Is *Nicotiana attenuata's* defense metabolite chlorogenic acid useful or harmful to its specialist herbivore *Manduca sexta*?

Master thesis

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1 ABSTRACT

1.1 English abstract

Plants produce various secondary metabolites. Several plants produce a phenolic compound chlorogenic acid (CGA).There has been a sizable amount of literature on the effect of chlorogenic acid on herbivore insects. Interestingly, it has been observed to exert positive, negative or no effects, highly depending upon the diet through which it was ingested. It was surmised that if CGA ingested through artificial diet, it exerts negative effect and if it is ingested through the plant, it exerts positive or no effect on *N. attenuata'*s specialist herbivore *Manduca sexta*. To study the effect of CGA on *M. sexta* when CGA is ingested through *N. attenuata,*we silenced *N. attenuata* hydroxycinnamoyl-CoA quinatetransferase (*Na*HQT; key enzyme in CGA biosynthesis) through virus-induced gene silencing (VIGS) and generated *N. attenuata* transient lines with suppressed CGA (*Na*HQT-VIGS). *M. sexta* larvae grown larger on *Na*HQT silenced plants than the larvae growing on wild type plants, suggesting that CGA ingested through *N. attenuata* is harmful to *M. sexta*. A midgut expressed carboxylesterase (COE) gene was down-regulated in *M. sexta* larvae feeding on *Na*HQT-VIGS plants. COE was also up-regulated in larval midgut in response to CGA when larvae fed on artificial diet containing CGA. To observe the correlation between *M. sexta* carboxylesterase (*Ms*COE) and CGA, we generated the *N. attenuata* plants with inverted repeats (*ir*) of *Ms*COE (irCOE lines). The transcript levels of COE are significantly decreased when larvae fed on *ir*COE and *Na*HQT-VIGS plants. Taken together, *Ms*COE is induced in response to CGA ingestion and may play a role in metabolizing CGA to reduce the negative effect of CGA on *M. sexta* larvae.

1.2 Deutsche Zusammenfassung

Pflanzen produzieren verschiedene Sekundärmetaboliten . Mehrere Pflanzen produzieren eine phenolische Verbindung Chlorogensäure (CGA). Es hat über die Wirkung von Chlorogensäure auf herbivore Insekten eine beträchtliche Menge an Literatur. Interessanterweise wurde beobachtet , positive , negative oder keine Wirkung ausüben , stark abhängig von der Ernährung durch die es eingenommen wurde. Es wurde vermutet , dass, wenn CGA eingenommen durch künstliche Ernährung, negative Wirkung ausübt , und wenn es durch die Anlage aufgenommen wird , auf spezialisierte Pflanzenfresser *Manduca sexta N. attenuata* ist es positiv oder übt keine Wirkung. Um die Wirkung der CGA auf *M. sexta* studieren, wenn CGA durch *N. attenuata* eingenommen , Schweigen wir N. attenuata Hydroxycinnamoyl -CoA quinatetransferase (*Na*HQT ; Schlüsselenzym in der Biosynthese CGA) durch Virus -induzierte Gen-Silencing (VIGS) und erzielte *N. attenuata* transiente Linien mit unterdrückter CGA (*Na*HQT - VIGS) . *M. sexta* -Larven größer geworden auf *Na*HQT Schweigen Pflanzen als die Larven wachsen auf Wildtyp-Pflanzen , was darauf hindeutet , dass CGA eingenommen durch *N. attenuata* ist schädlich für *M. sexta* . Ein Mitteldarm ausgedrückt carboxylesterase (COE) -Gen wurde in *M. sexta* Larven ernähren *Na*HQT - VIGS Pflanzen herunterreguliert . COE wurde auch in Larvendarmhochreguliert in Reaktion auf CGA wenn Larven auf künstliche Ernährung mit CGA zugeführt. Um die Korrelation zwischen *M. sexta* carboxylesterase (*Ms*COE) und CGA beobachten , erzielten wir die N. attenuata Pflanzen mit invertierten Wiederholungen (ir) von MsCOE (*ir*COE Linien). Die Transkriptspiegel COE sind deutlich zurückgegangen , wenn Larven auf *ir*COE und *Na*HQT VIGS - Pflanzen gefüttert . Zusammengenommen wird *Ms*COE in Reaktion auf CGA Einnahme induziert und kann bei der Metabolisierung von CGA um die negativen Auswirkungen von CGA auf *M. sexta* Larven reduzieren eine Rolle spielen.

2. INTRODUCTION

2.1 Introduction to phenolic compounds and CGA:

Phenolic compounds such as caffeic acid (CA), CGA, rutin may play important roles in plant herbivore interactions. From past several decades, particularly the interaction between herbivores and plant phenolics has been has been an intense area of study (Appel, 1993). During plant evolution the herbivores are considered as primary selective agent for phenolics (Harborne, 1979). The toxicity of cinnamic acid derivatives, CGA and the flavonoid glycoside, rutin are well documented (Sondheimer, 1964; Harborne, 1979, 1991; Isman and Duffey, 1983). These componds slow down the growth of lepidopterans such as *M. sexta*, *Helico verpazea*, *Heliothis virescens* when supplimented with artificial diet.

