Chapter-I
CHAPTER – I

GENERAL INTRODUCTION

1.1 Plant Metabolic Engineering

Plants are a vital source of nutrients and secondary metabolites that are used as pharmaceuticals, agrochemicals, biopesticides and food additives apart from they being the major source of carbohydrates, proteins, fats for food and shelter. Due to the increase in world population, there is a tremendous hassle on the available cultivable land to generate food and fulfill the needs wherein; modern technologies have been developed leading to plant improvement for better utilization of the land to meet the requirements (Rao and Ravishankar, 2002). Desired compounds are obtained by genetic manipulation of cells, tissues, organs or whole organism by the advanced tools in biotechnology. Recent advances in metabolic engineering have made it possible to not only increase the concentration of desired compounds, but also to introduce the novel biosynthetic pathways to a variety of species, allowing for enhanced nutritional or commercial value. To improve the metabolic engineering capabilities, new transformation techniques have been developed allowing for gene specific silencing strategies, stacking of multiple genes within the same region of the chromosome. The improvements in molecular biological research have given a new dimension to *in vitro* culture as well as for plant improvement, enhancing the yields of the products or producing novel products both in quantity and quality from genetically engineered plants.

One class of targets for nutrient engineering are the metabolic pathways that produce phytoalexins, flavonoids, and other molecules that are thought to play a role in the chemopreventive properties of vegetables and fruits. A different approach would be,
to express the pathway for a health-promoting molecule in a same/new host. With the advent of advanced molecular tools, plants could be genetically improved for high nutrient content, nitrogen fixation, biofuel production, photosynthetic efficiency and can also be used as bioreactors for production of biopharmaceuticals (Lau et al., 2014). Golden rice provides classic example of this strategy where vitamin-A content was increased along with the production of beta-carotene in the edible endosperm region (Paine et al., 2005). Metabolic engineering for the improved synthesis of plant natural products offers significant promise for decreasing production costs for commercial compounds and increasing the nutritional value of food crops. One of the major limitations to metabolic engineering of these systems is the lack of fully elucidated plant biosynthetic pathways. To accomplish this, three important classes of technologies namely transcriptomic (Usadel and Fernie, 2013) and metabolomic (Oksman and Saito, 2005) analyses; genome editing tools like TALENs (Joung and Sander, 2013), CRISPR/Cas 9 (Joung and Sander, 2014); synthetic biology parts such as promoters, transporters, multi-gene expression constructs and biosynthetic enzymes (Engler et al., 2014) when taken together, would enable the manipulation of plant metabolism at an extraordinary level, promising to decipher basic knowledge of plant metabolism into tangible benefits for agriculture.

1.2 Onion (*Allium cepa*)

Nature has a hand down on mankind with numerous herbs and herbal resurgence is budding from all corners of the globe. Certain substances present in medicinal herbs are recognized by the current and primeval civilizations for their remedial properties. Herbal medicines have turned out to be the most universal in recent past due to their less
adverse and non-toxic effects on mankind (Nath et al., 2010). Naturally occurring plant metabolites are the key resource for most modern medicines, where the products obtained will be tailored using the vital element as escort compound (Farooqi and Kumar, 2003).

Onion is one of the most important commercial condiment vegetable grown and consumed not only in India but also all over the world. It is the oldest cultivated crop and the pungent edible bulb of the lily family considered as a food of exceptional value for flavoring and seasoning. Onions are perennials, where the fleshy bulb that grows below the ground is used medicinally as well as for food. The green stems and leaves are hollow and can reach up to 3 ft (1m) in height. The plants bear small flowers that are usually white or purple. The onion is a hardy, bulbous, biennial plant, usually grown as an annual. It has superficial root system, a very short flattened stem at the base of the plant, which increases in diameter as growth continues. The leaves of the plant are long, linear, hollow, and cylindrical. Thickening of the leaf bases forms a bulb, when the plant reaches a certain stage of growth (Farooqi and Kumar, 2003).

The most important properties of onion embraces on antioxidant, anticancer, antimicrobial, asthma, cardiovascular compounds like sulfur, organo-sulfur, calcium and riboflavin from onions have a range of health benefits such as anti-carcinogenic, anti-platelet, anti-thrombotic, anti-asthmatic, anti-diabetic, fibrinolytic and hypo-cholesterolemic properties and other various biological actions including antibiotic effects. Though copious literature is available on onion and its curative effects on diabetes, thrombosis, cardiovascular and respiratory problems, it seems insufficient. Thus, more research has to be delved in, to comprehend the therapeutic and salutary effects of onion bulbs.
1.2.1 Origin and distribution

Onion is believed to have originated in Central Asia, perhaps in the Iran-Pakistan region. It has been cultivated since ancient times in the Middle East and India. It was a popular food in ancient Egypt, where it is depicted on tombs as early as 3200 BC and has been found in mummies. The Sanskrit equivalent for onion is “palandu”, which has been mentioned in the Garuda Purana. The great Indian sages, Maharishi Atreya and Lord Dhanwantri have described the use of onions in detail. It is referred to in the Bible and the Koran and also mentioned in the literature from Hippocrates, 430 B.C. down to present time. It derived its name from the Latin *Onio* and French *Oignon*.

