in the origin of T-DNA from *A. tumefaciens* to the host plant cell [14–16]. The Ti plasmid consists of T-DNA which delimits by 25 bp direct repeats sequences a.k.a. T-DNA border sequences. This T-DNA from Ti plasmid gets transferred and integrated in the host plant where it encodes two enzymes, and results into the production of a massive amount of auxin and cytokinin lead to the uncontrolled cell proliferation called crown gall disease. In addition, the production of some low-molecular-weight compounds like amino acid and opines (sugar phosphate derivatives) could be metabolized specifically depending upon the *Agrobacterium* strain. Thus, *A. tumefaciens* strain is solely responsible for the production of different types of opines and ultimately creating an ecological niche which suits to a particular strain of *A. tumefaciens* [17, 18]. The formation of tumor in the host plant's cell is the resultant of T-DNA integration, and this factor has become an intrinsic interest of researchers/scientists for strategic engineering of the plant's genome. The same property of T-DNA has been used widely nowadays to insert a foreign gene of interest for target manipulation without showing any adverse effect in the host plant. The elucidation of the fact that a wild/normal type of T-DNA region can be easily replaced by desired sequence has made *A. tumefaciens* stand out of the queue of other delivery systems. Furthermore, the remarkable feature of stable integration of T-DNA inside the host plant cell has inspired the promise that *A. tumefaciens* may utilize as gene vector (either in

homologous/heterologous system) in order to deliver target DNA sequence in the host plant cell.

In the early of 1980s, it was demonstrated that *A. tumefaciens* can be used as an efficient gene delivery system to produce a transgenic plant owing desired characteristics [19]. In addition, the transgenic plants have the ability to transmit the disarmed Ti plasmid to their progeny during the course of segregation, so less chances of a loss of desired gene. Apart from such attractive possibilities, the use of an antibiotic selection strategy makes the system efficient for the accurate selection of probable transgenic plants [20]. The eventual progress of using *A. tumefaciens* as a gene delivery vector for crop improvement has made it possible to use efficiently in the plant biotechnology sector, and the future began to look bright. During the 1990s, a monocot species, maize, was successfully transformed *by A. tumefaciens* [21]. In today's era, successful attempts have been made to transform horticultural cultivated flora and agronomically important species [17]. Recently, important plant species like soybean, canola, cotton, corn, potatoes, etc. have been transformed by *A. tumefaciens*, and improved varieties were released and successfully grown all over the world [22]. At present, apart from plant species, other organisms such as fungi, yeast, and mammalian cells were shown to be susceptible to ATMT [23]. However, nowadays the shift of plant molecular biology has been shifted toward more advanced experimentation which includes the elucidation of various aspects of molecular and biochemical mechanism/changes of T-DNA complex targeting the plant nucleus. Hence, based on the above presumed facts and present scenario, on how efficiently the *A. tumefaciens* could be used for genetic and metabolic manipulations, it can be considered as a bioengineer in advanced biotechnology.

4 An Overview on Expression of Transgene

For the perfect modification of target metabolite profile in respect to the utility of the particular plant, the expression of the gene of target enzymes or proteins has to be fine-tuned in a justified manner. For an appropriate expression of transgene in the host plant, the transcription factors need to be well identified. In context to the location and expression of metabolite, the role of promoters has been known to contribute significantly in engineering the specific host for a particular target. High level of transient expression can be obtained, but to get relatively stable transformed plants is not an easy task. Therefore, plant-specific promoters could be used to achieve a high-level as well as stable expression too. Promoters in metabolic engineering can be divided in three categories: constitutive promoters, organ-specific promoters, and inducible promoters.

The most commonly used constitutive promoter in plant genetic engineering is CaMV 35S promoter (cauliflower mosaic virus 35S) [24, 25]. In plant vectors the CaMV 35S promoter has been used extensively and is known to consist of more than one third of the full length sequence [26]. This promoter is of viral origin and has been thoroughly characterized in metabolic engineering of cell cultures and is able to

drive a high level of transgene expression in plant tissues [27, 28]. It has been interpreted that partial duplication from −343 to −90 base pair region of promoter can amplify the expression of the target gene up to tenfold [29]. As, in context to the fact that, CaMV 35S is of viral origin so, could be able to drive a high level of transgene expression *in planta.* However, in contrary, sometimes it can also be resulted into deleterious effects like co-suppression of gene which ultimately lead to gene silencing [30].

On the basis on the type of expression, there are various types of promoters used to target the expression of the gene in a specific manner like organ/tissue/developmental stage. These kinds of tissue-/organ-specific promoters have been characterized at large level in plant engineering system. The respective application of these types of promoters will not hamper the normal plant growth and development. However, at times, it can also lead to the synthesis of desired or value-added novel compounds. The use of the inducible promoter's strategy [31] is highly efficient in order to examine the resultant effect of expression of the transgene in complex biosynthetic routes. For example, pristinamycin is a polyketide antibiotic which is not found in plants normally used to remove the bacterial infection/contamination in the bioengineering of cell cultures.

Likewise, safeners are known as "herbicide antidotes" and the exposure to the safener would lead to the activation of In 2–2 promoter in shoot and root tissues. Safener can be used in the treatment of seed to control the weeds during the planting of material. The use of safener would also be useful in seed bioengineering or in case the expression of transgene is required for a small period of time after germination. In addition, by selecting an appropriate promoter, it could be possible to redirect the transgene expression toward an appropriate need [32] (Table 2).