The toxicity of CGA is due to its tendency to convert into the oxidized form enzymatically by polyphenol peroxidase, laccase or peroxidase under alkaline conditions or in the presence of transition metals (Felton et al., 1989; Appel, 1993). This oxidation leads to the formation of quinones that covalently bind to proteins, thus negatively affects the bioavailability of nutrients and also results in the formation of free radical oxygen species. These free radicals damage the proteins, lipids, nucleic acids (Felton et al., 1989, 1992; Appel, 1993; Summers and Felton, 1994).

CGA is synthesized in three distinct pathways (Hoffmann, L. et al., 2003; Niggeweg, R, et al., 2004; Villegas, R, Kojima, M, 1986). 1) The trans-esterification of quinic acid and caffeoyl-CoA via hydroxyl cinnamoyl-CoA by quinate hydroxyl-cinnamoyl transferase (HQT) activity (Zenk, M, 1974, 1979). 2) From p-coumaryol quinate to CGA by hydroxylation (Niggeweg, R, et al., 2004) and 3) by the activity of shikimate hydroxy cinnamoyl transferase (HCT) converts caffeoyl shikimic acid to caffeoyl CoA, a substrate of hydroxyl cinnamoyl CoA (Hoffmann, L. et al., 2003). The silencing of HQT gene does not affect the lignin formation, but the CGA level was reduced by 98% in tobacco and tomato; from the above silencing at least in these species first two pathways are responsible for the synthesis of CGA (Niggeweg, R, et al., 2004). On other side, a change in lignin amount and composition is due to a lowered HCT expression in tobacco, thereby third pathway implicating the lignin biosynthesis.

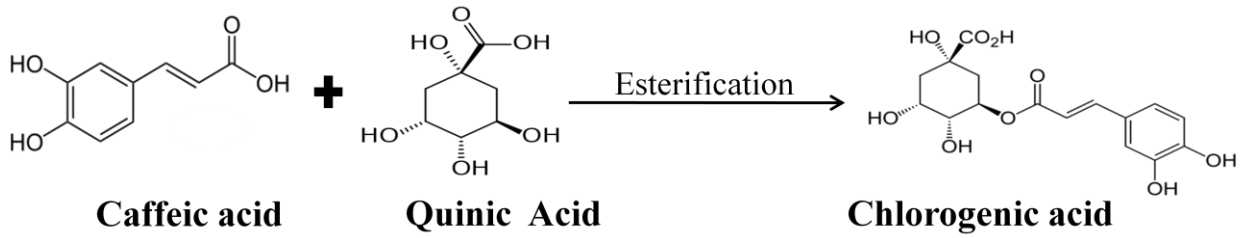

Figure 1: Biosynthesis of CGA in Phenylpropanoid pathway

2.2 Ultraviolet radiation and plant secondary metabolites

UV-B is the small fraction of the solar radiation that has effect on plant-herbivore interactions by regulating the levels of plant secondary metabolites. Based on these responses caused by UV-B, investigators defined them as "UV-specific" and "non-specific" (Patricia V. Demkure et al., 2010). The signaling pathways which are UV-B specific should not be activated by other environmental stressors such as wounding etc. This principle is frequently used for case, to figure out UV-B responses via the UV RESPONSE LOCUS 8 (UVR8)-CONSTITUITIVELY PHOTOMORPHOGENIC 1 (OP1) pathway in Arabidopsis (*Arabidopsis thaliana*; Browm et al., 2005; Brown and Jenkins, 2008; Favory et al., 2009), because UVR8-COP1 pathway is not activated by general stressors and does not lead to expression of "general stress markers" (Colix and Jenkins, 2008; Jenkins, 2009). Other researchers considered UV-B specific responses would be shown due to the result from the activity of a receptor which has maximum sensitivity in the UV-B region. This is considered as photo biological principle based on the wavelength specific responses. UV-B has been registered strongly for the characterized effects between plant and insect herbivores (Cladwell et al., 2003; Bassman, 2004; Roberts and paul, 2006). The best characterized UV-B radiation is to enhance the accumulation of phenolic compounds as role of defensive mechanism.

The group of lipid hormones known as jasmonates plays a role in defensive mechanisms against the herbivorous insects (Wasternack, 2007; Howe and Jander, 2008; Browse, 2009). The hypothesis of UV-B response on jasmonate mediated plant defense remains controversial because of the expression of proteinase inhibitor genes by germicidal UV-C radiation or very high doses of UV-B radiation in Solanum lycopersicon (Conconi et al., 1996; Stratmann et al., 2000). UV-A/UV-B failed to induce proteinase inhibitor in non wounded leaves. On the other

hand the proteinase inhibitor induced by UV-A/UV-B on mechanical damage of leaves but without increasing the levels of jasmonic acid (Stratmann et al., 2000). The expression of genes involved in fatty acid and oxylipin metabolism and the phenolic products accumulation was increased but not the expression of trypsin protease inhibitor (TPI) in non wounded *Nicotiana longifora* plants when exposed to solar UV-B radiation (Izaguirre et al., 2003).