Onion was introduced into the new world shortly after its discovery, and cultivation started as early as 1629. It is now distributed throughout temperate regions of the world including Europe, Asia, North America and Africa (Khare, 2002). It has several vernacular names such as onion in English, Cyvannulli in Malayalam, Erragadda or Ulligadda in Telugu, Vengayam in Tamil, Niruli in Kannada, and Pyaj in Hindi.

1.2.2 Nutritive value and composition

Onion has been described as the dynamite of natural foods. The outstanding characteristic of onion is its pungency, which is due to a volatile oil known as allyl-propyl disulfide. It contains vitamin B, trace of vitamin C and also traces of iron and calcium. Onions when compared with other fresh vegetable are relatively high in food energy, intermediate in protein content and rich in calcium and riboflavin. There is substantial disparity in composition between different varieties and it also varies with phase of mellowness and the length of storage. Onion has been accepted as an important source of valuable phytonutrients as flavonoids, (FOS) fructo-oligosaccharides and thio-sulphinates.
and other sulfur compounds (Slimestade et al., 2007). An investigation of a mature onion shows 86.6% moisture content, 1.2% protein, 0.1% fat, 0.6% fiber, 0.4% minerals, 11.1% carbohydrate principally in the form of sugars per 100gms of edible portion. Apart from calcium and riboflavin as mineral and vitamin, it also has phosphorus, iron, carotene, thiamine, and niacin in pocket-sized quantities. Its calorific value is 51.

1.2.3 Medicinal virtues

Onion is one of civilizations oldest medicines. It was apparent in early Mesopotamia to heal virtually every ailment. The physicians of primordial Egypt prescribed onions in various diseases. Dioscorides in the first century A.D. attributed many herbal remedies to them like stimulant, diuretic, expectorant and rubefacient. Onions should be taken with meals, preferably raw, as fried or cooked onions are comparatively difficult to digest. For therapeutic purposes, it is advisable to use onion juice instead of the whole onion, as it is an all-round medicine (Bakhru, 2011). The Allyl propyl disulfide and chromium present in it decrease fasting blood glucose levels; perk up glucose tolerance and lower insulin levels. Onions may be principally beneficial for women, who are at augmented risk of osteoporosis during the menopause as the compound gamma-L-glutamyl-trans-S-1-propenyl-L-cysteinesulfoxide (GPCS) inhibits the osteoclasts (the cells which break down bone) activity and fights osteoporosis (Sampath et al., 2010). Onions are also optional treatment for edema owing to their diuretic effect and its syrup is useful in extracting renal stones.

Interest in the potential health benefits of Allium includes antibiotic effects (Augusti, 1996; Briggs et al., 2001; Slimestade et al., 2007). Allium plants, which include onion, exhibit antibiotic activity against both Gram-positive and Gram-negative bacteria.
 Numerous in vitro, animal, and epidemiological studies indicate that onion or onion extract prevents cancer including gastrointestinal cancer, ovarian cancer, and skin cancer (Gonzalez, 2006; Troll, 1989; Wargovich, 1988). Onion has been experimentally documented to possess anti-diabetic potential. In a clinical study of alimentary hyperlipidemia, onion and onion essential oil prevented fat-induced increases in serum cholesterol and plasma fibrinogen and decreases in coagulation time and fibrinolytic activity. In animal studies, ingestion of onion significantly inhibited bone resorption (Morselli et al., 2000; Muhlbauer et al., 2002; Wetli et al., 2005). A meal of fried onions or a meal of fried onions with fresh cherry tomatoes increased resistance of lymphocyte DNA-to-DNA strand breakage. In pharmacologic and in vitro studies, onion and onion extract, alone and in combination with other products, have shown haemostatic effects including inhibited platelet aggregation, reduced plasma viscosity, decreased hematocrit, and increased fibrinolytic activity (Agarwal et al., 1997; Kalus et al., 2000). In a clinical study of subjects with arterial hypertension, an onion-olive oil maceration product significantly decreased systolic blood pressure and also a trend towards a decrease in diastolic blood pressure (Mayer et al., 2001).

### 1.2.4 Health benefits of onion

Onion, an exceptionally strong antioxidant is full of plentiful anticancer compounds. It has been particularly allied to inhibit stomach and intestinal cancers, thins the blood, lowers cholesterol, raises good-type HDL cholesterol, and wards off blood clots. The leaves of the plant are aphrodisiac, anti-spasmodic, anti-helminthic, alterative, carminative, digestive, diuretic, emollient, expectorant, mild laxative, stimulant and tonic. Onion possesses pain-killing property. It is beneficial in the treatment of eye when its juice is mixed with
honey. It is a valuable medicine for suppressing pain resulting from piles by consuming it
daily or by applying an ointment made of onion, turmeric and Indian hemp in hot sesame
oil (Bakhru, 1996).

