DISCUSSION

Flavonoids are aromatic amino acid-derived secondary metabolites with a multiple roles in plants. They act as antimicrobial agents, light protection pigments, feeding deterrents against pathogens and herbivores and are, among other pigments, crucial for flower coloration, attracting insects for pollination and seed dispersal (Izaguirre et al. 2007; Yu and Jez, 2008; Diaz Napal et al. 2009). Among the different classes of flavonoids, flavan-3-ols are of major importance. They increase the antioxidant potential, provide protection against stresses, act as UV filters and regulator of auxin transport. Flavonoid pathway with regards to flavan-3-ols biosynthesis has been exclusively studied in tea (Camellia sinensis) (Singh et al. 2008; Singh et al. 2009; Rani et al. 2012). Thus use of tea genetic resource for genetic engineering to increase flavan-3-ols content in alternate plants would be of great value. Here, a gene CsF3H encoding flavanone-3-hydroxylase from tea was overexpressed in tobacco to see its influence on flavan-3-ols content. In flavonoid biosynthesis pathway, just before the synthesis of flavan-3-ols, flavonol synthase catalyzes the synthesis of flavonols. Therefore, in this study silencing of NtFLS encoding flavonol synthase was also conducted to understand the role of flavonols as well as to study the effect of NtFLS silencing on flavan-3-ols content. The generated transgenic tobacco either overexpressing CsF3H or silencing NtFLS were characterised at morphological, biochemical and molecular levels.

5.1 Flavanone-3-hydroxylase and flavonol synthase of flavonoid pathway

Flavonoids are synthesized in a series of enzymatic steps beginning with chalcone synthase (CHS), which synthesizes naringenin chalcone from one 4-coumaroyl-CoA and three malonyl-CoA moieties. Activities of the enzymes chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavonoid 3’-hydroxylase (F3’H) result in the synthesis of dihydroflavonols. At this point the pathway splits into two branches, one leading to flavonols and other to anthocyanins/proanthocyanidins (PAs). The product of F3H activity, dihydroflavonols are further metabolized by either flavonol synthase (FLS) or dihydroflavonol reductase (DFR) (Martens et al. 2010). The products of FLS activity are flavonols, while the products of DFR activity are leucoanthocyanidins. Leucoanthocyanidins are the direct precursors of anthocyanidins synthesized by enzyme anthocyanidin synthase (ANS). Anthocyanidins form a branching point in the flavonoid
pathway synthesizing anthocyanins by the enzyme UDP glycosyl transferases (UGTs) and epicatechin by the enzyme anthocyanidin reductase (ANR; BANYULS, BAN) and catechin by the enzyme leucoanthocyanidin reductase (LAR). Catechin and epicatechin act as precursor for PAs (Xie et al. 2003). The studies on genetic engineering of flavonoid pathway has been conducted in many plant species. The overexpression or downregulation of specific genes leading to diversion of pathway towards particular flavonoids have been conducted in various plants (Han et al. 2010; Nishihara and Nakatsuka, 2011; Jiang et al. 2012). Flavan-3-ols are among the major flavonoid compounds found in higher plants (Winkel-Shirley, 2001). They are powerful antioxidants, and thus provide multiple health benefits to humans, including anti-inflammatory effects, immunity enhancement, as well as lowering risks of cardiovascular diseases and certain cancers (Santos-Buelga and Scalbert, 2000; Luximon-Ramma et al. 2006). They also have a role in providing protection against predation and provide astringent and bitter sensations to many fruits and fruit juices (Vidal et al. 2003; Obreque-Slier et al. 2010; Renard et al. 2011). Overall expression study of genes encoding flavonoid pathway enzymes in tobacco (Nicotiana tabacum L.) has identified lower expression for NtF3H and higher expression for NtFLS. Flavanone 3-hydroxylase (F3H), a 2-oxoglutarate-dependent dioxygenase initiates syntheses of the majority of flavonoid compounds, including flavonols, anthocyanins, and PAs (Springob et al. 2003). Among different plant species, tea contains uniquely high concentrations of polyphenolic compounds, which mainly consist of flavan-3-ols: (+)-catechin (Cat), (-)-epicatechin (EC), (+)-gallocatechin (GC), (-)-epigallocatechin (EGC), (+)-catechin gallate (CG), (-)-epicatechin gallate (ECG) and (+)-gallocatechin gallate (GCG). These flavan-3-ols together constitute more than 30% of the dry weight of tea leaves (Kimura et al. 2002; Mamati et al. 2006). Almost all the genes of flavonoid biosynthetic pathway in tea has been cloned and characterized in order to understand their role in flavan-3-ols accumulation (Singh et al. 2008; Rani et al. 2009; Rani et al. 2012). Earlier studies have reported higher CsF3H expression and activity in tea leaves. This higher expression and activity has been reported to be correlated with increased biosynthesis of flavan-3-ols during different developmental stages in tea leaves (Singh et al. 2008). Higher expression of CsF3H in actively growing tissues has also been reported in Citrus paradisi and Petunia hybrida (Pelt et al. 2003). Ectopic expression of apple F3’H has been reported to alter flavonoids content in Arabidopsis mutants (Han et al. 2010). Since expression of gene encoding NtF3H in tobacco was found to be minimum and also F3H occupies the central
role in synthesis of flavan-3-ols/ PAs, overexpression of the gene CsF3H from tea in the transgenic tobacco could enhance flavan-3-ols content.

The higher accumulation of flavan-3-ols in plants has earlier been achieved by overexpression of the structural and regulatory genes of main pathway (Han et al. 2012; Yuan et al. 2012) and by downregulation of the genes of competing pathways (Nakamura et al. 2006; Jagtap et al. 2011; Jiang et al. 2012). The techniques like antisense and sense suppression, virus induced gene silencing (VIGS) and RNA interference (RNAi) have been applied for silencing of genes (Hoffmann et al. 2006; Nakamura et al. 2006; Nagamatsu et al. 2007; Jagtap et al. 2011; Jiang et al. 2011; Jiang et al. 2012). RNAi-based silencing method using intron-containing self-complementary hpRNA (ihpRNA) constructs, appears to be the most effective (Smith et al. 2000; Wesley et al. 2001). ihpRNA based post-transcriptional gene silencing (PTGS) has the potential to efficiently silence genes in a range of plant species like *Medicago*, soybean, petunia, tobacco, cyclamen and many more plants (Davies et al. 2003; Subramanian et al. 2005; Wasson et al. 2006; Nakatsuka et al. 2007; Boase et al. 2010). Gene silencing machinery in plants appeared to be much more specific than in animals (Schwab et al. 2006). *Arabidopsis* plants transformed with a CHS-ihpRNA construct showed pronounced silencing that was resulted in the lesser production of flavonoid pigments (Wesley et al. 2001). RNAi also provides an attractive new reverse-genetics tool for the study of gene function (Waterhouse and Helliwell, 2003).