5 Engineering Primary Metabolism Using ATMT

5.1 Modulation in Primary Metabolites and Aspect of Engineering *In Planta*

The importance of modulating the primary metabolic pathway is a worthy strategy to provide strength at innate immunity level *in planta* to cope up with certain environmental imbalances and pathogen attack. Basically, the plants depend upon the innate immunity to protect themselves from pathogen attack. This innate immunity depends upon the induced defense responses [51]. The primary metabolic pathway undoubtedly plays an undeniable role in providing the cellular energy requirements which in turn help in strengthen the defense system in plants [52, 53]. The injury from ecological stress like pathogen attack, freezing, drought, and flooding often seems to be linked with the overproduction of free oxygen radicals. Superoxide dismutases (SODs) are a class of metalloproteins known to be actively involved in the detoxification of these free oxygen radicals, by breaking down into hydrogen peroxide and molecular oxygen. An overexpression of SODs was done in alfalfa to combat over winter stress [54]. Likewise, alfalfa is also found to be sensitive to aluminum

Table 2 Different plant-based promoters used in transformation experiments and engineering purposes *in planta*

Promoter	Name	Origin	Reference
Constitutive promoter	35S	Derived from viral origin, well characterized in plant metabolic engineering, highly expressed in plant vascular tissues with less expression in meristems tissues	[28]
	Ubiquitin	Plant origin promoter showing high constitutive expression but developmentally regulated	[33]
	Actin	Plant origin promoter showing high constitutive expression but developmentally regulated. Belongs to multigene family and highly expressed in between the tissues	[34–36]
Tissue/ Organ specific	StMCPI/patatin promoters	StMCPI and patatin promoters drives tuber-specific gene expression StMCPI is independent of hormonal and environmental fluctuations and the latter has high expression in sucrose treated leaves and in tubers	[37, 38]
	B-conglycinin promoter	The promoter regulates embryo-specific gene expression	[39, 40]
	OsGT1 promoter, ZmZ27 promoter, Opaque-2 promoter,	These are endosperm-specific promoters and expression is highly regulated by different developmental stages	[41, 42]
	Lhch3 promoter	A leaf-specific promoter derived from *Arabidopsis* and highly regulated by light	[43]
	Lat52 promoter	Lat52 promoter is a pollen-specific promoter and developmentally regulated with high expression during maturation of pollen	[44]
Inducible promoters	Apase promoter	Phosphatase-inducible expression in roots	[45]
	Safener inducible promoter, In 2–2 promoter	Maize In 2–2 promoter is stimulated by benzene sulfonamide herbicide safener	[46]

(*continued*)

Table 2 (continued)

Promoter	Name	Origin	Reference
	Pristinamycin responsive promoter	The promoter based on recombinant transcription factor fusion between VP16 and repressor of pristinamycin (Pip) transactivating domain of herpes simplex virus	[47]
	Ethanol-inducible promoter, glucocorticoid-inducible promoter, ecdysone-inducible promoter	The model is based upon the interaction between specifically designed transcription factor and inducer that can lead into activation of a synthetic promoter	[48–50]

(Al) toxicity, which leads into small roots and low yield. A multipurpose gene named *malate dehydrogenase* was incorporated into Alfalfa which resulted into the production of acetate, succinate, citrate, oxalate, and malate and with a concomitant increase in Al tolerance [55].

Although interminable progress has been made to enlighten the complex cascade steps underlying the developmental defense pathways, to date very little knowledge has been generated about the actual role and involvement of primary pathways in regulating the plant defense mechanisms. In addition, the linked pathways provide a connecting phase between secondary and primary metabolism as illustrated in Fig. 1. However, it has been well documented that the role of primary metabolism is to provide the elementary compounds and precursors for secondary metabolism routes. Based on the convincing experimental evidences only, we have chosen the modulated/engineered pathway studies involving essential primary metabolites like amino acid, carbohydrates, etc.

5.1.1 Essential Amino Acid Metabolism

Engineering the amino acid production pathway to enhance the accumulation of important amino acids like methionine, lysine, threonine, and tryptophan in fruits, food, and crops has been accomplished successfully [56]. Proline has been considered as osmotic protectant and plays a major role in both biotic and abiotic stresses in plants, so engineering the proline pathway has provided a way to cope up with these stresses *in planta* [57]. Transgenic rice with enhanced lysine content was developed by overexpression of seed stored in the β-phaseolin gene from *Phaseolus vulgaris* [58]. Another example includes the production of essential amino acid AmA1 (*Amaranth Albumin 1*) from *Amaranthus hypochondriacus* which leads to the increase in 2.5–4-fold higher content of methionine, tyrosine, and lysine transgenic potatoes [59].