2.3 *Manduca sexta*:

M. sexta is more common in North American continent and it belongs to the family Sphingidae. It feeds on he leaves of plants belonging to the family Nightshades (Solanaceae). It is also known as tobacco horn worm, because it develops a horn which is reddish brown in color located dorsally on the terminal abdominal segment. Especially in neurobiology *M. sexta* is specimen organism for conducting experiments due to its short life span and easily accessible nervous system. It has a short life cycle of 4 to 7 weeks. Eggs of *M. sexta* are spherical in shape and translucent green in color (Villanueva, Raul, 2009). *M. sexta* can grow up to 6 to 8 centimeters during its life cycle. *M. sexta* haemolymph is green in color due to the presence of a blue green protein pigment called insecticyanin. During its life cycle, larval stage under goes molting process (Ecdysis) which indicates the larval development from one stage (instar) to other. Normally they undergoes to five larval instars. Later it undergoes to pupae stage for 12 to 17 days depending on the conditions for growth. Adult moth known as Carolina sphinx emerges from pupal case during eclosion (Weston Tripp, Matt Edward, 2000).

The larvae mostly feed on leaves, naturally the plants increases the production of secondary metabolites as a defensive mechanism to overcome the damage due to insect herbivores. The insects also produce enzymes or other chemical compounds to metabolize and detoxify the plant secondary metabolites which are hazardous to them.

2.4 Carboxylesterases (COE):

Carboxylesterases (CES; EC 3.1.1.1) comprise a multigene family and pervasive in all living organisms. These enzymes are broadly distributed in insects (Cambell, P. M et al., 1997; Heidari, R, 2004, 2005, 2007; Strode, C, 2008). Caboxylesterases efficiently catalyzes the hydrolysis of a variety of ester and amide containing compounds to their component alcohols and acids by the addition of water. They are involved in variety of physiological functions such as

detoxification, metabolism and also involved in insect development and behavior (Gilbert, L. I. et al., 2000; Oakeshott, J. G. 1999; Taylor, P and Radic, Z. 1994). The endogenous compounds such as acyl-glycerol, acyl-carnitine and acyl-CoAs are also hydrolyzed by caboxylesterases. The mechanism of hydrolysis reaction occurs in one or two steps ranging across general acid/base, electrostatic and covalent catalyses (Oakeshott, J. G. et al., 2005; Voet, D. Voet, J.G. 1990).

2.4.1 Classification of Carboxylesterases:

The classification of carboxylesterases is difficult due to their overlapping substrate specificity. Earlier, these enzymes are classified in to A, B, and C type esterases based on their interaction with organo phosphorous compounds (OPs) (Aldridge, W. N. 1953, 1993). According to this classification, most of the insect esterases are classified as B-type because they are inhibited by OPs. A-type esterases are not inhibited by OPs and also hydrolyse uncharged esters and C-type enzymes that neither hydrolyze nor inhibited. The Reiner and Aldridge (1972) scheme has been used to classify eukaryotic esterase isozymes detectable after native PAGE (polyacrylamide gel electrophoresis) relied on the application of inhibitor sensitivities to subdivide the nonlipase group of catalytic ester hydrolysis (Aldridge, W.N, Reiner, E. 1972).

Further insect esterases can be subdivided into 14 major branches and set down into three big groups at the higher level on the classification scheme of Oakesholt et al., 2005. The first group (I-N) is intracellular class which contains membrane associated, secreted, non-catalytic proteins and AchE clade involved in neuro developmental process. The second group (D-H) is Secreted catalytic class composed of secreted catalytic proteins as well as β-esterases. The third groups (A-C) neuro developmental class mainly composed of intracellular catalytically active proteins involved in dietary and detoxification functions and has broad substrate specificities.

2.4.2 Mechanism of carboxylesterase hydrolysis:

Carboxylesterases, hydrolyzes their substrate in two steps. First, the nucleophilic attack on the cabonylcarbon of the substrate by the oxygen of a serine residue in the active site of an enzyme, which displace the alcohol product and forms a stable acyl-enzyme. In the second step, similar nucleophilic attack was made by water (Myers, M. et al., 1988; Ollis, D. L. et al., 1992; Satoh, T., Hosokawa, M. 2006; Wheelock, C.E. et al., 2008) displacing the serine residue to release the

acid product of the reaction and regenerates free enzyme. During both nucleophilic substitution reactions the "oxyanion hole" reduces the tetrahedral transistion state of energy in which the charge is placed on to oxygen take in by hydrogen bonding to the main-chain nitrogens of the active site (Oakeshott, J.G. et al., 2005, Voet, D. Voet, J.G. 1990). This may be the xenobitic resistance of carboxylesterase in insects.

The insects have evolved predominantly against xenobiotic resistance by increasing the detoxification systems and/or reducing xenobiotic target site sensitivity (Li, X. et al., 2007). The up-regulation of esterase genes has been implicated the resistance mechanisms of species in the orders of Lepidoptera, Diptera and Hemiptera (Field, L.M., Devonshire, A.L., 1998, 2000; Hemingway, J. et al., 1998, 2002). *Bombyx mori* has the largest number of insect caboxylesterases (Quan-you yu et al., 2009). Midgut is the first barrier of xenobiots preoral toxicity, in which carboxylesterases may be involved in eliminating or detoxifying the secondary metabolites in the diet (Quan-you yu et al., 2009).