Gene expression can be controlled for the enzymes acting at branching point in the pathway allowing the substrate to be used for particular flavonoid synthesis. One such branch point in flavonoid pathway is shown by dihydroflavonols. These dihydroflavonols can be used either by FLS to form flavonols or by a series of enzymes to form anthocyanins/ PAs. Reducing FLS transcript abundance through antisense or increasing DFR transcript abundance has restored anthocyanin biosynthesis in flower limb of white petunia (Davies et al. 2003). Similarly, introduction of an antisense FLS construct into *Nicotiana tabacum* has increased anthocyanin content of the petals up to three times (Holton et al. 1993). Increased anthocyanin production was also resulted from introduction of an antisense FLS construct into lisianthus (Davies et al. 1997). Thus, competition between DFR and FLS for dihydroflavonol substrates commonly influences the level of anthocyanin production in plants. Here in the present study, expression of gene encoding NtFLS enzyme was found to be maximum in tobacco. Therefore by silencing NtFLS, diversion of flavonoid pathway towards accumulation of anthocyanins/ PAs was assumed. RNAi based approach was used to silence gene encoding NtFLS in tobacco in order to see
the influence on flavan-3-ols/ PAs formation. Altering the balance between DFR and FLS enzyme activities, through genetic modification, could be a useful strategy for diversion of pathway towards specific flavonoid type.

5.2 Altered flavonoid content and transcript level of genes encoding flavonoid enzymes in transgenic tobacco plants

Metabolic pathway-engineering is aimed at either enhancing the production of target compounds or reducing the synthesis of harmful/ useless compounds in a multi-step biosynthetic pathway. In order to use such a powerful tool, knowledge about the genetic and molecular basis of the entire pathway is absolutely essential. Secondary metabolic pathways in plants are receiving increasing attention as their study offers innovations in agriculture and horticulture besides being important in chemical and pharmaceutical industries. In this context, one of the most thoroughly analysed secondary metabolic pathway in plants is the flavonoid biosynthetic pathway. Metabolic engineering of flavonoid biosynthetic pathway has been pursued to analyse the change in flavonoid content of various transgenic plants (Akagi et al. 2009; Hong et al. 2012; Kovinich et al. 2012; Kumar et al. 2013).

Here in present study, the role of a tea flavanone 3-hydroxylase (CsF3H) through overexpression and a tobacco flavonol synthase (NtFLS) through silencing in transgenic tobacco was explored in overall regulation of flavan-3-ols accumulation and flavonoid pathway flux. The CsF3H overexpressing transgenic tobacco as well as NtFLS silenced transgenic tobacco lines were observed for increased level of flavan-3-ols (catechin, epicatechin and epigallocatechin) content. Further, both overexpressing and silenced transgenics were found to have decreased level of anthocyanins. Thus, the potential of whole pathway has been diverted towards flavan-3-ols production. Hence, overexpression of CsF3H and downregulation of NtFLS has modulated the utilization of anthocyanidins towards flavan-3-ols in transgenic plants. Earlier studies have reported higher expression and activity of gene encoding CsF3H in tea leaves. This higher expression and activity was correlated with increased flavan-3-ols in tea leaves (Singh et al. 2008). Hence, also observed to be responsible for increase in flavan-3-ols content of transgenic tobacco overexpressing CsF3H. NtFLS is the other regulatory enzyme catalyzing the branching step of flavonoid pathway towards flavonols production. It competes with DFR for the common substrate towards the formation of flavan-3-ols/ anthocyanins. Here in the present
study, post-transcriptional silencing of gene encoding NtFLS has reduced flavonols (quercetin) to a minimum level and has diverted the carbon flux of the pathway towards the flavan-3-ols instead of anthocyanins production. In contrast to this, introduction of antisense FLS construct in Nicotiana and Lasianthus has been reported to increase the anthocyanin production (Davies et al. 1993; Davies et al. 1997). This difference in results could be due to the utilization of RNAi based NtFLS silencing in the present study. PTGS is considered to be the most effective of all other silencing techniques (Smith et al. 2000; Wesley et al. 2001). Hence, the results showed that the competition between NtDFR and NtFLS enzymes also influences the level of anthocyanin production in plants.

Similar to the above results, transgenic tobacco plants overexpressing MdANR gene have exhibited higher level of PAs and lower level of anthocyanins in their flowers (Han et al. 2012). Overexpression of CsANR has also increased flavan-3-ols and decreased anthocyanins in transgenic tobacco (Kumar and Yadav, 2012; Pang et al. 2013). Increase in flavan-3-ols was also observed by overexpression of a grape berry transcription factor VvMYB5b in tobacco (Deluc et al. 2008). In contrast to this, transgenic tobacco overexpressing PtrDFR1 has exhibited higher level of anthocyanins and lower level of PAs in their flowers (Huang et al. 2012). An increase in anthocyanin content has been reported in petunia by altering the competition between DFR and FLS through antisense silencing of FLS (Nielsen et al. 2002; Davies et al. 2003). Red coloured flowers have also been reported in tobacco by suppression of two endogenous genes encoding F3H and FLS and by simultaneously overexpression of a gene from gerbera encoding DFR. Such genetic manipulation was resulted in elevation of pelargonidin level in tobacco and imparted red colour to the flowers (Nakatsuka et al. 2007). Silencing of gene NtCHI encoding CHI has been reported to reduce pigmentation and changed flavonoid content in tobacco flowers (Nishihara et al. 2005). F3H enzyme has been reported as critical for controlling anthocyanin biosynthesis in tobacco flowers (Zhang et al. 2012). In the same way, overexpression of apple F3H has been reported to cause higher accumulation of anthocyanins in tobacco flowers (Nakatsuka et al. 2007). Ectopic expression of BAN in tobacco petals and Arabidopsis leaves was resulted in loss of anthocyanins and accumulation of condensed tannins, suggested the interaction between anthocyanidin and PAs synthesis (Xie et al. 2003).