Onions are known to contain anthocyanin and flavonoids. Mechanisms of action
include free radical scavenging, chelation of transition metal ions, and inhibition of
oxidases such as lipoxygenase (Udayan and Venkatesh, 2005). The anti-oxidative effects
in onion such as inhibition of lipid peroxidation and lowering of low-density lipoprotein
(LDL) cholesterol level have been allied with condensed risk of neurodegenerative
disorders, several cancers, cataract formation, ulcer development and cardiovascular
diseases. 3-mercapto-2-methylpantan-1-ol (3-MP) in onion inhibits peroxy-nitrite-
induced cytotoxicity, intracellular tyrosine nitration and intracellular reactive oxygen
species (Evans, 1983).

Onions are anti-coagulant food having a truly wonderful ability to counteract the
detrimental clot-promoting effects of eating fatty acids. It acts as an effective remedy for
cholera. Onion ground with pepper mixture allays thirst, vomiting, diarrhea and
restlessness when consumed by a cholera patient. Research studies have proved that the
onions affect the liver’s metabolism of glucose or release of insulin or prevent insulin’s
destruction. The probable hypoglycemic substances in onions are allicin and allyl propyl
disulfide. Onion is a mucus clearing food and has been for cold, cough, bronchitis and
influenza. Presence of essential oils like catechol, protocatechnic acid, thiocyanate,
thiopropiono aldehyde and other micronutrients in onion avoid the peril of developing
heart diseases and heart stroke. Intake of raw onion helps in healing tooth disorders.
Its juice can be consumed, applied for curing ear infections, skin disorders, rheumatic
diseases, urinary infections and bleeding piles (Bakhru, 2011). The aphrodisiac properties of onion increases libido and strengthens reproductive organs for sexual impotence. Other pharmacological activities of onion include inhibition of carcinomas, immune-suppression and neuro-protective effects. It is highly effective against pathogenic gram-positive bacteria and dermatophytic fungi and also promotes other beneficial microorganisms.

Organosulfur compounds such as di-allylsulfide, thiosulfinates and flavonoids have been the spotlight of much research pertaining to antioxidant activity, cancer prevention, coronary heart disease, and many other factors relating to human disease. Researchers using epidemiological data have shown association between increased onion consumption and lower risk of certain cancers, lipid and cellular oxidation and subsequent damage to cellular function and overall health. Many promising aspects relating to high daily intake of onions have been explicated. However, it is perceptible that more research is still needed in order to clearly identify \textit{in vivo} health benefits from onion consumption in the human diet.

\textbf{1.3 Flavonoids}

Flavonoids, an amazing array of compounds represent one of the largest and most studied classes of phenylpropanoid derived plant specialized metabolites. They are formed from the aromatic amino acids phenylalanine & tyrosine and malonate. It consists of 15 carbon atoms arranged in three rings giving rise to a flavan nucleus (Figure 1.1). Many classes of flavonoids are present classified based on the functional group and the side chain. They are of mixed biosynthetic origin with one ring being shikimate-derived and the other being derived from polyketide.
Flavonoids are highly important class of compounds having a lot of significance, as catalysts in the light phase of photosynthesis, regulators of ion channels involved in phosphorylation, stress protectants in plant cells by scavenging activity. Another interesting fact is that due to its favourable UV-absorbing properties, flavonoids scavenge UV generated ROS and protect the plants. Flavonoids also have a range of medicinal properties like antioxidant, enzyme inhibition, anti-inflammatory, vascular, oestrogenic, cytotoxic antitumour, anti-bacterial activities and also reduce the risk of coronary heart diseases. Some flavonoids can act as anti-spasmolytic agents by relaxing smooth muscles and hepatoprotective agents (Middleton and Kandaswami, 1992). The flavonoid intake in diet is considerably high as compared to those of Vitamin C, Vitamin E and carotenoids (Yamasaki et al., 1997). These are present in all terrestrial plants as glycosylated derivatives and are found in all plant organs including flower, fruit, stem, leaf and root. These large compounds are ubiquitous in food plants occurring as glycosides and contain several phenolic hydroxyl groups on their ring structures attributing to the brilliant shades of blue, scarlet, and orange, in leaves, flowers and fruits (Peterson and Dwyer, 1998). Flavonoids play different roles in the ecology of plants. Due to their attractive colors,
flavones, flavonols, and anthocyanidins may act as visual signals for pollinating insects. Because of their astringency, catechins and other flavanols can represent a defense system against insects harmful to the plant.

Flavonoids act as catalysts in the light phase of photosynthesis and/or as regulators of ion channels involved in phosphorylation. They can also function as stress protectants in plant cells by scavenging ROS produced by the photosynthetic electron transport system. Many flavonoids are found to be strong free radical scavengers and antioxidants (Birt et al., 2001; Duthie and Dobson, 1999; Fukumoto and Mazza, 2000; Klahorst, 2002; Unno et al., 2000). Excessive free radical production and lipid peroxidation in vivo are known to cause many kinds of disease such as atherosclerosis, cancer and chronic inflammation. Some flavonoids have been reported to possess a variety of biological activities, including antiallergic, anti-inflammatory, antiviral, anti-proliferative, antioxidative, anti-diabetic, hepato- and gastro-protective, antiviral, anti-neoplastic and anti-carcinogenic activities in addition to having effects on mammalian metabolism (Ren et al., 2003; Zand et al., 2002). They have received considerable attention because of their beneficial effects as antioxidants in the prevention of human diseases such as, cancer and cardiovascular diseases, and some pathological disorders of gastric and duodenal ulcers, allergies, vascular fragility, and viral and bacterial infections.

Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants, and also the diverse consumption of humans (Barberan and Clifford, 2000). The dietary intake of flavonoids has been estimated to vary from 100 to 1000 mg/day (Aherne and Obrien, 2002). Flavonoids are heat stable, but easily lost due to cooking and
frying (Hertog, 1993). It is usually thought that flavonoids are absorbed by passive diffusion after the glycosylated flavonoids are converted to their aglycones. The colon microflora would play an important role in this conversion. The bioavailability of certain flavonoids differs markedly depending on the food source where the absorption of quercetin from onions has been shown to be fourfold greater than from apples or tea (Hollman, 1997).

1.4 Naringenin

Naringenin whose IUPAC name 5, 7-dihydroxy-2-(4-hydroxy phenyl)-chroman-4-one (Figure 1.2) suggests that it is a flavonone; a class of flavonoids having a lot of importance and biological effects in living organisms. It is a yellow crystalline powder with a melting point of 247-250°C. The chemical formula is C_{15}H_{12}O_{5} comprising a molecular mass of 272.257g/mol.

![Figure 1.2 Structure of Naringenin](image)

Naringenin has a bioactive effect on human health as an antioxidant, radical scavenger, anti-inflammatory, carbohydrate metabolism promoter and immunity system modulator. It also works as an anti-ulcer agent and estrogen antagonist, which inhibit the
action or biosynthesis of estrogenic compounds. Naringin, an aglycone derivative of naringenin and naringenin exert antioxidant activity in human body. Recent advances divulge that it has a role in curing obesity. It is predominantly found in grape and citrus fruits and a small amount in other vegetables.

Naringenin, a bioflavonoid exhibits anti-estrogenic activity (Jacob and Kaul, 1973; Miksicek, 1993; Ruh et al., 1995) that may be responsible for the decreased incidence of breast cancer in women consuming a large amount of phytoestrogens (Adlercreutz et al., 1992) and could exert cholesterol-lowering properties by inhibiting cholesteryl ester synthesis (Borradaile et al., 1999). It partially deactivates the Fenton reaction (Cheng and Breen, 2000), restores glutathione-dependent protection against lipid peroxidation in α-tocopherol deficient liver microsomes (Van Acker et al., 2000) and inhibits malonaldehyde production induced either by ascorbic acid in rat brain mitochondria (Ratty and Das, 1988) or by autoxidation in rat brain homogenates (Saija et al., 1995). Naringenin may modulate cytochrome P450-dependent monooxygenase, the primary enzyme involved in the metabolism of many xenobiotics such as drugs, carcinogens and environmental pollutants (Ueng et al., 1999). The main sources of naringenin are citrus fruits and tomato (Davies and Graeme, 1981; Kawaii et al., 1999).

1.5 Isoflavonoids

Isoflavonoids are flavonoid polyphenolic compounds with 3-phenylchromen-4-one backbone (Figure 1.3). These are a class of organic compounds and well-known group of phytoestrogens abundantly produced in the Fabaceae members. These are produced via a branch of general phenyl propanoid pathway. Isoflavones and their derivatives that are a large group of secondary metabolites act as phytoalexin compounds which are involved
in plant-pathogen and plant-animal chemical warfare (John, 2000). They also have a natural role in root nodulation and *Rhizobium* nitrogen fixation. The interest in isoflavones originates from the discovery in 1940 that isoflavones in subterranean Clover were the cause of infertility effects observed in Western Australian Sheep (Bennetts et al., 1946).

**Figure 1.3 Isoflavan Ring**

Isoflavones such as genistein and daidzein were able to prevent the growth of estrogen-receptor positive and negative breast cancer cells *in vitro* (Heber, 2008). Most members of the *Fabaceae* family like soybean, green bean, alfalfa sprout, mung bean, red clover blossom, red clover sprout, kudzu root, *Psoralea* contain significant quantities of isoflavones and have been studied for their estrogenic activity (Bouea et al., 2003). In plant tissue, they mostly occur as glycosides or their respective malonates or acetyl conjugates, rendering them even more water-soluble.

Isoflavonoids (or isoflavones) are a type of phytoestrogens, or plant hormones, that have a chemical structure similar to human estrogen. The health benefits believed to be provided by isoflavonoids come from the weak estrogenic activity of these molecules in the human body (Jung et al., 2000). Isoflavonoids are found in soybeans, chickpeas,
and many other legumes; however, soybeans are unique because they have the highest concentration of the two most beneficial isoflavonoids, genistein and daidzein (Eldridge and Kwolek, 1983; Tsukamoto et al., 1995). They help prevent the buildup of arterial plaque, which reduces the risk of coronary heart disease and stroke (FDA 1999); help reduce breast cancer (Peterson et al., 1991), help prevent prostate cancer by delaying cell growth (Messina and Barnes, 1991), fight osteoporosis by stimulating bone formation (Civitelli, 1997) and even relieve some menopausal symptoms (Nestel et al., 1999). There has been increasing interest in health-protecting and health-promoting effects of these compounds and in their possible use in human medicine (Cos et al., 2003).