2.5 *Agrobacterium tumifaciens*

Agrobacteruim is a unique plant species bacteria involved in plant-fungal-animal transmission. It is a soil born bacterium closely related to *Rhizobium*. It causes crown gall disease in plants (dicots). Virulent strains of *A.tumifaciens* contain a 200kb tumor inducing plasmid (Ti plasmid) and infect plants through wounds (Dubey, R.C, 2008). *Agrobacterium* infection has been extensively utilized as a vector for transferring desired genes into plant cells. Even though transgenic soyabean (*Glycine max*) plants have been obtained, the seed legumes are the important species that have not responded well by this transformation process (Gupta, P.K, 2006). The success in this approach for gene transfer has resulted from improvement in tissue culture technology. When compared to dicotyledons, monocotyledons could not be successfully utilized for *Agrobacterium* mediated gene transfer, except in *Asparagus*. The reasons for this are not fully understood, because of T-DNA transfer does not occur at cellular level (Gupta, P.K, 2006).

2.5.1 T-DNA transfer process

T-DNA transfer process starts with the event of nicking the Ti plasmid at two specific sites, each between the third and fourth base of the bottom strand of each 25bp repeat. This event

initiates DNA synthesis from the nick in the right hand 25bp repeat sequence in 5'-3' direction, thus displacing a single T-DNA strand (Gupta, P.K, 2006). The T-DNA single strand forms a complex with protein *vir E* and gets transported to the plant nucleus. The endonuclease that encoded by *vir D* operon that produces the nick in the border sequences. The gene products of the operon *vir B* play a role in directing T-DNA transfer extracellularly. The several products of *vir B* operon have been identified in bacterial envelope (Grierson, D, 1991). Other than the role of Ti plasmid, the genes located on the chromosome of *Agrobaterium* also help in virulence. These genes are involved in the synthesis and secretion of glucons, cellulose fibrils and cell surface proteins. These loci play a role in virulence and also *Agrobacterium* mediated gene transfer.

2.6 Tobacco rattle virus (TRV)

The genus *Tobravirus* (Family: Virgaviridae) was subdivided in to 3 subgroups. 1) Tobacco rattle virus (TRV), 2) Pea early browning virus (PEBV) and 3) Pepper ring spot virus. The Tobacco rattle virus group name is originated because, when the wind blows through a heavily infected tobacco field a rattle like sound is produced by TRV-infected dried leaves. Geographically TRV has been recorded throughout the Europe, North America, New Zealand and Japan. TRV is a bipartite plant virus (Webstter, R.G, Granoff, A, 1994).

2.6.1 Virus structure and transmission

The virus genome is composed of two single stranded RNA molecules, RNA 1 and RNA 2, which are separately encapsulated by polypeptide subunits. The virion particles are of similar diameter (20 to 23 nm) and different lengths with rigid rod-shaped. The L (large) particles encapsidates RNA 1 and have similar size (180-210nm). Whereas, the size of S (small) particles (50-110nm) varies between different strains even in one subgroup and therefore, the difference in size of encapsidated RNA 2 depends on size of the S particles (Webstter, R.G, Granoff, A, 1994).

Naturally Tobraviruses are transmitted by root feeding nematodes, Trichodorus. The virus does not replicate in nematode but can persist there for months to years. TRV particles are associated with the lining of pharynx and esophagus of nematodes. When nematode feed on a new plant the release of virus particles from the nematode is thought to occur by a change in pH of the saliva.

TRV probably be the widest of any plant virus that infects more than hundred species including several common weed species (Webstter, R.G, Granoff, A, 1994). The disease symptom on leaves varies from all kinds of yellow markings (blotching, mottling, mosaic and ringspot) to necrosis. Symptoms on underground plant parts include corky arcs in potato tubers, necrotic spots in hyacinth bulbs which lower the vigor, yield and value of crops.

2.7 Mechanism of RNAi

The RNAi was popularized by work in *Caenorhabditis. elegans*. When long dsRNA were injected into a worm's gonod, the standard way of introducing transgenes to worms they blocked the expression of endogenous genes in a sequence specific manner. In eukaryotes most protein coding genes are transcribed by RNA polymerase II, which generates premRNAs better then to process to form mature mRNAs. These mRNAs are then transported from the nucleus to the cytoplasm, where they are translated. RNAi can regulate endogenous gene expression. RNAi can be certain motion by genomically encoded by short regulatory RNAs known as microRNAs (Hannon, G., 2002). In worms and flies RNAi can be activated by endogenous transposition. In plants and cultured insect cells, RNAi also has a role in antiviral defense in which viral dsRNA was targeted for destruction by the RNAi machinery. When lond dsRNAs enter the cell they were recognized and cleaved by dicer, which is a member of the RNase III family of dsRNA specific endonuleases. Cleavage by dicer creates short dsRNAs, they are characterized by 20-25bp long with two nucleotide 3' overhangs. These are called small interfering RNAs or siRNAs (Borchardt, A.;,Tuschl, T., 2002). siRNAs can form a ribonucleotide complex called RISC (RNAi silencing complex). This RISC includes slicer, argonaute protein with RNase. RISC first mediates the unwinding of the siRNA duplex. A single stranded siRNA is coupled to RISC then binds to mRNA in a sequence specific manner. The binding mediates target mRNA cleavage by slicer. The side of the cleavage falls in the middle of the siRNA complementarity region. The cleaved mRNA can be recognized by the cell as being aberrant and then destroyed. This prevents translation from occurring, silencing the expression of the gene from which the mRNA was transcribed.