Further, the influence of overexpression and silencing on the diversion of flux was studied at the transcript expression level of genes encoding flavonoid pathway enzymes. Significant increase in the transcript level of genes encoding NtDFR and NtANS was
observed in overexpressing and silenced transgenics. This upregulation in transcript expression of genes encoding NtDFR and NtANS might be responsible for the observed higher flavan-3-ols and lower anthocyanins in transgenic plants. Also, transcriptional control might be playing an important role in regulating the overall activity of flavonoid biosynthesis enzymes (Abeynayake et al. 2012). Similar modulation of flavonoid biosynthetic pathway gene expression has earlier been reported upon overexpression/silencing of genes from the same flavonoid pathway (Xie et al. 2004; Takahashi et al. 2006; Xie et al. 2006; Rosati and Sinoneau, 2008; Kovinich et al. 2012). Higher expression of gene encoding DFR and ANS was reported in pigmented flowers of Capsicum annum (Stommel et al. 2009). However, overexpression of cranberry ANS gene produced no effect on anthocyanin content of transgenic tobacco flowers (Polashock et al. 2002).

Hence, concluded that the higher accumulation of flavan-3-ols in CsF3H overexpressing and NtFLS silenced transgenics could be due to the utilization of anthocyanidins for flavan-3-ols production rather than for anthocyanins formation.

5.3 Increased antioxidant potential of transgenic tobacco plants

Flavonoids are involved in large number of plant functions. They act as non-enzymatic antioxidants and play an important role in compromising oxidative stresses (Han et al. 2009). Flavonoids are antioxidant phytochemicals belonging to the polyphenolic family and are extensively present in fruits, vegetables, grains, and beverages (Deng et al. 1997; Rice-Evans, 2001). Previous studies have provided evidence for the ROS (reactive oxygen species) scavenging activity of flavonoids. They are known to scavenge peroxyl radicals and chelate iron ions. Under this category anthocyanidin, flavanone, flavone, flavonol and PAs are the main players (Nesi et al. 2000; Winkel-Shirley, 2001). Flavan-3-ols are nearly the end products of flavonoid biosynthesis and like other flavonoids possess scavenging ability in vitro (Rice-Evans et al. 1997; Aron and Kennedy, 2008). Most of work done so far on flavonoids as antioxidative molecules has been validated through in vitro experiments (Deng et al. 1997; Harborne and Williams, 2000; Rice-Evans, 2001). However, studies about their effect as antioxidants in planta are lacking. The secretion of flavan-3-ols from Centuria maculosa provides the protection to the plant against pathogen attack (Bais et al. 2006). The strong antioxidant and free radical scavenging properties of flavan-3-ols make them ideal to protect the plants against oxidative stresses. Thus the
increased flavan-3-ols content in CsF3H overexpressing and NtFLS silenced transgenics may be responsible for the improved antioxidant potential.

Also, the response on transcript expression of genes encoding antioxidant enzymes and activity of antioxidant enzymes were analyzed in CsF3H overexpressing and NtFLS silenced transgenics. The activities of antioxidant enzymes glutathione reductase (GR), catalase (CAT) and ascorbate peroxidase (APx) showed the similar increasing trend as that of transcript expression of genes encoding these enzymes in CsF3H overexpressing and NtFLS silenced transgenic lines. In contrast to this, transcript expression of gene encoding glutathione S-transferase (GST) and activity of GST showed the reverse trend in both overexpressing and silencing transgenics. This has suggested the transcriptional regulation of APx, GR and CAT and post-transcriptional regulation of GST enzymes in CsF3H overexpressing and NtFLS silenced transgenic lines. Results have further suggested that higher mRNA production does not always mean for higher activity of the protein synthesized by similar mRNA. There could be either the rate of such mRNA degradation was higher or inactive protein enzyme was produced. The transcript expression of genes encoding antioxidant enzymes and activity of antioxidant enzymes APx, GR, CAT and GST in wild tobacco seedlings with in vitro catechin treatment showed similar trend as was observed with CsF3H overexpressing and NtFLS silenced transgenic seedlings. The significant increase in enzymatic activities of all the enzymes in tobacco seedlings was observed with lower dose of catechin exposure as compared to its higher dose. The decrease in enzymatic activities at higher concentration of catechin exposure could be causing negative regulation of these enzymes (Pelletier et al. 1999). Thus, in vitro and in vivo experiments suggest that flavon-3-ols (catechins) are interacting with GR, APx, and CAT at mRNA level and leading to increase in their enzymatic activities. In contrast to this, flavon-3-ols are directly increasing the activity of GST without influencing its mRNA level. It has been shown earlier that rapid development of higher peroxidase and CAT activities under stress is a trait of tolerant plant species or genotypes, enabling them to protect themselves against oxidative stress (Zhang et al. 2007). Therefore, an increase in the activity of H2O2-scavenging enzymes is crucial for an effective defence against oxidative stress (Gossett et al. 1994). Similarly, increase in APx, GR and CAT activity of antioxidant enzymes has been observed in CsDFR and CsANR overexpressing transgenic tobacco plants (Kumar et al. 2013).

The next question arises that how these flavonoids interact with antioxidant enzymes to increase the free radical scavenging activities of plant. Perhaps this is one of
the greatest challenges to provide the molecular evidence through which these flavonoid compounds exert beneficial activity in plants. Flavonoids are synthesized mainly in the cytosol, in multi-enzymatic complexes linked to the endoplasmic reticulum (ER) membrane and from here they are transported to their subcellular destinations (Hrazdina and Wagner, 1985). Owing to their potent redox activities, this subcellular trafficking is tightly regulated to avoid undesired chemical or enzymatic reactions. It can be presumed that inside the plants either these flavonoids undergo modification by glycosylation or prenylation, or undergo conjugation with glutathione and specific flavonoid-conjugate transporters, move across the membranes and through intracellular transport and then reached to the vacuoles. From there, these compounds get remobilised and bind to the targets or receptors directly or indirectly to activate antioxidant pathway genes (Dixon and Pasinetti, 2010).

Hence, increased flavan-3-ols and enhanced level of antioxidant enzymes expression and activity in CsF3H overexpressing and NtFLS silenced transgenics could potentially be contributing towards improvement in their antioxidant potential.