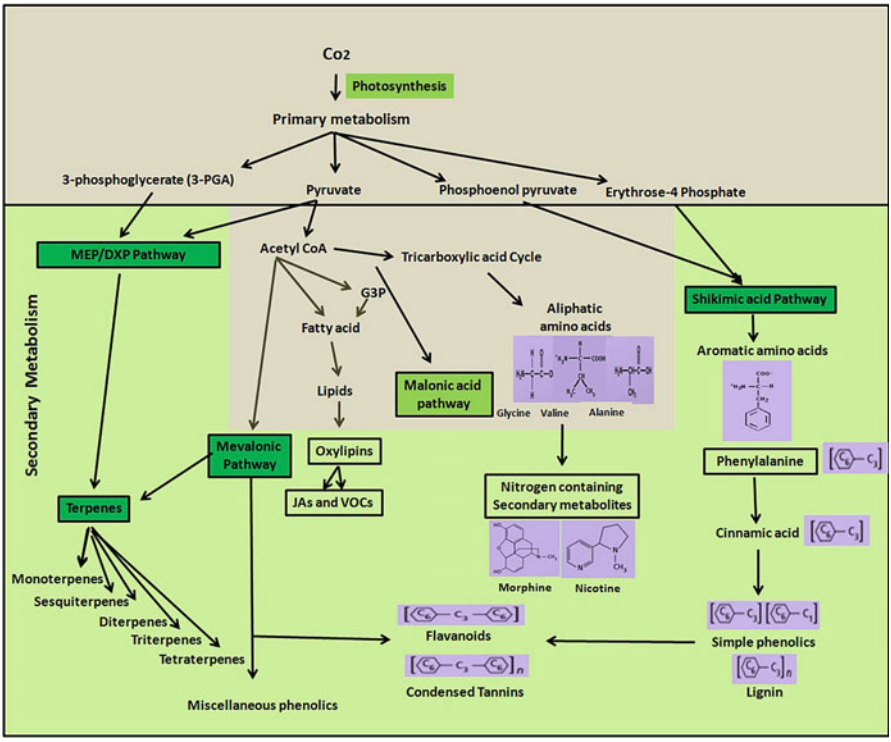

Fig. 1 Interconnection between primary and secondary metabolism *in planta*

In contrast, the lysine content in *Arabidopsis* was either increased by five times through knockout expression of lysine catabolism pathway or twelve times by modulation of bacterial feedback-insensitive dihydrodipicolinate synthase (DHPS) transgene [60].

5.1.2 Carbohydrate Metabolism

In plant kingdom, carbohydrate metabolism involves reversible sequential conversion of sugars into starch and cellulose as photosynthetic products. Starch is an example of stored carbohydrate which accumulates stably in roots, tubers, and seeds while transiently in aerial part like leaves. In the last few years, attention has been given to engineer the starch content and to modify the properties by coordinating relative proportion of amylose and amylopectin content. ME has been used in an attempt to produce novel starch using a heterologous expression of bacterial enzymes by changing the frequency and number of branching chains [61]. Engineering primary metabolite includes enhancing the adenylate pool to increase starch content along with the increase in yield of tubers in potato [62] and overexpression of potato sucrose transporter to enhance the sugar uptake in transgenic pea seeds [63]. Similar experiments have been carried out in rice and wheat species in order to modify the starch content along with seed biomass. A gene

from maize was introduced for modification of ADP-glucose pyrophosphorylase (shrunken 2) which resulted in the production of 20% and 40% of higher seed biomass, respectively, than control plants [64, 65]. More recently, applying an antisense waxy (*Wx)* gene under *Wx* promoter was expressed in transgenic rice which led to production of low-level amylose in rice grain [66], and in contrast, applying the same approach to isoamylase-encoding gene has led to the increase in content of amylopectin in rice grain [67]. Strategies have been applied in sequential coordination of the enzymes/genes involved in the starch synthesis, along within the branches/skeleton in monomer unit. This stepwise engineering has successfully led to the production of high-amylopectin and high-amylose starch contents in transgenic potatoes [68]. A novel method for engineering of starch content has been explored, in which starch-binding domains were used to target recombinant enzymes during starch metabolism [69].

Apart from the engineering of starch, manipulations in cellulose biosynthetic pathway have been done. The cellulose is an essential source of fiber and pulp and will provide a way for value addition in industries. However, in addition, it serves as precursors for many commercially exploited products/polymers. Although the biosynthetic pathway for cellulose has not been fully understood to date, various enzymes/gene have been identified and characterized like Korrigan cellulases, sucrose synthase, etc. These studies led to the determination of consecutive aspects of metabolically progressive underlying mechanism operating *in planta.* Furthermore, the suppression of sucrose synthase belonging to Ces-A family of cellulose synthases has shown the simultaneous inhibition in the accumulation of fiber in transgenic cotton [70]. However, in *Arabidopsis*, dominant negative experiments and antisense approach including Ces-A genes have revealed the role of Ces-A proteins in the formation of primary and secondary cell wall [71, 72].

6 Engineering Secondary Metabolism Using ATMT

6.1 Modulation in Secondary Metabolites and Aspects of Engineering *In Planta*

Secondary metabolites are the outcome/products/compounds derived from secondary metabolism operated in plants. These metabolites are bioactive in nature and known to be actively involved in defense mechanism to overcome the fluctuations in the ecological environment and to protect itself from the pathogen attack. These compounds are produced in minute quantity/as per requirement depending upon the type of pathogen attack. However, being able to cope up with the disturbing ecological parameters and pathogen attack, these compounds are of intrinsic interest of scientists/researchers.

Plant secondary metabolites are categorized into three major groups: terpenes, phenolics, and nitrogen-containing compounds. In the modern era, scientists have often started to engineer the secondary metabolism for their intrinsic interests. Likewise, efforts have been made in engineering the pathways (terpenes, phenolics, and alkaloids) which proved out to be very beneficial for diverting the target metabolite flux as illustrated in Table 3.