In plants the aberrant RNA that results from the RISC mediated cleavage can also serve as template for RNA dependent RNA polymerase (RdRP). This process relies on unprimed RNA synthesis in which the aberrant RNA is used as a template. The resulting dsRNA is a

substrate for dicer activity which generates more siRNAs. In some organisms with endogenous RNAi mechanisms for example fungi, plants, worms and mammals RNAi also involves in another amplification step. In this step single stranded siRNA is not associated with RISC, bind to target mRNA in a sequence specific manner and serve as a primer for RdRP to polymerize the antisense RNA strand. Such specificity is intrinsically sensitive to natural sequence variation. The dsRNA molecule that is created serves as a substrate for dicer, which cuts into siRNAs. In turn these can either unwind and prime RNA dependent RNA polymerization or together with RISC mediate the cleavage of target mRNA. This amplification is coupled with RNAi spreading between cells is thought to underline germline transmission of RNAi in worms. RNAi spreding is also described in plants but not mammals.

3. Aim

To find whether *Nicotiana attenuata*'s CGA is useful or harmful to *Manduca sexta.*

Background

Earlier it was that CGA exerts negative effect on herbivores. *Helicoverpa zea, Helicoverpa armigera* and *Manduca sexta* has retarded larval growth when fed on artificial diet with CGA (Isman and duffey, 1982; Kimmins et al., 1995; Osier et al., 1996). In case of *Manduca sexta,* no effects are observed when CGA ingested through phenylalanine-ammonia- lyase (PAL) over or under expressing tobacco foliage (Felton et al., 1997). At the same time this literature states that CGA exerts positive or no effects on the herbivores. So mostly it has been seen that CGA ingested through the synthetic diet exerts negative effect. Whereas, CGA ingested through the plant causes positive or no effects. It must be noted that several exceptions exist in both these cases. But all these literature agrees on one point, that the effect of CGA is highly dependent on diet with which it is ingested. As *M. sexta* is a specialist herbivore for *N.attenuata,* our group members found that a particular midgut expressed COE is induced upon CGA ingested through *N. attenuata*. Then to find the role of this COE in M. sexta's CGA metabolism they tried to silence it using PMRi. They could efficiently silence it and observed that the COE silenced larvae were growing slower than the controls. Another interesting observation was that, these larvae excreted very high proportion of CGA which they ingested. To find the role of COE in *M.sexta* an *invitro* assay was conducted using the crude midgut extracts of wild type plants fed larvae. The gut extract was incubated with CGA and it was found that CGA concentration

decreased after the reaction and caffeic acid (CFA) and quinic acid (QA) (which were not added to reaction) were found in this reaction. In other containing COE inhibitor CA and QA were found very low suggesting that COE cleaved CGA to CA and QA. From this assay it was not clear whether CGA was cleaved by our candidate COE. Because COEs are known to induce in a genral response to several xenobiotics, secondly in lepidopterans many COEs occur. As we know from the literature that CGA from plants exerts positive effects so we were wandering that COE silencing will increase the availability of CGA in larva. But if that was true why the mass of COE silenced larvae was lower than the controls. It should actually be higher with the help of this hypothesis we wanted to answer our ultimate question that whether *N. attenuata*'s CGA exerts positive or negative effects on *M. sexta*.

4. MATERIALS AND METHODS

4.1 Plant germination and growth conditions

Seeds of *N. attenuata* (31st generation) were germinated on agar plates containing Gamborg's B5 medium as previously described by Krugel *et al*., 2002. Seeds were sterilized for 5 min in aqueous solution containing dichloroisocyanuric acid with 0.1 g/ 5 ml (DCCA: Sigma, St. Louis, MO, USA), supplemented with 50 µl of 0.5% (v/v) Tween-20 (Merck, Darmstadt, Germany). Seeds were washed 3 times with sterile water before incubation for 1 hr in sterile liquid smoke (House of Herbs, Inc.; Passaic, New Jersey, USA) solution, 50x diluted in deionized water and supplemented with 50 µl of 0.1 M gibberellic acid, GA3(Roth, Karlsruhe, Germany). After this treatment, seeds were washed 3 times with sterilewater. 25 seeds were transferred individually to a petri dish containing germination medium of Gamborg's B5 with minimal organics and 0.6% (w/v) phytagel (Sigma). Plates were maintained in a growth chamber (Snijders Scientific, Tilburg, Netherlands) at 26^{o}C for 16 h light (155 µmol s-1 m-2) and 24°C for 8 hr dark in a period of 10 days.

4.2 Preparing and infiltration of *Agrobacterium*

Agrobacterium GV3101 containing pTV00 (EV), pTVHQT, pBINTRA constructs are inoculated in a Pre-culture of 10 mL YEP medium with 50mg/L kanamycin antibiotic. These cultures were grown overnight at 28^{o}C and 200 rpm to attain an OD_{600} of 0.4-0.6. After attaining the desired

OD the cultures were centrifuged at 4000 rpm for 12 min at 20^{o}C. Supernatant was discarded and pellet was resuspended in a solution of 5mL 10mM MgCl2 and 5 mL 10mM MES. Inoculums are prepared by mixing 5 mL pBINTRA-MES-MgCl2 and 5 mL MES-MgCl2- solution of each construct (pTV00, pTVHQT).

NOTE: 5 mL of *Agrobacterium* culture is sufficient to inoculate 3 to 4 plants. So, based on the number of plants to be silenced the culture volume can be prepared.