### 1.6 Genistein

Genistein, 3-(4-hydroxyphenyl) chromen-4-one (Figure 1.4) is one of the preciously known isoflavone. It was first isolated from Dyer’s Broom, *Genista tinctoria* in 1899. It is found in a number of plants such as Lupin, Fava Beans, Soybeans, Kudzu and Psoralea. Its chemical formula is C\textsubscript{15}H\textsubscript{10}O\textsubscript{5} with a molecular mass of 270.24 g/mole. It is a central intermediate in the biosynthesis of more complex isoflavonoids.

![Figure 1.4 Structure of Genistein](image)
Genistein is an important isoflavone showing many biological properties like antioxidant and anti-heleminthic activities. It is involved in plant-animal warfare, acts as phytoestrogen and cancer chemoprotective agent. It also exhibits inhibitory action of several tyrosine kinases and topoisomerases, stimulates autophagy. Recently reported health benefits include relief of menopausal symptoms, reduction of osteoporosis, improvement in blood cholesterol levels and lowering the risk of certain hormone related cancers (Jung et al., 2000).

Genistein is biosynthetically the simplest of the isoflavonoid compounds of the Leguminosae. Most of the studies have focused on the pharmacological activities of genistein as a tyrosine kinase inhibitor, its chemoprotectant activities against cancers and cardiovascular disease, and its phytoestrogen activity. Natural sources from which isoflavonoids have been isolated, including genistein and closely related analogs like biochanin A (Balasubramanian and Nair, 2000) and their glycosides are listed in an excellent review by Dewick (1994). Many isoflavonoids exhibit broad-spectrum antimicrobial activity and are therefore believed to help the plant fight microbial disease. Genistein may function both as a phytoalexin and as a phytoanticipin. Genistein shares structural features with the potent estrogen estradiol-17b (Barnes et al., 2000), particularly the phenolic ring and the distance (11.5 Å) between its 40- and 7- hydroxyl groups. An early epidemiological confirmed breast cancer indicated that soy consumption was directly correlated with reduced risk of cancer (Lee et al., 1991) and the effects appeared to be dietary rather than genetic.

Inhibition of the growth of human stomach cancer cell lines in vitro by genistein and biochanin A apparently involves stimulation of a signal transduction pathway leading
to apoptosis (Yanagihara et al., 1993). Genistein also appears to improve plasma lipids, resulting in lowered LDL cholesterol, the ratio of total cholesterol to HDL cholesterol, and the ratio of LDL to HDL cholesterol, in pre-menopausal women (Merz Demlow et al., 2000). A study has indicated that isoflavone-rich soy protein may attenuate bone loss in the lumbar spine of post-menopausal women, and that this effect is due to isoflavones rather than to soy protein (Alekel et al., 2000). An isoflavones rich diet may help approximately two thirds of post-menopausal women to better cope with hot flushes, in addition to potentially reducing the risk of cardiovascular disease, which is elevated post-menopause. Genistein also inhibits DNA topoisomerase and tyrosine protein kinase (Akiyama et al., 1987) as well as possessing antioxidant and cell cycle inhibitor activity. Genistein blocks EGF-mediated tyrosine phosphorylation in vivo in human epidermal carcinoma cells. When specifically targeted to the B-cell-specific receptor CD-19 by conjugation to a monoclonal antibody, genistein selectively inhibited CD-19-associated tyrosine kinase activities, resulting in death of human B-cell precursor leukemia cells (Uckun et al., 1995).

1.7 Isoflavone Synthase (IFS)

Isoflavone synthase (IFS) is the crucial enzyme required for the biosynthesis of isoflavonones from flavonones. IFS catalyses the oxidation of 7, 4’-dihydroxyflavanone (liquiritigenin) or 5, 7, 4’-trihydroxyflavanone (naringenin) to daidzein or genistein respectively (Jung et al., 2000). The biosynthetic skeleton of isoflavonoids is constructed by a subfamily of cytochrome p450 or CYP93C designated as 2-hydroxyisoflavonone synthase (IFS) (Akashi et al., 1999; Steele et al., 1999). Taxonomically, this enzyme is
primarily limited to leguminous members. The reaction occurs in two steps, where the 2-hydroxylation and aryl ring migration of flavonone substrates to yield a 2-hydroxy isoflavonone and then followed by a dehydration step to produce isoflavones.