Table 3 *Agrobacterium tumefaciens*-mediated genetic transformation (ATMT) resulting in significant alteration in primary and secondary metabolites in plant

Host plant	Gene	Isolated from	Inferences	Ref
Soybean (Glycine max L.)	*Cnx1 gene (GmCnx1)*	*Soybean*	Nitrate reductase (NR) and aldehyde oxidase (AO) were found approximately 2.6–3.9-folds higher than non-transgenic control plants	[90]
Tobacco	*PsnSuSy2 gene*	*Populus simonii* × *Populus nigra*	Increase in the cellulose content and fiber length and secondary cell wall was significantly thicker than normal plants	[91]
Withania somnifera	*Sterol glucosyltransferase (SGTL1) gene*	*Withania somnifera*	Increase in glycosylated withanolide and sterols	[92]
Artemisia annua	*HDR and ADS genes Artemisinic aldehyde Δ11 (13) reductase (DBR2) gene*	*A. annua*	Facilitates higher production of artemisinin content than non-transgenic lines	[93]
Artemisia annua L.	*HMG-Co A reductase gene (hmgr); amorpha-4,11-diene synthase (ads) gene*	*Catharanthus roseus (L) G. Don; A. annua*	Transgenic line was found to contain 7.65-fold higher (1.73 mg/gDW) artemisinin than the non-transgenic plant	[94]
Artemisia annua	*Cytochrome P450 monooxygenase (cyp71av1) and cytochrome P450 reductase (cpr) genes*	*A. annua*	38% higher accumulation of artemisinin content	[95]
Scutellaria baicalensis	*Phenylalanine ammonia-lyase isoforms (SbPAL1, SbPAL2, and SbAPL3) and one gene-encoding cinnamate 4-hydroxylase (SbC4H)*	*S. baicalensis*	The baicalin and baicalein contents in roots were 22 and 107 times higher than those in flowers	[96]

(*continued*)

Table 3 (continued)

Host plant	Gene	Isolated from	Inferences	Ref
Potato cultivar Désirée plants	*StAN11, a WD40-repeat gene*	*Chieftain (Solanum tuberosum L.).*	Dihydroflavonol reductase (DFR) was increased and StAN11 regulated anthocyanin biosynthesis	[97]
Barley cultivar Golden Promise	*Overexpression of cytokinin dehydrogenases (CKX)*	*Barley*	Cytokinin (CK) homeostasis mechanism in transgenic plants	[98]
Vicillin in cultivar Pusa 16	*γ-TMT gene*	*Perilla frutescens*	Elevated level of α-tocopherol	[99]
Panax ginseng	*Overexpression of CYP716A47 and CYP716A53v2 gene from CYP716A subfamily genes RNAi of CYP716A52v2 gene*	*Panax ginseng*	Overexpression of CYP716A52v2 greatly increased the content of oleanane-type ginsenoside (ginsenoside Ro), whereas RNA interference against CYP716A52v2 markedly reduced it	[100]
Atropa belladonna	*H6H gene*	*A. belladonna*	Increase in contents of hyoscyamine and scopolamine in roots, stems, leaves, and fruits of transgenic; content of scopolamine in transgenic line C8 was 2.17 mg × g^{-1} DW that was 4.2-folds of the non-transgenic ones (0.42 mg × g^{-1} DW)	[101]
Withania somnifera	*Squalene synthase*	*W. somnifera*	Fourfold significant increase in squalene synthase activity and 2.5-fold enhancement in withanolide; production of withaferin A was also observed	[102]

(*continued*)

Table 3 (continued)

Host plant	Gene	Isolated from	Inferences	Ref
Eucommia ulmoides Oliver.	*EuIPI gene*	*Eucommia ulmoides*	Three to fourfold increase in the total content of trans-polyisoprenes	[103]
Artemisia annua	*3-Hydroxy-3-methylglutaryl CoA reductase (HMGR) gene*	*Catharanthus roseus (L.) G. Don*	Increase of 22.5% artemisinin content	[104]
Cotton (G. hirsutum)	*Phytochrome B (PHYB) gene*	*Arabidopsis thaliana*	Highly increased in plant growth, with 35% more yield than normal plant. Moreover, decrease in apical dominance and increase in boll size were also seen to contribute in higher yield mass of transgenic cotton	[105]
Transgenic potato (Solanum tuberosum)	*Phytochrome B*	*Arabidopsis*	Greater biomass production, resulting in extended underground organs with significant increase in tuber yields	[106]
Coptis japonicus	*3′-Hydroxy-N-methylcoclaurine 4′-O-methyltransferase (4′OMT) gene*		Improvement of berberine yields (1.5-fold)	[107]
Chinese cabbage	*CYP79B2, CYP79B3, and CYP83B1*	*Arabidopsis*	Accumulation of higher levels of glucobrassicin, 4-hydroxy glucobrassicin, and 4-methoxy glucobrassicin	[108]
Eleutherococcus senticosus Rupr. and Maxim. plants	*PgSS1 gene (a squalene synthase gene*	*Panax ginseng*	Phytosterols (beta-sitosterol and stigmasterol) as well as triterpene levels was increased by 2–2.5-folds	[109]
Solanum tuberosum	*b-1,3-Glucanase class III (Glu-III)*	*Solanum tuberosum* cv. Igor	*In planta* protein production	[110]

(*continued*)

Table 3 (continued)