Agrobacterium was infiltrated to dorsal (underside) part of a leaf using 1 mL syringe without needle. After completion of infiltration the plants were covered with plastic racks for 2 days to avoid the exposure of plants to light. Stable transgenic inverted repeat *Ms*COE (*ir*COE) *Nicotiana attenuata* were generated by transforming the recombinant pRESC8 transformation vector containing 350kb fragment of *M. sexta* COE gene in an inverted repeat orientation. Transgenic lines were screened (Gase et al., 2011) for single insertions. These lines are also VIGSed with same vectors. As a positive control to check gene silencing is successful or not, *N.attenuata* plant VIGSed with pTVPDS (phytoene desaturase, causes bleaching due to the depletion of carotenoids) was used.

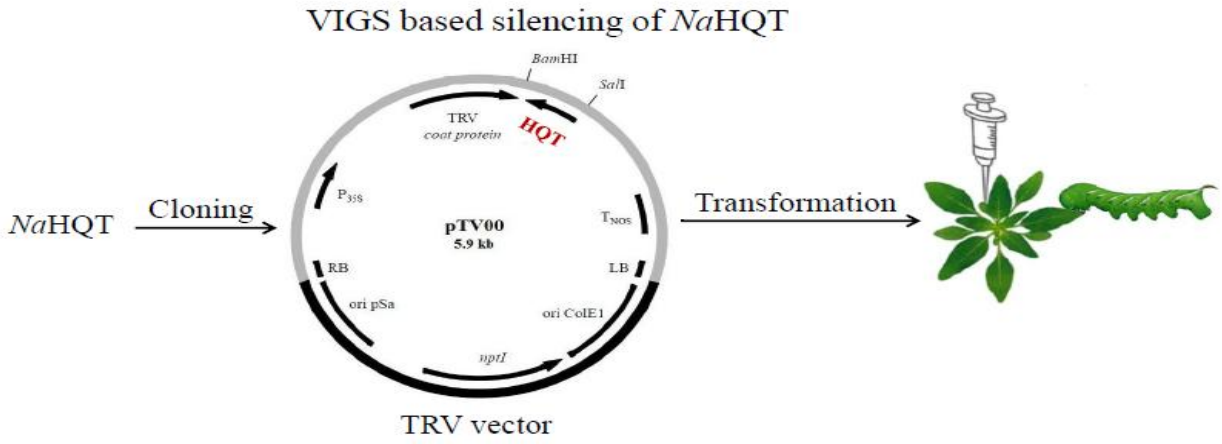

Figure 2: Schematic representation of generation of *Na*HQT-VIGS plants.

4.3 UV-B treatment:

To observe the growth and development of *N. attenuata* plants under UV-B conditions growth chamber (Snijders Scientific, Tilburg, Netherlands) was used. *N. attenuata* plants VIGSed with EV and HQT lines were kept in chamber with UV-B source. Around one meter distance is maintained between UV-B radiating source and plants. The provided intensity of UV-B radiation is more compared to the natural UV-B radiation emitted from sunlight. To reduce the damage of plants from high intensity of UV-B radiation a mesh like structure was arranged between plants and source. The plants were subjected to UV-B radiation for 5 min twice a day (morning and evening sessions). This was repeated for 5 consecutive days.

4.4 Secondary metabolite extraction and analysis by HPLC

For analysis of secondary metabolites in *N. attenuata* plants, five biologically replicates with 2 seedlings pooled for each replicate were harvested at five different time points. Chlorogenic acid (CGA) was analyzed using an HPLC-DAD method (Keinanen *et al*., 2001). 100 mg of leaf material was homogenized with ceramic beads by Genogrider 2000 (SPEX Certi Prep) at 500 strokes per minute for one minute and then extracted with 1 mL extraction solution (40% methanol, 0.5% acetic acid) per 100 mg sample fresh weight. After centrifugation at 13,000 rpm for 20 min at 4°C, the supernatants was collected and 1µL of supernatant was injected into an Agilent 1100 HPLC (Agilent HPLC 1100 Series,Palo Alto, CA) installed with a Chromolith FastGradient RP-18 (5032 mm; Merck,Darmstadt, Germany) endcapped 50 x 2 mm HPLC column (Lot No. HX802433 Merck,Darmstadt, Germany) attached to a Gemini NX RP18, 3µm, 2 x 4.6 mm precolumn (Phenomenex, Aschaffenburg, Germany) with a column oven set at 40°C. The mobile phase consisted of a mix of solvent A (0.1 % formic acid and 0.1 % ammonium hydroxide solution in water (pH 3.5) and solvent B (methanol) was used in a gradient mode (time/concentration min/% for A: 0:00/100; 0.50/100; 6.50/20; 10:00/20; 15:00/100) with a flow rate 0.8 mL/min. Under these conditions, chlorogenic acid, eluted at RT 3.0-3.1 detected at 320 nm. The peak areas were integrated using the Chromeleon chromatographic software (version 6.8; Dionex, Sunnyvale, USA) and the amount of metabolites in plant tissue was calculated using external dilution series of chlorogenic acid standards.

4.5 Statistical analysis

Statistics were performed using the SPSS software version 17.0. Statistical significance was evaluated using one-way analysis of variance (ANOVA) at a 0.05 level and means were compared by the LSD post-hoc test. For analysis of differences in plant performance the *t* test was used with the one-tailed distribution of two sets of samples with equal variance. The number of replicates (*n*) used in each experiment are given in the Figure legends.