5.4 Characterization of CsF3H overexpressing transgenic tobacco plants

5.4.1 Specific activity of Camellia sinensis flavanone-3-hydroxylase

Hydroxylation of (2S)-flavanone to (2R,3R)-dihydroflavonol is a key step in the biosynthesis of flavonols, anthocyanidins and catechins (Ebel and Hahlbrock, 1982; Heller and Forkmann, 1988). F3H enzyme was initially characterized in Petroselinum crispum and Matthiola incana (Forkmann et al. 1980; Britsch et al. 1981). Earlier F3H enzyme activity was not linked to F3H locus in genome because loss of function mutants was not found in M.inacana. Later on, Forkmann and Stortz (1981) correlated the F3H locus with capacity for enzymatic 3- hydroxylation of flavanones to dihydroflavonols using the extracts of Antirrhinum majus. Subsequently purification of F3H from A. majus has shown that it is a soluble protein belonging to the family of 2-oxoglutarate dependent dioxygenases and requires ascorbate and ferrous ions as cofactors for its activity (Forkmann and Stortz, 1981). The enzyme was also purified from petunia and exhibited low specific activity, high stereo specificity and a narrow substrate specificity (Britsch and Grisebach, 1986; Britsch et al. 1992). Attempts to purify flavanone 3-hydroxylase from parsley cell cultures were unsuccessful because of the low activity of hydroxylase and its extreme instability under the usual purification conditions. But later on, the purification of
enzyme was made possible by using anaerobic conditions in the presence of ascorbate and by addition of 2-oxoglutarate and ferrous ion to the buffers (Britsch and Grisebach, 1986). cDNA clones encoding F3H protein have been isolated from *Antirrhinum majus, Hordeum vulgare, Malus domestica, Camellia sinensis, Citrus paridisii, Forsythia X intermedia* and *Zea mays* (Martin *et al.* 1991; Meldgaard, 1992; Davies, 1993; Singh *et al.* 2008). However, little biochemical characterization has been reported for any of the corresponding proteins. In the present study, ORF of gene encoding CsF3H was expressed in expression vector to produce recombinant protein. Similar to the earlier reports, CsF3H recombinant protein was also found to exhibit activity in the presence of naringenin, oxoglutaric acid and ferrous sulphate and resulted in the formation of dihydrokaempferol. Thus indicated that CsF3H is encoding a functional protein.

5.4.2 *CsF3H* overexpressing tobacco transgenic lines exhibited tolerance to salt stress and *Alternaria solani*

Since CsF3H overexpressing transgenic tobacco were found to have increased level of flavan-3-ols and showed higher antioxidant potential, they were evaluated for their response to salt stress and *Alternaria solani*.

5.4.2.1 Better tolerance against salt stress

Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions. Salt stress is one of the major abiotic factors faced by the plants (Seigler, 1998). It decreases the crop yield by altering plants metabolism, reducing water potential, ion imbalance and other osmotic effects (Sekman *et al.* 2007). Salt stress is known to impair the cellular electron transport within the different subcellular compartments and results in the generation of ROS (Ali and Alqurainy, 2006). The concentration of ROS under normal growth conditions is low (Polle, 2001), whereas under stress conditions their concentration is high (Karpinski *et al.* 2003; Laloi *et al.* 2004). ROS can interact with a number of other molecules and metabolites such as DNA, pigments, proteins, lipids, and other essential cellular molecules which lead to a series of destructive processes (Lamb and Dixon, 1997; Mittler, 2002). The degree of damage caused by them depends upon balance between their formation and scavenging by antioxidant system (Hernández and Almansa, 2002). The antioxidant system includes enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx) and
glutathione reductase (GR) (Asada, 1999; Kiddle et al. 2003; Gomez et al. 2004; Mateo et al. 2004). So producing crops tolerant to salt stress is a need of the hour.

In the present study, the transcript expression level of Csf3H was found to be clearly induced in tea leaves exposed to 50 mM and 150 mM NaCl stress. Whereas, exposure to other stresses like drought, ABA and GA3 has been observed previously to decrease the expression of Csf3H significantly (Singh et al. 2008). This has indicated the important role of F3H protein in response to salt stress. Various physiological factors contributed might be contributing towards the tolerance of Csf3H overexpressing transgenics to salt stress.

5.4.2.1 Reduced ROS accumulation in transgenic tobacco

The accumulation of ROS in Csf3H overexpressing transgenic lines was found to be appreciably reduced under salt stress conditions compared to control tobacco. This was evident through histochemical localization of ROS based on staining with DAB for H2O2 and NBT for O2− (Shulaev and Oliver, 2006). Thus indicating lesser oxidative damage in Csf3H overexpressing transgenics. Also, lesser damage is supported by the lower electrolyte leakage (EL) and malondialdehyde (MDA) content in transgenics during the salt stress. MDA is a secondary end product of polyunsaturated fatty acid oxidation, which is widely measured to know the extent of lipid peroxidation, as an indicator of oxidative stress (Lin and Kao, 2000). MDA concentration was increased progressively with the progression of salt exposure time, indicating that the degree of stress and the level of lipid peroxidation was aggravated with stress time. In the present study, Csf3H overexpressing transgenic tobacco lines and control lines showed different level of leaf MDA content under salt stress. A significant increase in MDA content in the leaf due to salt was observed in the control plants than in the transgenic plants. The lesser MDA content in Csf3H overexpressing transgenics under stressed and unstressed conditions, may be due to higher accumulation of flavan-3-ols. Phenolic compounds inhibiting lipid peroxidation has also been documented earlier (Takamah, 1983). In fact, polyphenols enhance the defense system against oxidative stress and retard or inhibit lipid auto-oxidation by acting as radical scavengers (Lopez-Berenguer et al. 2009). Consequently, polyphenols play an important role in alleviating toxic effects of salt ions. This means higher antioxidant potential of transgenic tobacco has protected plants from oxidative damage and therefore MDA did not increase in response to the salt treatments. Polyphenol synthesis and accumulation is generally stimulated in response to biotic or abiotic stresses.
Increase in polyphenol content in different tissues under increasing salt exposure has also been reported in a number of plants (Parida and Das, 2005). Similarly, an enhancement in polyphenol and antioxidant activity levels was observed in the leaf extract of Mentha pulegium under salt stress (Oueslati et al. 2010). The in vitro application of catechins has significantly reduced the MDA content in sweet pepper under salt stress (Yiu et al. 2012). Furthermore, enhanced activity of antioxidant enzymes in CsF3H overexpressing transgenics might be responsible for observed salt stress tolerance in transgenic lines. Activation of genes encoding ROS-scavengers by flavan-3-ols binding to the targets/receptors has also been reported previously (Dixon and Pasinetti 2010). An exogenous application of catechin in sweet pepper plant has been reported to inhibit the salinity-induced enhancement of O$_2^-$ generation and H$_2$O$_2$ content. Hence, mitigated the oxidative stress response due to increase in ROS scavenging activity of antioxidant enzymes (Yiu et al. 2012). A close correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in numerous crops such as pea, cotton, rice and sugarbeet (Gossett et al. 1994; Dionisio-Sese and Tobita, 1998; Hernández et al. 2000; Jbir et al. 2001; Shalata et al. 2001; Muscolo et al. 2003). Significant enhancement in the accumulation of caffeic acid and chlorogenic acid was observed in artichoke leaves in response to salinity and suggested that these phenolic compounds were perhaps stress-induced (Rezazadeh et al. 2012).