Host plant	Gene	Isolated from	Inferences	Ref
cv. Desiree and cv. Sante´				
Arabidopsis thaliana	*Fatty Acyl Hydroxylase cDNA*	*Castor Bean (Ricinus communis L.)*	Accumulation of higher amount of ricinoleic, lesquerolic, and densipolic acids in seed	[111]
Arabidopsis thaliana	*SfN8DT-1 gene*	*Sophora flavescens*	Higher accumulation of prenylated apigenin, quercetin, kaempferol as well as 8-prenylnaringenin	[112]
Artemisia annua	*AaPYL9 gene*	*Artemisia annua*	Drought resistance and improved artemisinin content	[113]
Catharanthus roseus	*Deacetylvindoline-4-O-acetyltransferase (DAT) gene*	*Catharanthus roseus*	Increased yield of vindoline in transgenic plants	[114]
Atropa belladonna	*Hydroxylase gene*	*Hyoscyamus niger*	Improved alkaloid content	[115]
Rice	*Dammarenediol-II synthase*	*Panax ginseng*	Synthesis of dammarane-type sapogenin 20(S)-protopanaxadiol (PPD) and dammarane-type sapogenin 20(S)-protopanaxatriol (PPT)	[116]
Bacopa monnieri	*Cryptogein gene*	–	Accumulation of saponin in transgenic plants maximally up to 1.4–1.69%	[117]
Lycopersicum esculentum Mill.	*Stilbene synthase*	*Vitis vinifera*	Biosynthesis of two novel compounds named as trans-resveratrol and trans-resveratrol-glucopyranoside	[118]
Arabidopsis	*Myo-inositol oxygenase and an L-gulono-1,4-lactone oxidase*	*Arabidopsis*	Enhancement of Vitamin C content	[119]

(*continued*)

Table 3 (continued)

Host plant	Gene	Isolated from	Inferences	Ref
Panax notoginseng	*Squalene synthase (SS)*	*Panax notoginseng*	Enhancement in the biosynthesis of ginsenosides, saponins	[88]
Panax ginseng	*PgDDS. Dammarenediol-II synthase*	*Panax ginseng*	Production of a tetracyclic triterpenoid; dammarenediol-II	[120]
Panax notoginseng (Burk) F. H.	*PnFPS, PnSS, PnSE1, PnSE2, and PnDS*	*Panax notoginseng*	Biosynthesis of triterpene saponin	[87]
Kale (Brassica oleracea var. acephala)	*AtMYB12 transcription factor*	*Arabidopsis thaliana*	Several fold increase in both total phenolics content and flavonol accumulation	[121]
Lycopersicum esculentum	*S-linalool synthase (LIS) Gene*	*Clarkia breweri*	Enhancement in aroma and flavor compounds	[81]
Rose	*3',5'-Hydroxylase (F3'5'H), a key enzyme for delphinidin biosynthesis*	-	Accumulation of delphinidin; an anthocyanin results in blue color	[122]

6.1.1 Terpenes

The terpenoids family constitutes the major class of secondary metabolites. The family consists of more than 40,000 natural compounds, constituting both primary as well as secondary metabolites. Many efforts have been made to engineer the terpenoid metabolic pathway *in planta*. Many of the primary metabolite syntheses via terpenoid pathway consist of photosynthesis pigments, plant-derived phytohormones, and ubiquinones which are essential for respiration [73] (Fig. 1). Attempts have been made to produce monoterpenes in transgenic plants; this strategy is easier than to modify the terpenoids containing complexed longer chains [73]. To date, progress have been made to identify the formation, localization, stabilization, and subcellular compartmentalization of the target products as the plant system is highly complexed and consists of specialized structures for proper storage and transportation of volatile and hydrophobic compounds. The plant species which do not contain these specialized secretory structures made it difficult to introduce new pathways [74]. The strategy of overexpression of pathway enzyme in plant species proved to be fruitful in enhancing the desired substrate. Wu and coworkers provided opportunity to encourage the production of pharmaceutically or industrially relevant plants for commercialization at large scale [75, 76].

The biosynthetic pathway is started from acetyl-CoA or glycolytic intermediates. These are synthesized by the fusion of five-carbon isoprene units. Terpenes are produced by condensation of five-carbon elements that may have branched carbon skeleton of isopentane units. The terpenes are also known as isoprene units; at high temperature terpenes may decompose to give isoprene unit. Therefore, the terpenoid group is sometimes called as isoprenoids. The terpene group is categorized on the basis of number of five-carbon units, like monoterpenes (10-C), sesquiterpene (15-C), and diterpenes (20-C). In addition, triterpenes with 30 carbons, tetraterpenes with 40 carbons, and polyterpenoids with [C5]n carbons (where $n > 8$) have also been reported. There are two terpene biosynthetic pathways available *in planta*, i.e., mevalonic acid pathway, in which three molecules of acetyl-coA are condensed in stepwise manner to yield mevalonic acid which is a 6-C compound (the mevalonic acid is then pyrophosphorylated, decarboxylated, and followed by dehydration to produce isopentenyl diphosphate (IPP2)), and the MVA pathway which is known to yield triterpenes and sesquiterpenes which occur in the endoplasmic reticulum [73]. IPP is also discovered to be involved in the formation of intermediates in the carbon reduction cycle of photosynthesis through channeling individual set of chemical reactions known as methylerythritol phosphate (MEP) pathway which occur in the chloroplast/plastid [77, 78]. Larger units are formed via condensation of different sets of carbon ring like 10-carbon compound geranyl diphosphate (GPP), then 15-carbon compound farnesyl diphosphate (FPP), then 20-carbon compound geranylgeranyl diphosphate (GGPP), and finally 30-C compound triterpenes and the 40-C compound tetraterpenes, carotenoids [73]. These two leading pathways have been in focus for metabolic engineering so far [79].

To date, examples of metabolic engineering of monoterpenoid are like (S)-limonene synthase gene from *Clarkia breweri* in petunia resulting in the formation of linalool, which showed repulsion to aphids [80]. So far, the same gene under the control of a fruit-specific promoter encourages the accumulation of linalool in fruits of tomato [81]. Transgenic tomatoes expressing phytoene synthase resulted in the fourfold increment in the production of carotenoid than normal plants. Although, in the case of transgenic tomato expressing phytoene desaturase, no enhancement was observed in the overall carotenoid content, the content of β-carotenoid was threefold higher than the normal plants [82].