4.6 Bioassay with Artificial diet

This assay was done to find whether only CGA induces COE or other secondary metabolites. Artificial diet of 10g was taken in a plastic box for each secondary metabolite used. The concentrations of metabolites are CGA, caffeic acid and quinic acid are 300µg/g, nicotine 1mg/g, rutin 600µg/g. These metabolites are dissolved in appropriate solvents like CGA in ethanol and others in water. Artificial diet with water is used as control. 10 neonates of *M.sexta* were placed for each metabolite. RNA was extracted from midgut of larvae after 24 hr of feeding and qPCR analysis was done to check the *Ms*COE transcript levels.

4.7. RNA extraction

The frozen midgut samples were grinded using a geno grinder, aliquoted in labeled tubes and stored at -80 °C. RNA extractions from the grinded samples were done by phase extraction and lithium chloride (LiCl) precipitation method (Kistner and Matamoros 2005). The midgut samples of *M.sexta* were used for RNA extraction. Initially the samples were mixed in 600 µL of RNA extraction buffer (2 % CTAB (hexadecyltrimethylammonium bromide), 2 % PVP (polyvinylpyrrolidone 360.000), 100 Mm Tris-HCL pH 8.0, 25 mM EDTA, 2 M NaCl previously warmed at 65 0C) containing 2 % β-mercaptoethanol and immediately 600 µL of phenol-choloroform-isoamylalcohol (25:24:1 pH 8.0) was added. The tubes were mixed well and incubated for 10 min at 55°C with frequent shakings. The phases were then separated by centrifugation at 1200 rpm for 10 min at room temperature. The upper phase was collected and re-extracted with 600 µL of phenol-choloroform-isoamylalcohol (25:24:1 pH 8.0). The upper phase was collected again and RNA was precipitated by 8 M LiCl giving final concentration of 2 M to LiCl in the mixture. The samples were then kept on ice overnight. On the following day samples were centrifuged for 20 min at 4°C, the supernatant was then discarded and the pellet

was washed with 1mL cold 2 M LiCl with centrifugation for 20 min at 4°C . The pellet was washed again twice in 800 µL of ice-cold 80 % ethanol with centrifugation for 20 min at 4°C. The pellet was dried and dissolved in 12-15 µL of DEPC water depending upon the size of the pellet. The concentration and the purity of extracted RNA were measured with Nanodrop (Ratio A260/A280 ˜ 2.0 and Ratio A260/A230 ˜2) (NanoDrop Technologies, Wilmington, DE, USA). RNA was then diluted to get the concentration of 166.7 ng/ µL.

4.8 Reverse transcription and quantification of transcript levels by q-PCR

Reverse transcription (RT) was done in 96-well microtiter plate using oligo (dT) and Superscript II reverse transcriptase according to the manufacturer's instruction (www.invitrogen.cow). 6 µL of the diluted RNA solution (166.7 ng/ µL) was added to the wells of the plate. The master mix (denature and reaction mix) were prepared for a 20 µL reaction/well. 4 µL of the denature mix was added to each RNA sample. The plate was centrifuged briefly, covered with a SealMat and heated at 65°C for 5 min on PCR machine. The denatured RNA was immediately chilled on a pre cooled Alu-Rack for 1 min. To each of the well 10 µL of the reaction master mix was added and centrifuged to the well bottom.

The cDNA was diluted 1:4 with MilliQ to minimize pipetting errors in qPCR and centrifuged down. For quantification of the relative expression levels of selected genes, real-time q-PCR was performed on a Mx3005P qPCR system (Stratagene, Santa Clara, CA, USA, http://www.stratagene.com) with qPCR Core Kit for SYBR® Green I (Eurogentec, Seraing, Belgium, http://www.eurogentec.com) following the manufacturer's instructions. 16 µL of the SYBR® Green master mix were added to 4 µL of sample well and dilution series of cDNA (six progressive 1:4 including undiluted sample) and blank (MilliQ water).

Note: Actin is used as a house keeping gene for gene of interest belonging to plant, because of its stability and level of expression. Ubiquitin is used as a house keeping gene for gene of interest belonging to insect.

5. **Results**

5.1 CGA induces COE

qPCR analysis of RNA isolated from midgut of *M. sexta* larvae fed on artificial diet containing *N.attenuata*'s secondary metabolites like CGA, CFA, QA, nicotine and rutin showed significant rise in COE transcripts in larvae feeding on CGA containing diet. The transcript levels of COE were increased by 3 folds. The COE transcript levels in larvae fed on other metabolites were similar to the levels of COE transcripts in control larvae fed on artificial diet Fig. 3.