Hence, better antioxidant system, decreased electrolyte leakage and MDA content could be responsible for the observed tolerance to salt stress in CsF3H overexpressing transgenic tobacco lines.

**5.4.2.1.2 Enhanced chlorophyll content in transgenic tobacco**

Chlorophyll content in plants is directly correlated to the healthiness of plant (Zhang et al. 2005). The resistance of photosynthetic systems to salinity is associated with the capacity of the plant species to effectively compartmentalise the ions in the vacuole, cytoplasm and chloroplast (Croser et al. 2001). The decrease in chlorophyll content under stress is a commonly reported phenomenon and in various studies, this may be due to membrane deterioration (Djanaguiraman et al. 2006). A decline in leaf chlorophyll content was observed in all plants in response to salt stress but it was less in transgenic plants overexpressing CsF3H. Therefore, a negative correlation was observed in leaf MDA and chlorophyll contents under salt stress. Similar decrease in MDA content and increase in chlorophyll content has been observed in Arabidopsis overexpressing arginine
decarboxylase gene (PtADC) from *Poncirus trifoliata*. The PtADC overexpression has conferred tolerance to *Arabidopsis* against drought, cold and osmotic stresses (Wang et al. 2011b). Chlorophyll content of the *Vetiveria* was not much affected by the exposure of 100 mM salt because of the increased polyphenolic content (Mane et al. 2011). Overexpression of an ethylene responsive factor (ERF) gene *W6* from *Triticum aestivum* L. in *Nicotiana tabacum* has increased chlorophyll content and improved the salt tolerance of transgenic tobacco (Lu et al. 2008b). In the same way, transgenics poplar hybrid plants overexpressing chloride channel gene (GmCLC1) from soybean were observed for increased photosynthetic pigments when subjected to salt stress (Sun et al. 2013). Also, *Arabidopsis CBF3*-overexpressing transgenic oat showed elevation in chlorophyll content in response to salt stress (Oraby and Ahmad, 2012).

Thus from the above reports, it has been concluded that higher chlorophyll content in *CsF3H* overexpressing transgensics compared to control might have contributed towards their tolerance to salt stress.

### 5.4.2.1.3 Increased root growth and degree of pectin methyl-esterification in transgenic tobacco

Most of the stresses on plants lead to change in composition and ultra structure of cell wall. Pectins constitute the major portion of primary cell wall. The main component of pectin is homogalacturonan (HGA) having variable amount of methyl ester groups. Pectins are secreted in to cell wall in a highly methyl esterified form and subsequently de-esterified by action of pectin methyl esterase (PME) (Willats et al. 2001). These PMEs are known to be involved in regulation of many processes in plant physiology. Regulation of PME activity by PME inhibitors may present a way to investigate the role of PMEs in cell wall modification during different stresses. It has been shown earlier through *in vitro* studies that green tea flavan-3-ols are strong inhibitors of PMEs (Lewis et al. 2008). Their inhibitory action may be due to synergistic effect of different flavan-3-ols (Morre et al. 2003). These flavan-3-ols inhibit enzyme activity either by direct competitive interactions (Persson et al. 2006) or by altering regulation of gene expression encoding PME at the genetic level (Ahmed et al. 2004). The higher content of flavan-3-ols in *CsF3H* transgenic tobacco seems to be responsible for the observed decrease in pectin methyl esterase activity of transgenic lines compared to control plant under normal as well as under salt stress conditions. *Arabidopsis* PME inhibitor, AtPMEI-2 has been shown to inhibit PME activity (Raiola et al. 2004). PME activity is inversely related to the degree of pectin
methyl-esterification. Significantly higher pectin methyl-esterification observed in CsF3H transgenic lines compared to control tobacco plant in response to salt stress conditions as determined histologically and immunologically could be due to lower PME activity. In vitro exposed wild tobacco seedlings to flavan-3-ols EC and EGC in the presence of NaCl showed similar trend of increase in pectin methyl-esterification through decreasing PME activity as was observed in CsF3H transgenic lines. This in vitro experiment on wild type plants has supported the increase in pectin methyl-esterification due to flavan-3-ols accumulation in CsF3H transgenic lines. The pectin methyl-esterification is reported to be increased during cell elongation phase and decreased as cell elongation ceased (Parre and Geitmann, 2005). The presence of high PME activity in cold-acclimated leaves of oil rape seeds has been reported to contribute towards leaf rigidity, while low PME activity in de-acclimated leaves of rape seeds has contributed towards cell wall loosening and their growth promotion (Solecka et al. 2008). Also, longer primary roots in CsF3H overexpressing transgenics compared to control under salt stress conditions is indicating for a positive correlation between flavan-3-ols accumulation and root growth. The enlarged root system is very important for providing tolerance against stresses in plants (Werner et al. 2010). It also increases the ability of plants to survive under nutrient deficient conditions (Coque and Gallais, 2006). Alteration in root growth, root length and lateral root density in flavonoid pathway mutant plant has indicated the flavonoid mediated modulation in their root morphology (Buer et al. 2010). Increase in root growth was observed in Vetiveria zizanioides (L.) accumulating polyphenols under the influence of 50 and 300 mM salt stress (Mane et al. 2011). Enhanced root elongation and tolerance to drought stress has also been observed in Arabidopsis overexpressing pepper PME inhibitor protein, CaPMEII (An et al. 2008). In CsF3H overexpressing transgenic tobacco lines, the higher degree of pectin methyl-esterification due to increase in flavan-3-ols might be responsible for longer root system compared to control plants in response to salt stress. Similar enhanced root system has been observed previously in Arabidopsis overexpressing arginine decarboxylase gene (PtADC) from Poncirus trifoliate. Overexpression of PtADC gene has conferred tolerance to plants against drought, cold and osmotic stresses (Wang et al. 2011b). The presence of vigorous root system due to trehalose accumulation has also conferred tolerance to transgenic rice against salt stress (Garg et al. 2002). Earlier study has shown that elevation of PME activity induced dwarfism in transgenic tobacco plants (Hasunuma et al. 2004). The increase in the amount of pectins, especially in those having a low degree of methyl-esterification, has been regarded as a symptom of the defense
strategy and plant adaptation against elevated levels of heavy metals in the soil (Krzesłowska, 2011). Higher degree of pectin methyl-esterification has also been reported in response to lead in *Allium cepa* root cells (Wierzbicka, 1998) and to cadmium in *Linum usitatissimum* hypocotyls (Douchiche et al. 2007), *Salix viminalis* (Vollenweider et al. 2006), and *Oryza sativa* (Xiong et al. 2009). An aluminium induced increase in pectins was detected in roots of *Cucurbita maxima* (Le Van et al. 1994), *Triticum aestivum* (Tabuchi and Matsumoto, 2001; Hossain et al. 2006), *Zea mays* (Schmohl and Horst, 2000; Schmohl et al. 2000) and *Solanum tuberosum* (Schmohl et al. 2000).