Attention has been given to carotenoid biosynthetic pathway, which led to the synthesis of pigments which takes part in photo protection system and light harvesting complex. The carotenoid pathway is started with the coupling reaction of two geranyl phosphate molecules resulted in the production of geranylgeranyl pyrophosphate (GGPP). This GGPP get converted into phytoene in the presence of phytoene synthase enzyme and eventually into Z-carotene in the presence of phytoene desaturase enzyme in a stepwise manner to yield lycopene and finally either into β-carotene (provitamin A) in the presence of β-cyclase enzyme or δ-carotene and then to α-carotene by enzymes ε-cyclase and β-cyclase, respectively

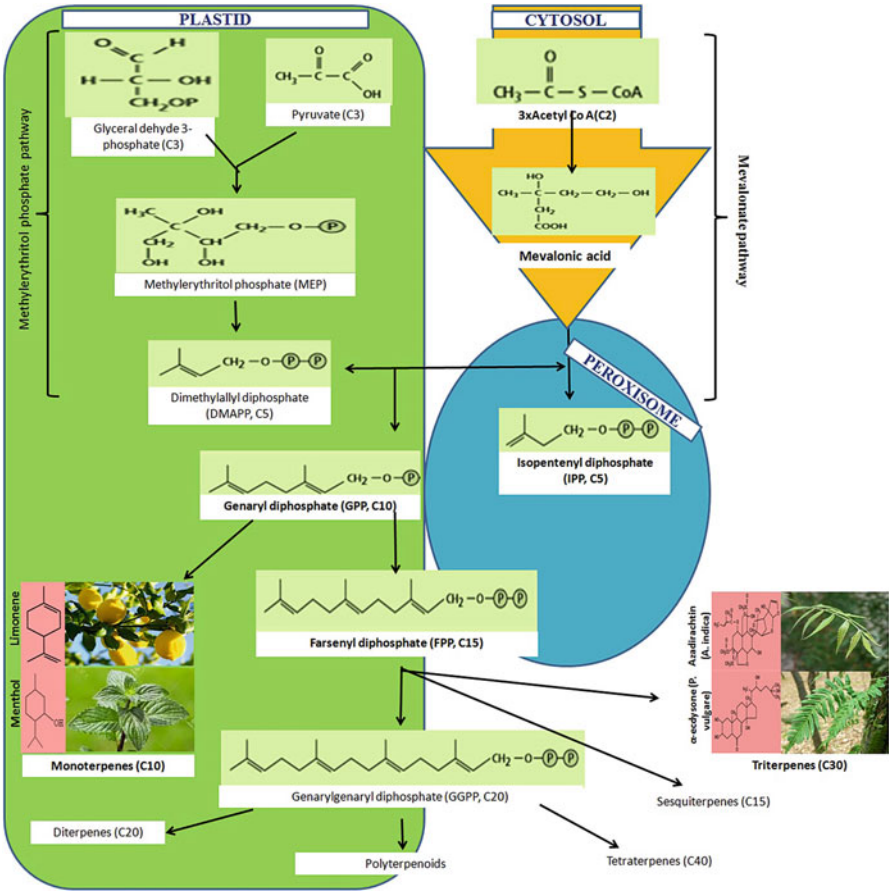

Fig. 2 Basic outline of terpene biosynthetic pathway *in planta*. Biosynthesis of terpenes proceeds via MVA and MEP pathways

[83]. Golden rice is supposed to be the best example which consists of two foreign genes: a bacterial phytoene desaturase and the daffodil phytoene synthase [84]. In addition, the overexpression of mono-/sequiterpene synthase FaNES1 from strawberry in *Arabidopsis* resulted in the production of nerolidol (CIS) derivatives named 4,8-dimethyl-1,3(E), 7-nonatriene [85].

Consistent with the notion, secondary metabolites, synthesis via terpenoid pathway such as monoterpene (C10), sesquiterpene (C15), diterpene (C20), and triterpene (C30), are actively involved in the ecological and physiological advantage to plants (Fig. 2). Basically, terpenes provide resistance to pathogen-related infection and are known to have antimicrobial, antifungal, and insecticidal properties. Terpenes are also having the ability to attract pollinators, repel pests, etc. In addition, apart from the use of terpenoids in flavor, fragrance, and medicines, they also contribute to agricultural sector. The benefit in manipulation in the inherent

characteristic of plant in improvement of aromas and fragrance in horticultural sector and as a beneficial source of cosmetics/pharmaceutical has led the researcher to invest their time to engineer the terpenoid pathway [73]. The strategies for manipulation of terpenoid pathway have been reviewed by many workers [73, 74]. Studies like heterologous expression of genes were also done in order to upregulate/overexpress the desired gene. Likewise, a heterologous expression in tomato results in the reduction of a monoterpenoid which promotes the biosynthesis of undesirable flavor and color in mint species [74] and enhanced the production of aroma in ripening fruit [81]. The manipulation of ketocarotenoid biosynthetic pathway in medicinally important plants like tobacco and tomato species through the heterologous expression of bacterial gene also resulted in beneficial outcomes [86]. For example, tomatoes with β-carotene were further co-transformed with hydroxylase gene and β-carotene ketolase gene [86]. In addition, a crtW gene from bacteria was also introduced in tomato and tobacco leaves, and similarly, ketocarotenoid biosynthetic pathway was observed to be operated in leaves and nectary tissues of tobacco, respectively [86].