Isoflavone synthase (IFS) is the entry point enzyme of isoflavonoid biosynthesis, and therefore the key step for engineering isoflavone production into plants (non-legumes) that lack the pathway. To demonstrate proof of principle for the genetic manipulation, IFS from licorice, soybean and red clover was introduced in Arabidopsis thaliana, corn, tomato, rice and tobacco resulted in successful genistein accumulation in which the phenylpropanoid pathway was activated by C1 and R transcription factors showing that heterogenous IFS was able to take host flavanone intermediates as substrates (Akashi et al., 1999; Jung et al., 2000; Kim et al., 2003; Yu et al., 2000). The Figure 1.5 represents the phenyl propanoid pathway showing the conversion of Flavonoids to Isoflavonoids.

![Phenylpropanoid Pathway](image)

**Figure 1.5 Phenylpropanoid Pathway (Jung et al., 2000)**
1.8 Conventional Cloning

The human life has revolutionized in production of better products in terms of quality and quantity with the advent of recombinant DNA technology. Recombinant technology includes, insertion of a desired gene in a particular gene and its amplification from the whole genome of an organism. The isolated gene of interest is cloned into a vector that forms a recombinant DNA. When the recombinant DNA is inserted into a host system, the host system will produce a particular protein, which can be used after a proper downstream processing. Many methods are available to clone the desired gene into the expression vector where conventional cloning is the most commonly used.

The basic cloning workflow by the conventional way includes isolation of target DNA fragment, ligation of inserts into an appropriate cloning vector, transformation of recombinant plasmids into suitable host for propagation and screening of hosts containing the intended plasmid. To increase the efficiency of molecular cloning, many specialized tools and methodologies were developed by means of the properties of unique enzymes (Figure 1.6). TA cloning exploits the benefit of Taq polymerase and ligation independent cloning uses T₄ DNA polymerase that generates single stranded DNA overhangs. In recent times, GC Cloning has come into pipeline, where non-proof reading DNA polymerases like Taq, TfiI and Tth add a single 3’-G to blunt DNA molecules by means of PCR or G-tailing reaction. The 3’-G overhang on the DNA insert is then ligated to vectors containing a single 3’-C overhang. (http://www.atzlabs.com/pdf/GC_Cloning_Kit_lifetech_india.pdf).
1.9 Gateway Cloning

The current day transgenic study in biological systems relies to a huge scope on DNA cloning technologies. Cloning technologies and its immense use in research investigations have become very important in the field of biology for the analyses of recombinant genes. The robust site-specific recombination based Gateway cloning forms a platform and it is a vital asset in molecular biology for efficient cloning, modular assembly and expression in diverse perspectives (Karimi et al., 2007). Binary vectors are used in genetic transformation, which vary in size, origin of replication, bacterial selectable markers and overall structure. Conventional cloning involves wieldy handling of binary vectors with restriction and ligation reactions. Efforts were also made in the construction of smaller vectors with emphasis on required unique restriction sites in the T-DNA region (Tzfira et al., 2005). The modern Gateway cloning has a considerable advancement because of its arrangement of DNA fragments in a predefined order,
orientation, and reading frame regardless of their sequence. The recent Gateway cloning technology developed originally by Life Technologies (Hartley et al., 2000) and marketed by Invitrogen has been approved by a huge society facilitating the assembly of expression units in a large variety of *in vivo* and *in vitro* systems. This review gives an overall idea on the site-specific Gateway recombination cloning system.

Gateway system exploits the principle of site-specific recombination allowing the integration and excision of λ phage back and forth of a bacterial chromosome by itself (Katzen, 2007). This etiquette requires two enzymes BP Clonase and LR Clonase to catalyze the BP and LR reactions respectively shuttling the sequences between plasmids bearing flanking compatible recombination attachment (*att*) sites. BP clonase enzyme mix (Invitrogen, Life Technologies) consisting of the phage integrase and integration host factor transfers a DNA fragment of interest flanked by two *att*B sites into a donor vector p(DONR) carrying two *att*P sites (Figure 1.7). Recombination takes place between the matching *att*B and *att*P sites and the DNA fragment is inserted into the donor backbone resulting in an entry clone, which is flanked, by two L sites. LR clonase enzyme mix (Invitrogen, Life Technologies) consisting of integrase and integration host factor and the phage excisionase catalyse the LR reaction, where the key substrates are entry clones. The DNA fragment of interest flanked by two *att*L sites is transferred into a destination vector carrying two R sites (Figure 1.7). Recombination again takes place between the matching *att*L and *att*R sites resulting in a novel expression clone flanked by *att*B sites.

Assembly of entry clones can also be done by restriction and ligation of DNA fragments in vectors, where *att*L sites flank multiple cloning sites. But, in most times entry clones are not directly used, as the *att*L sites are too long to be placed as spacers
between DNA sequences of interest compared to the engineered \textit{attB} sites that are short in length without any translation initiation and stop codons. The inventors of Gateway system engineered variants of the original \textit{attB}, \textit{attP}, \textit{attL} and \textit{attR} sites so that they will react specifically facilitating directional cloning (Cheo et al., 2004; Sasaki et al., 2004).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{gateway_cloning}
\caption{Gateway Cloning showing BP and LR Reaction (Invitrogen)}
\end{figure}