Thus the results clearly demonstrate that the elevation of endogenous flavan-3-ols levels has conferred tolerance to *CsF3H* overexpressing transgenic tobacco against salt stress by improving root growth, antioxidant system and increasing pectin methyl-esterification.

### 5.4.2.2 Increased protective ability against *Alternaria solani*

Early blight disease caused by necrophytic fungus like *Alternaria solani* is one of the major biotic stress for crops belong to Solanaceae family (Franc and Christ, 2001). The infestation by *A. solani* causes significant loss to the crop yield (Van der Waals et al. 2001; Kapsa and Osowski, 2003; Patel et al. 2004). Therefore, development of transgenic crops tolerant towards *A. solani* can be a promising option. In this regard, leaves of transgenic tobacco plants overexpressing *CsF3H* showed reduced lesion diameter after inoculation with *A. solani* as compared with inoculated leaves of control tobacco plant. It has been shown earlier that pectin methyl-esterification of cell wall decreased the accessibility to pectin degrading enzymes and hence considered to increase resistance against pathogens (Boudart et al. 1998). A role of pectin methyl-esterification of the cell wall in plant disease resistance has been reported in several pathosystems. For instance, highly methylesterified pectin has been related to resistance of potato (*Solanum tuberosum*) cultivars to Erwinia soft rot (Marty et al. 1997). Overexpression of PME inhibitor AtPMEI1 in *Arabidopsis* has conferred tolerance to fungus *Botrytis cinerea* (Lionetti et al. 2007). Potatoes with higher pectin methyl-esterification were found to be resistant to Erwinia soft rot (Marty et al. 1997). *Phaseolus vulgaris* accumulating pectic fragments with higher degree of methyl-esterification was resistant towards *Colletotrichum lindemuthianum* (Boudart et al. 1998). Similarly, *Triticum aestivum* with different methyl ester distribution in HGA was found to be tolerant towards a fungus *Puccinia graminis* f. sp. tritici (Wietholter et al. 2003). Since endo-polygalacturonase of fungus prefers unesterified pectins rather than methyl esterified
pectins as their carbon source (Kars et al. 2005), it is likely that higher degree of pectin methyl-esterification in CsF3H transgenics due to inhibitory action of flavan-3-ols on PMEs, restricted the growth of fungus *A. solani*.

Also improved antioxidant system due to high level of flavan-3-ols in CsF3H overexpressing transgenics might have favoured plant resistance to *A. solani*. The role of antioxidant enzymes in different defensive mechanisms have been shown through many studies. Leaf rust infection has caused the activation of CAT and GST enzyme activity in resistant wheat genotypes (Ivanov, 2005), while stripe rust infection has stimulated peroxidases, CAT and other antioxidant enzymes in resistant cultivars of wheat (Asthir et al. 2010). Similarly, upregulation in the enzymatic activities of AP₅, GR, CAT and GST has been observed in different lines of wheat and flax upon infestation with powdery mildew (Ashry and Mohamed, 2011; Kovacs et al. 2011). Therefore, higher degree of pectin methyl-esterification and improved antioxidant system might be responsible for providing resistance against *A. solani* to CsF3H overexpressing transgenics tobacco.

### 5.5 Characterization of *NtFLS* silenced transgenic tobacco plants

The flavonols act as regulator of auxin transport and have role in plant reproduction and fertility (Mo et al. 1992; Kuhn et al. 2011). Downregulation of *NtFLS* gene encoding flavonol synthase in tobacco might have affected these processes. Therefore, the influence of *NtFLS* silencing was also studied on such plant functions.

#### 5.5.1 *NtFLS* silencing reduced pollen growth and fertility

Flavonols, a class of flavonoids have especially been shown to have strong stimulatory effect on pollen development, germination, pollen tube growth, and seed set (Mo et al. 1992; Ylstra et al. 1992, 1996). We have observed through *in vitro* as well as *in vivo* studies a strong inhibition in pollen tube growth of self-pollinated *NtFLS* silenced tobacco that has ultimately resulted in a seed set arrest. This could be due to decrease in flavonol content of *NtFLS* silenced transgenic tobacco achieved through silencing of gene encoding *NtFLS*. All strong *NtFLS* silenced transgenic tobacco lines have yielded fruits with significantly less number of seeds. The *in vitro* and *in vivo* experiments conducted on these silenced transgenic tobacco revealed that 1µM quercetin was sufficient to rescue the inhibited pollen germination and pollen tube growth. All these observations support the hypothesis that flavonols particularly quercetin is essential for pollen germination in
tobacco. Earlier, petunia plants with antisense *CHS* or sense *CHS* cosuppression have produced white flowers with male sterile character due to complete blockage of flavonoids production (Van der Meer *et al*. 1992; Napoli *et al*. 1999). The male sterility in these mutants was due to the failure to produce a functional pollen tube. Addition of kaempferol at the time of pollination could restore the male sterility in petunia flowers (Mo *et al*. 1992). These mutant plants were conditional male sterile as flavonoid deficient pollens did not function in self-crosses but were partially functional on wild-type stigmas containing flavonols (Mo *et al*. 1992). This has led to the assumption that *CHS*-deficient pollens lack factors that are required for pollen tube growth. In these cases, such stigmas were functionally complemented with flavonols (Taylor and Jorgensen, 1992). Similarly, inability of pollens from male sterile petunia white anther (*wha*) mutant to germinate normally have been found to be complemented by flavonol addition (Napoli *et al*. 1999).