Metabolic engineering is not only meant to overexpress/downregulate the particular compound, but it could also provide a platform to enhance the yield of desired product up to various folds. Successful achievements regarding ME were discussed in Table 3. Pharmaceutically important terpenoids like diuretic glycyrrhizin, artemisinin (antimalarial drug from Artemisia), taxol (anticancerous; from *Taxus* species), and perilla alcohol have been isolated from plants. Although various bioactive compounds were derived from endangered species belonging to a threatened environment [76], the synthesis of these compounds by chemical methods is inefficient and also prohibitively costly [75]. Wu and workers have evaluated various factors that required for production of high amount of terpenoids in *Nicotiana* and were able to identify the various strategies for improving the yield up to 1000-fold [75]. Recently, Niu and coworkers have characterized PnFPS, PnSS, PnSE1, PnSE2, and PnDS genes, which are found to be responsible for the accumulation of triterpene saponins in *Panax notoginseng* [87]. However, it is noteworthy that the overexpression of squalene synthase (SS) in the same species leads to the production of ginsenosides [88]. Experiments have been set to manipulate the terpene pathway as a wish that the production of resultant/desired/novel product could be achieved via manipulation/engineering of the terpene synthase genes [89].

6.1.2 Alkaloids/Nitrogen-Containing Compounds

Alkaloid is one of the largest groups consisting of more than 12,000 low-molecular-weight compounds [123]. The nitrogen atom present in these compounds is a part of heterocyclic ring that consist of both nitrogen and carbon atom, which does not have nitrogen in peptide or amide group [124]. Alkaloids are derived from common amino acids, in particular tyrosine, lysine, and tryptophan, while some alkaloids are derived from ornithine, an intermediate in arginine biosynthesis also. Alkaloids are known for their striking pharmacological activities on vertebrate animals. The group consists of biogenically unrelated and structurally diverse molecules. Alkaloids which consist of similar structured compounds are the derivative of related

biosynthetic pathways; however, many alkaloids are thought to be evolved from unique biosynthetic origins [125]. The substrate starting compound decides the type of structural class to which the alkaloid belongs [125]. Amino acids act as the starting precursor molecule for some alkaloid production; however, few are derived from purine derivatives also. Approximately 12,000 known alkaloids are known to have potent biological activities and can be exploited at commercial levels like stimulants, pharmaceuticals, poisons, and narcotics. The alkaloids are majorly known for their role in plant pathogen interaction, as they provide defense mechanism enabling the plants to cope with adverse ecological conditions by binding the cellular targets in antagonistic organism [126, 127]. Emphasis has been given to manipulate caffeine biosynthetic pathway in such a way that three *N*-methyltransferase genes were overexpressed, and the enhancement in caffeine content led to the increased tolerance from pests in tobacco plant [128, 129]. Additionally, in *Nicotiana*, the overexpression of methyltransferase genes from coffee results in 0.2–5 μg of caffeine/g fresh weight of leaf sample [129]. The various biosynthetic pathways/regulatory enzymes/genes involved in the production of alkaloid have been reviewed by Hashimoto and Yamada [130]. Genetic manipulations and characterization involving overexpression/suppression have been done with major emphasis on the key regulatory genes like PMT, ADC, and ODC which are involved in nicotine biosynthetic pathways [131, 132]. An antisense downregulation of berberine bridge enzyme in poppy leads to the reduction of benzophenanthridine alkaloids with concomitant increase in several amino acids [133]. In addition, the production of pharmaceutical important compounds derived from indole alkaloid biosynthetic pathway in *Catharanthus roseus* has always been in focus to overexpress the compounds (vinblastine and vincristine) having anticancer properties and may be applied as a new approach for manufacturing drugs [134]. Despite having various chemical diversities and metabolic complexities in alkaloid biosynthesis, efforts have been made which significantly contribute advancement in revealing the role and importance of alkaloid compounds. Table 3 represents some of the examples of significant metabolic manipulations in alkaloids biosynthetic pathway via ATMT approach.

6.1.3 Phenolics Compound

Large varieties of secondary products are produced in plants that consist of a phenol group. The phenolic compound consists of hydroxyl group as a functional aromatic ring. The group is chemically heterogeneous consisting of approximately 10,000 individual compounds. Phenolics are the derivatives of phenylpropanoid pathway, shikimate pathway, and pentose phosphate pathway via a phenylalanine as a precursor compound [135, 136]. This is an essential pathway in plants which provides the majority of carbon flux and the plant obtained approximately 20% of the total metabolites through phenylpropanoid pathway [137]. The pathway yields majority of product which includes flavonoids, condensed tannins, anthocyanin, lignins, lignans, and other miscellaneous phenolics. The shikimic acid pathway is responsible for the conversion of simple carbohydrate precursors to aromatic amino acids; these precursors are derived from the pentose phosphate and glycolysis pathway

[138]. The majority of secondary phenolic metabolites in plants are derived by the enzyme phenylalanine ammonia-lyase (PAL) which converts the phenylalanine to yield trans-cinnamic acid via the elimination of an ammonia molecule. PAL is perhaps the key enzyme present at a branch point between primary and secondary metabolism and yields majority of plant secondary metabolites. Flavonoids are compounds originated from phenylalanine and known to share a common pathway with lignan and lignin. 4-coumaroyl-Co-A is the first precursor for flavonoid biosynthesis which gets converted into chalcone in the presence of chalcone synthase enzyme. The stepwise conversions lead to the production of a variety of compounds which provide pigments like anthocyanin, phytoalexins (defense compounds), and many regulatory compounds [139]. During the early 1990s, the pathway has been focused for metabolic engineering to transfer multigene to target plants. Flavonoids are phenolic compounds with high antioxidant activity and therefore optioned as a favorite target for metabolic engineering. Manipulations like stacking of anthocyanidin synthase and dihydroflavonol 4-reductase transgenes in *Forsythia* by a series of sequential transformation events have been targeted to utilize the idea of sequential manipulation *in planta* [140].