Several laboratories have implemented the use of this technique due to its efficient, flexible and reliable qualities. It can also be employed for over expression of genes using multisite Gateway binary vectors for two or three fragment recombination under the influence of a strong inducible or tissue specific promoter (Karimi et al., 2007). For studies involving cis-regulatory sequence analysis, translation fusion, subcellular localization, multisite Gateway system forms an excellent plinth to clone and analyse the expression patterns (Citovsky et al., 2006). There are even binary vectors that are engineered and designed for expressing tagged proteins preferably by placing a tag at the N terminus of the characterized protein when post-translational modifications occur at or near its C terminus essential for functionality (Underwood et al., 2006). Gene silencing,
genomic fragment recombination, gene stacking, protein-protein interaction can also be comprehensively unstated using these recombinational Gateway expression cassettes (Alvarez et al., 2006; Burch Smith et al., 2004; Chen et al., 2006; Karimi et al., 2007; Puig et al., 2001; Robertson, 2004; Schwab et al., 2006).

1.10 Stable Gene Expression in Plants

In the present era, genetic transformation of plants has become a broadly used technology that serves several purposes in the meadow of commerce and research. The use of transgene technology allows the improvement of certain plant traits including disease resistance, stress tolerance, enhanced nutrition, male sterility (Lanfranco, 2003) and also for the production of various high-value proteins (Giddings et al., 2000). Transgenic plants are also recurrently used in elemental research as a tool to study gene function by overexpressing the target genes (Lloyd, 2003). However, all these applications are hampered by high inter-transformant variation of transgene expression often resulting in a majority of less useful transformants with low-level transgene expression.

Basic and applied research involving transgenic plants often requires consistent high-level expression of transgenes. This can be achieved by stable gene integration in the nuclear genome. The possibility to stably integrate genes into the genome of cells has an imperative brunt on many biomedical ensures long term reproducible as well as defined gene expression while, transiently expressed genes are advantageous for the faster analysis of expression and protein production. Stable research includes the development of pharmaceutical products. Stable gene expression paves the way for analysis of gene function and regulation, large-scale protein production, drug discovery and gene therapy. The hall ark of stably transformed cells is that the foreign gene
becomes part of the genome and is therefore replicated. Descendants of these transformed cells therefore will also express the new gene, resulting in a stably transformed organisms. Gene transfer are of two types; direct and indirect (Rao et al., 2009). The direct methods are the physical method of gene transformation such as, electroporation, biolistic mediated transformation; while indirect are the biological method i.e. the application of Agrobacterium tumefaciens to transform the plants.

1.10.1 Agrobacterium mediated gene transfer

Plant genetic transformation method utilizes the natural engineer, Agrobacterium tumefaciens to transfer the foreign gene into the plants. It is a gram-negative soil borne pathogen that colonizes wounded plant cells and induces the formation of crown gall that produces special amino acid derivatives called opines, which the bacteria are able to use as a carbon and nitrogen source (Smith and Townsend, 1907). It has wide range of hosts including dicots and monocots to gymnosperms and some fungal species like yeasts, ascomycetes and basidiomycetes (DeCleene and DeLey, 1976). A. tumefaciens transfers a part of its DNA called Transfer-DNA (T-DNA) into the plant cell after infection (Figure 1.8). The T-DNA encodes enzymes that synthesize auxins and cytokinins for unregulated cell proliferation, and opines from standard amino acids (Gelvin 2003). The major advantage of using Agrobacterium is the stable integration of the transgene in the host plant chromosome, stable expression, low copy number and also large DNA fragments can be transferred (Ko and Korban, 2004; Lopez et al., 2004). It is most commonly preferred method for the development of transgenic crops (Rivera et al., 2012; Tzfira and Citovsky, 2006). There was a significant increase in the number of successful reports on the production of broad range of transgenic plant species using A. tumefaciens during the past two decades (Estrella et al., 2005; Tzfira and Citovsky, 2006).
1.10.2 Biolistic mediated gene transfer

A number of direct DNA transfer methods have been developed to genetically transform plants recalcitrant to Agrobacterium-mediated transformation (Twyman et al., 2002). Among these methods, particle bombardment has become the most successful, because it is based on purely mechanical principles and is therefore not dependent on the biological factors that restrict the Agrobacterium “host range”. Particle bombardment works with any plant species, variety, and explant, leaving the regeneration of fertile plants rather than the DNA transfer process itself as the only significant bottleneck (Altpeter et al., 2005). The technique involves the acceleration of small DNA-coated metal particles (either gold or tungsten) into plant tissue with sufficient force to break through the cell wall and membrane (Figure 1.9). Some of the particles reach the nucleus, where the DNA is
released, probably by a simple diffusion mechanism (Altpeter et al., 2005). Notably, the foreign DNA entering a bombarded cell is naked, double-stranded, and competent for both transient episomal expression and integration into the genome.

![Figure 1.9 Schematic representation of the working methodology of Gene Gun (Williams et al., 1991)](image)

**1.11 Transient Gene Expression in Plants**

Stable and transient gene expression systems are the two methods available for the plant based secondary metabolite production (Plant Metabolic Engineering). Unfortunately, the labour intensive and time consuming process for generating large number of transgenic lines has been a barricade for stable transformation studies (Cazzonelli and Velten, 2006).