The silencing of *CHS* gene in tomato was resulted in parthenocarpy. But it was not identified whether the cause of this phenomenon is the lack of flavonols (Schijlen *et al*. 2007). However, our study has provided the first report on the specific role of flavonol (quercetin) in pollen germination through *in vitro* and *in vivo* experiments in *NtFLS* silenced transgenic tobacco. In addition, this study has also documented the application of *FLS* silencing as a novel approach to obtain less seeded fruits.

### 5.5.2 *NtFLS* silencing reduced free IAA content and altered plant morphology

*NtFLS* silenced transgenic tobacco plants were found to be smaller in height, showed delayed flowering and produced lesser number of flowers/ fruits. It has been known earlier that plant hormones play an important role in regulating fruit development. Indole acetic acid (IAA), one of the endogenous auxin in the plants is considered to be a critical determinant of plant growth control (Casimiro *et al*. 2003; Friml, 2003; Grebe, 2004). A possible direct role of flavonoids in auxin distribution has been proposed earlier by several research groups (Brown *et al*. 2001; Taylor and Grotewold, 2005). Loss of CHS activity in *Arabidopsis* has caused an increase in polar auxin transport (Brown *et al*. 2001). Additional evidence that flavonoids act as auxin transport inhibitors has also been obtained from experiments with *CHS* silenced *Medicago truncatula* plants. The flavonoid-deficient roots of these plants have shown an increase in their auxin transport relative to wild type. These plants were also unable to initiate root nodulation (Wasson *et al*. 2006). Amongst the flavonoids, flavonols have been considered to be highly active in inhibiting auxin transport (Kuhn *et al*. 2011; Hassan and Mathesius *et al*. 2012). Flavonols can compete
with the auxin transport inhibitor 1-N-naphthylphthalamic acid for binding to proteins involved in auxin transport (Jacobs and Rubery, 1988; Noh et al. 2001; Murphy et al. 2002). The kaempferol overaccumulator tt7 mutant has been found to be affected in auxin transport (Peer et al. 2004). Hence, several experiments have hinted that flavonols are involved in modifying auxin transport (Peer and Murphy, 2006). To confirm this consideration, free IAA levels were estimated in NtFLS silenced tobacco. Reduction in flavonol (quercetin) content upon NtFLS silencing has increased the IAA transport towards the roots, thereby decreasing free IAA content in shoot apical region of NtFLS silenced lines. This lower IAA content could be responsible for delayed flowering and inflorescence with less number of flowers. Further, decreased IAA content could also be the reason for short and kinked shape of the pollen tubes in NtFLS silenced lines compared with the control pollen tubes. The results suggest that IAA is one of the most important hormones regulating pollen tube growth. Results further provided the in planta confirmation to assumption of earlier study that IAA plays an important role in the regulation of pollen germination (Wu et al. 2008). Thus, the delayed flowering, less number of flowers, inhibition in pollen germination and less seeded fruits in NtFLS silenced tobacco could be under quercetin-mediated free IAA regulation.

5.6 Effect of flavonoids application on growth and development of tobacco seedlings

In vitro grown wild tobacco seedlings were used to study the influence of EC and Quer on growth and development as well as on flavonoid and antioxidant system. The exogenous application of two flavonoids EC and Quer at 50 and 100 μM concentration has upregulated the expression of genes encoding flavonoid biosynthetic pathway enzymes such as NtPAL, NtCHI, NtF3H and NtFLS in tobacco shoots compared to untreated control shoots. The upregulation in gene expression of some of these enzymes was also found with the lower dose of these flavonoid exposure. The expression of genes influenced by lower dose of flavonoid exposure can be categorized as early responsive genes and other can be late responsive. The decrease in expression level of NtCHS at 100 μM EC exposure in tobacco shoots might be due to negative regulation at higher dose (Pelletier et al. 1999). The transcript expression of NtPAL, NtCHI, NtF3H and NtFLS showed downregulation in flavonoid exposed root of wild tobacco. Thus increased expression of genes encoding flavonoid pathway enzymes in flavonoid exposed tobacco shoots has
suggested higher accumulation of flavonoids in the aerial part of the seedlings. There was higher levels of total flavonoids content in the shoot than in root region of such treated seedlings. More free IAA accumulation was observed in shoot region compared to root region of exposed tobacco seedlings, suggested the flavonoid mediated regulation of IAA accumulation. Flavonoids have been known to act as endogenous regulators of auxin transport (Brown et al. 2001; Taylor and Grotewold, 2005). Under this aglycone flavonols like quercetin and kaempferol have been reported as highly active inhibitors of auxin transport than glycosides (Hassan and Mathesius, 2012). Hence, free IAA retention in shoot portion of tobacco seedlings might be responsible for various observed morphological and developmental events.

Auxin is required at several developmental stages including lateral root formation. Lateral root primordial are unable to divide if excised from the primary root and its division can be rescued if supplemented with exogenous auxin (Dubrovsky et al. 2001). Further it has been reported that higher levels of auxin (IAA) in the aerial part or shoot of a plant resulted in inhibition of lateral and adventitious roots formation (Reed et al. 1998). Similar inhibition in lateral and adventitious root formation was also observed in the exogenously flavonoid exposed wild tobacco seedlings. While application of IAA to growing plants stimulates lateral root development (Blakely et al. 1982; Muday and Haworth, 1994). Conversely, growth of tomato roots on agar containing auxin-transport inhibitors, including NPA, has decreased the number of lateral roots (Muday and Haworth, 1994). A negative correlation was found between the degree of branching in root systems and the amount of NPA-binding activity present in roots in different species of plants (Lomax et al. 1995). Thus, these evidences support the fact that free IAA is necessary for lateral root formation. While inhibition of these lateral and adventitious roots is mediated by the blockage of free IAA transport resulted from flavonoid accumulation.