Recently, strategies were applied to enhance the flower pigments that have contributed successfully to metabolic engineering [141]. In addition, nowadays, overexpression strategy has led to significant results like by overexpressing chalcone isomerase gene from *Petunia* which resulted in 20-fold increase in the flavonoid level in tomato paste and 80-fold increase in the flavonoid content in the tomato peel [142]. The flavonol synthase and chalcone synthase

Genes were also observed to significantly upregulate the flavonol biosynthesis in tomato fruits [143]. *Lycopersicon esculentum* (tomato) with a number of nutritional utilities (like flavonoid content, carotenoid content, ascorbic acid, anthocyanin pigments, etc.) has always been a favorite species for researchers/scientists to engineer these many qualities into quantities through ATMT approach. Some of the significant results were shown in Table 4.

6.1.4 Lignins

Like flavonoids, lignins are the derivatives of phenylalanine and have been classified as phenylpropenes. Lignins are highly complexed polymers which consist of ferulic acid, caffeic acid, and sinapic acid monomers joined to each other. Lignin is an essential component of the cell wall which provides rigidity to the plant architecture. Recently, ME have illustrated the utilization of multiple gene transfer in tree species to modify the lignin content and architecture of the desired plant [176]. Similarly, suppression/downregulation of multiple genes involved in the lignin biosynthetic pathway in tobacco leads to low lignin content without much change in the morphology of the transgenic plants [177, 178]. The transfer of tobacco LIM regulatory protein which is involved in lignification pathway and controls various enzymatic reactions in plant has also been reported [179]. In addition, two Myb-like transcription factors derived from *Eucalyptus gunnii* were expressed in xylem and determined the action sites of cinnamyl alcohol dehydrogenase (CAD) and hydroxycinnamoyl-CoA reductase (CCR) genes that are known to encode stepwise enzymatic reactions

Table 4 Improvement of organoleptic trait/quality tomato using genetic transformation

Character	Gene inserted	Phenotype of engineered fruit	References
Size	fw2.2	Size increased	[144]
Parthenocarpy	IAA9, Arf8	Induction of parthenocarpy	[145, 146]
Flavor	Thaumatin	Improved flavor	[147]
Firmness	β-galactosidase	Enhanced firmness	[148]
	EXPIA	Decreased firmness	[149]
	PME	Shelf life is reduced	[150]
	PG	Decreased softening	[151]
Flavor and aroma	LeAADC2, LeAADC1A	Enhanced/reduced 1-nitro-2-phenylethane, 2-phenylethanol, and phenylacetaldehyde	[152]
Nutritional quality			
Trait	**Targeted Gene**	**Fruit quality**	**References**
Ascorbic acid	ADCS and GCHI	Increase in fruit folate content	[153, 154]
	GME, GalLDH	reduction/enhancement in fruit ascorbic acid content	[155–157]
Soluble solid content	LCY-B, CYC-B	Enhancement in β-carotene and lycopene content	[158–160]
	Crt Y, Crt I, Crt B	Increase in carotenoid content	[82, 161]
	PSY-1	Increase in carotenoid content	[162]
	Dxs	Increase in carotenoid and phytoene content	[163]
	Lin5	Reduction in accumulation of sugars	[164]
Flavonoid	CHI, F3H, CHS, FLS	Flavonoids increased	[165]
	MYB12	Accumulation of flavonols	[166]
	Ros1, Del	High accumulation of anthocyanins	[167]
	STS, FNSII, CHS, CHI, CHR	Accumulation of deoxychalcones, resveratrol, and flavones	[168]
	CHI	Increase in fruit peel flavonol content	[142]
Carotenoid	FIBRILLIN	Increase in volatiles and carotenoid content	[169]
	Spermidine synthase	Increased in lycopene content	[170]
	CHY-B, LCY-B	Zeaxanthin and β-cryptoxanthin	[171]
	COP1LIKE, DET-1, CUL4	Enhanced flavonoid and carotenoid content	[172–174]
	CRY-2	Increase in carotene content	[175]

essential for the synthesis of p-coumaryl alcohol [180]. ME regarding lignin content in plants was beautifully reviewed by Boudet et al., Anterola and Lewis, and Boerjan et al. [180–182]. Moreover, Bell-Lelong et al. have observed that *Arabidopsis* C4H, a lignin-associated promoter, was much efficient than general promoter like

CaMV35S [183]. However, using lignin-specific promoter could prove more effective for manipulations or engineering purposes.

7 Conclusions

In today's era, ME strategies have been made focus to coordinate between a multipoint or multilocus gene to control the metabolic flux through cascading secondary metabolite pathways. The expression of target gene/loci and its localization is now starting to supersede using the specific promoters in aspect of particular compound. As discussed above, various points in a given biosynthetic pathway could be controlled either by using antisense or overexpressing genes/enzymes or via application of the transcriptional regulators to overrule the negative role of endogenous genes/enzymes. Recently, the era has moved forward in the area of ME by using various advanced approaches like proteomics, genomics, and metabolomics in order to expand the knowledge of pathways and routes *in planta*. The production of Golden rice has changed the global view and led the researchers/scientists to think and focus on using the *Agrobacterium* as a natural genetic manipulator so far. Hence, using ATMT strategy can help in multilocus genetic manipulation and remodeling the genome of target plants.

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