The advent of *Agrobacterium* based transient high expression vector system was reported for a wide range of plant species (Sainsbury et al., 2009). These vector systems were first intended for stable integration (Zambryski et al., 1983) and later found to be useful for transient expression to achieve very high levels of recombinant products (Kapila et al., 1997). Some of the other findings also make agroinfiltration the preferred
option over stable transformation to analyze gene silencing (Schob et al., 1997), promoter analysis (Yang et al., 2000) and to explore various genetic and physiological factors (Wroblewski et al., 2005). Transient expression is more preferable because of its ease to confirm the correct assembly and retention of biological activity of the target products quite rapidly (Johansen and Carrington, 2001). This system is also proficient in expressing multigenes simultaneously within the same cell, having greater inferences in congregation of intricate multimeric proteins using plants (Vaquero et al., 1999).

1.11.1 Agroinfiltration

Agroinfiltration is a technique, where the leaves of plants are infiltrated with the suspension of Agrobacterium harbouring gene of interest cloned in the binary vector. It has been proved that transient expression in leaf epidermal cell occurs between 2-6 days (Sainsbury et al., 2010) (Figure 1.10). The high level expression of recombinant protein is achieved using the specially designed vectors or by using suppressor of gene silencing gene (Huang et al., 2009).

![Timeline and Steps Involved in Agroinfiltration Technique](image)

**Figure 1.10 Timeline and Steps Involved in Agroinfiltration Technique (Modified from Varghese, 2014)**
1.11.2 Transient expression vectors

1.11.2.1 pEARLEY- Gateway compatible vector system

pEARLEY gate vectors are gateway compatible plant destination vectors that are widely used for stable expression of recombinant proteins. pEARLEY vectors are derived from pFGC5941 (http://www.chromDB.org), a pCAMBIA (http://www.cambia.org) binary vector backbone. This vector is better to study the protein expression, as it comes with a series of tags that could be handy for recombinant protein detection and purification. This vector possess cauliflower mosaic virus 35S promoter with C terminal His/HA tag (Figure 1.11). Keith et al., (2006) reported high levels of transient expression of GUS protein in pEARLEY with four different epitope tag combinations in tobacco, maize, soybean, rice, tomato, cotton and Arabidopsis. Immunoblot detection of the tagged recombinant proteins proved that all four epitope tags were readily detected in all the species tested.

1.11.2.2 pEAQ- HT vector system

pEAQ-HT vector system is useful for rapid transient protein production in plants without the use of viral replication machinery (Sainsbury and Lomonossoff, 2008). pEAQ-HT expression system is derived from the conventional binary vector pBIN19 (Bevan, 1985) with the disabled version of CPMV-HT (Cow Pea Mosaic Virus-Hyper Translatable) region (Canizares et al., 2006; Sainsbury et al., 2008) resulting in high level protein expression with a C terminal histidine tag fusion. In CPMV-HT system, the gene of interest is cloned between modified 5’ untranslated region (UTR) and 3’UTR of CPMV RNA 2. The gene will fuse to the main initiation codon AUG at position 512 of CPMV RNA 2, because the upstream of this site was essential for replication of RNA 2 by
the RNA-1- encoded replication complex (Rohll et al., 1993). So, this site was positioned immediately to the upstream of 3’UTR to create a molecule that mimics RNA 2 (Canizares et al., 2006). Furthermore, Sainsbury et al., (2008) demonstrated the suitability of this system for the production of heteromeric protein. This vector also proved its efficiency in accumulating large quantities of mRNA, when the suppressor of gene silencing (P19) was co-infiltrated. The RNA 2 leader sequence is also referred as hypertranslatable leader sequence (HT) because it enhances the translation efficiency (Figure 1.12).

1.12 Objectives of this Study

The use of plants for medicinal purposes dates back thousands of years but genetic engineering of plants to produce desired biopharmaceuticals is much more recent. As the demand for biopharmaceuticals increase, it would be wise to ensure that they will be available in significantly larger amounts, on a cost effective basis. Hence, this study aims to understand the flavonoid and isoflavonoid biosynthesis and to use molecular approaches to express the rate limiting genes involved in its metabolic pathway. Isoflavone Synthase (IFS), a key enzyme involved in the biosynthesis of isoflavonoids is
focused in the present research work. Thus, incorporation of this *Glycine max (Gm) IFS* gene in onion, which is the most economically important vegetable with high medicinal properties should increase the synthesis of highly expensive biopharmaceutical compound isoflavonoid genistein from the flavonoid naringenin. **To accomplish this, we have formulated the following specific objectives:**

1. To clone the *Gm IFS* gene into plant gene expression vector through Gateway and conventional cloning technologies.

2. To optimize *in vitro* system for the local commercial onion cultivar ‘Bellary’.

3. Stable and transient gene expression of the *Gm IFS* in onion calli and green leafy vegetables respectively.

4. To analyse and compare the production of isoflavonoids through HPLC in both WT and Transgenic lines.