Effect of flavonoid exposure was further studied on vascular system of tobacco seedlings. Variation in dosage of flavonoid applications has altered the vascular system of a leaf in a predictable fashion. This could be because of the free IAA retention in aerial portion of the seedlings induced by increased flavonoid accumulation. Numerous parallel vessels extended towards the root apex were found only in the seedlings exposed to 100 μM EC. This might be due to the inhibition of basipetal free IAA transport from root apex towards the root base. Also, this could be a result from more flavonoid accumulation in the root tip only and lesser in the root as a whole. Similar responses have been reported to be evoked by three synthetic auxin transport inhibitors TIBA, NPA and HFCA in
Chapter 5          Discussion

Arabidopsis. Later experiment had revealed that auxin transport is required for vascular tissue continuity and the restriction of vascular differentiation to narrow strands (Mattsson et al. 1999).

Exposure to higher dose of EC and Quer has inhibited plant growth to a much higher level as compared to their lower dose exposure and untreated control. This finding is supported by the fact that NPA has restricted leaf auxin translocation and concurrently reduced leaf size in Arabidopsis and bean (Keller et al. 2004). Although, the exact mechanism of auxin in controlling leaf expansion remains unclear. The increase in auxin level has been observed to have negative effects on leaf expansion. Decreased cell size of leaf epidermis, palisade and spongy parenchyma was observed in tobacco seedlings exposed to 100 μM EC and Quer. This decrease in cell size could be due to flavonoid-mediated free IAA retention in the leaf of seedlings treated with higher doses of EC and Quer. Such changes in leaf cell size have earlier been reported through various studies. Transgenic petunia overproducing auxin has developed epinastic, smaller and narrower leaves than non-transgenic control plant (Klee et al. 1987). Similarly, Arabidopsis mutants sur1 and sur2 overproducing auxin have resulted in less expanded leaf (Boerjan et al. 1995). Also, the application of exogenous auxin to bean (Phaseolus vulgaris) and Arabidopsis leaf blades have inhibited long-term blade elongation (Keller et al. 2004). A low auxin concentration has been documented to drive cell elongation and cell enlargement in tobacco cell culture (Zazimalova et al. 1995; Winicur et al. 1998). Hence, higher level of flavonoid in the aerial region was resulted into inhibition of basipetal IAA transport from aerial region to root of the tobacco seedlings. This inhibition in free IAA transport was found to be responsible for the decrease in root system and several morphological and anatomical alterations in flavonoid exposed tobacco seedlings. Flavonoids are co-localized to the tissues that transport auxin (Murphy et al. 2000; Peer et al. 2001) and to the plasma membrane (Peer et al. 2001) where auxin transport inhibitor binding site is localized (Dixon et al. 1996). These characteristics make flavonoids suitable endogenous regulators of auxin transport.

Furthermore, influence of exogenous application of Quer and EC was also studied on the transcript level of genes encoding antioxidant enzymes and activities of antioxidant enzymes like GR, APx, CAT and GST in tobacco shoot and root. EC was effective in increasing the expression and activity of these antioxidant enzymes at lower concentration, while Quer was effective at higher concentration. Earlier literature has suggested differential level of expression of genes encoding antioxidant enzymes in various parts of
the plant (Mohanpuria et al. 2007; Gill and Tuteja 2010). Our results document that expression of genes encoding antioxidant enzymes is dependent on the concentration and nature of flavonoids.

Few biochemical studies conducted earlier have also documented the interaction of flavonoid and antioxidant enzymes. An increased antioxidant activity in phenolics and flavonoids rich fractions of *Convolvulus arvensis* (Elzaawely and Tawata, 2012) and *Vernonia blumeoides* leaves (Aliyu et al. 2011) have been reported. A positive relationship between increased flavonoids and antioxidant enzyme activity has been reported in seeds of *Aframomum sceptrum* (Erukainure et al. 2011). The GPx activity has been reported to be activated by the action of flavonoids, quercetin and catechin (Nagata et al. 1999). The quercetin and its derivatives have been found to prevent oxidative cell damage by either reducing the activity of glutathione peroxidase or increasing glutathione level (Cruz et al. 1998). Flavonoids have also been reported to protect the cells from glutathione depletion with the cooperation of ascorbic acids (Skaper et al. 1997). Taken together, flavonoids might be regulating antioxidant system by acting at transcriptional and post-transcriptional levels.

5.7 Conclusions
The present study has addressed some important findings.

1. Overexpression of *CsF3H* gene encoding flavanone 3-hydroxylase and downregulation of *NtFLS* gene encoding flavonol synthase has increased flavan-3-ols accumulation and decreased anthocyanins content in transgenic tobacco through significant upregulation in genes encoding NtDFR and NtANS enzymes of flavonoid pathway.

2. Transgenic tobacco plants show improved antioxidant potential as well as enhanced transcript expression of genes encoding antioxidant pathway enzymes and their activities.

3. Increased root growth, chlorophyll content and decreased MDA content, electrolyte leakage along with the improved antioxidant system and pectin methyl-esterification has provided tolerance to salt stress and fungus *Alternaria solani* in *CsF3H* overexpressing transgenic tobacco.
4. Reduction in *NtFLS* transcript level as well as quercetin content was achieved through post-transcriptional silencing of gene encoding NtFLS in silenced transgenic tobacco.

5. The pollen germination and pollen tube growth was reduced in *NtFLS* silenced transgenic tobacco. This could be the reason for the production of fruits with significantly less number of seeds.

6. The pollen germination in *NtFLS* silenced tobacco was found to be rescued upon quercetin supplementation, thus documented the role of flavonols particularly quercetin in pollen germination as well as in the regulation of plant fertility.

7. The lesser quercetin content in *NtFLS* silenced transgenic tobacco has increased the polar free IAA transport towards the root. This has decreased endogenous concentration of free IAA in apical region of shoot. Thus, the delayed flowering, inhibition in pollen germination and less seeded fruits in *NtFLS* silenced transgenic tobacco was found to be under quercetin-mediated free IAA regulation.

### 5.8 Future prospects

The information generated during the course of present study can be used to carry out future work as follows:

1. The strategy of overexpression of *CsF3H* can be used to engineer flavonoids in variety of plant species for improving nutritional and health related traits.

2. The multivariate functions of *CsF3H* can further be exploited for understanding the physiological significance of flavan-3-ols in plant disease resistance and abiotic stress tolerance.

3. The present study presented the cross talk between flavonoid and antioxidant pathways. This would help in tailoring the crops for enhanced flavan-3-ols production for making them resistant to oxidative stresses.

4. The mechanism behind the role of quercetin in increasing the pollen germination can be explored further.

5. The role of flavonols in seed set can be explored in two ways 1) the problem of seed set can be rescued by imparting flavonols to the plants, and 2) direct