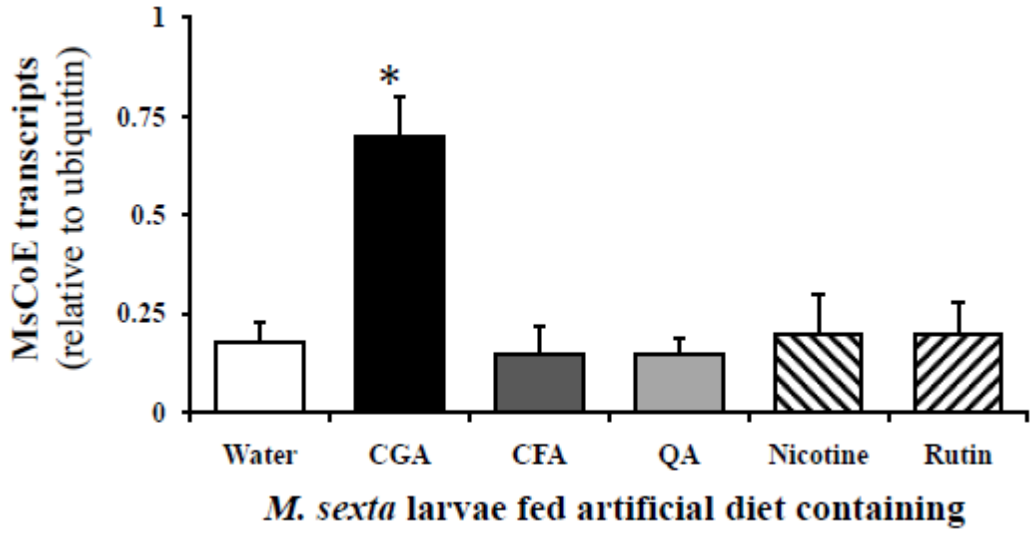

Figure 3: COE gene expression is induced in larval midgut in response to CGA ingestion.
Concentrations of metabolites in artificial diet are CGA, CFA, QA 300µg/g, nicotine 1mg/g, rutin 600µg/g.

This bioassay with artificial diet and qPCR analysis states that only CGA induces COE in *M.sexta*.

5.2 Effect of *Na*HQT expression on CGA level

The foliar level of CGA in *N. attenuata* is strongly correlated with the expression of *Na*HQT. The natural concentration of CGAin *N.attenuata* is 180-220µg/g FM of leaf. The concentration of CGA is reduced by 50-70% (reduced to 60-100µg/g FM of leaf) in *Na*HQT suppressed plants (WT+HQT) when compared to WT+EV plants Fig. 4B.

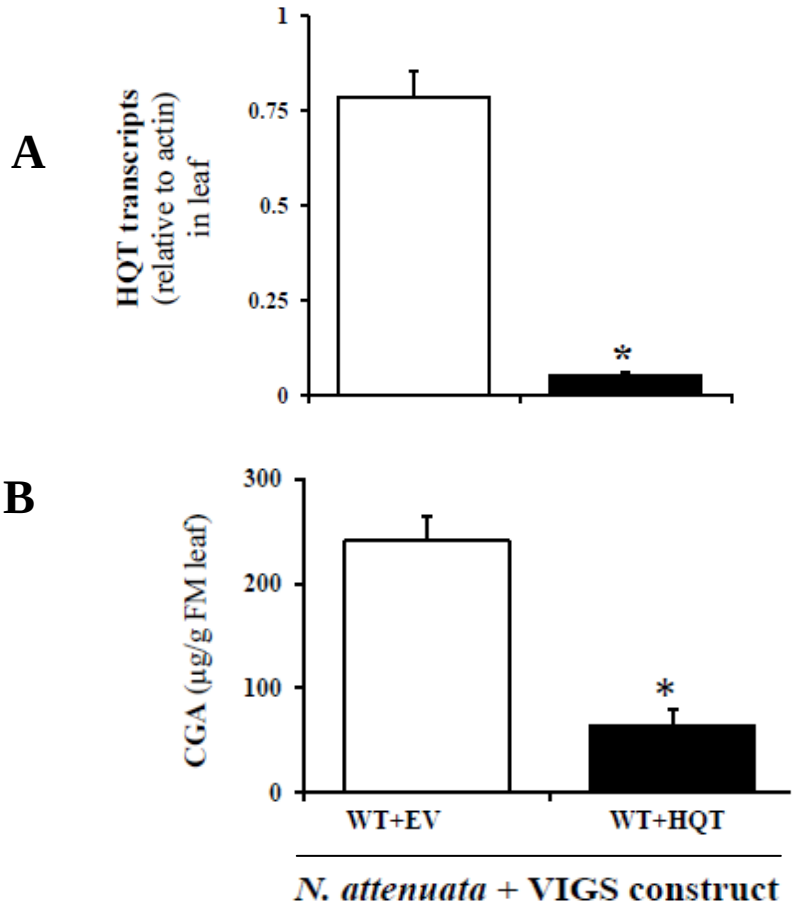

Figure 4. Silencing efficiency of *Na*HQT. (A) qPCR analysis of HQT transcripts in wild type plants and *Na*HQT silenced plants. (B) The significant decrease in CGA level in *Na*HQT plants after successful silencing through VIGS. n= 35 WT+EV, 50 WT+HQT.

The silencing efficiency of *Na*HQT was determined by RT-PCR. In Fig. 4A the transcript levels of *Na*HQT were significantly reduced by 7 folds in *Na*HQT suppressed plants. This observation shows the relation between the *Na*HQT expression and CGA biosynthesis in *N. attenuata* plants. There was no change in the plant growth and development due to the silencing of *Na*HQT.

5.3 Effect of chlorogenic acid on larval mass of *M. sexta*

According to the reports of Bietal (1997), the concentration of CGA in plants with over and under expressing PAL is siginicantly different but these activities do not influence the weight gains of *M. sexta*. In our observations significant differences were found among the *M. sexta* larval mass when they fed for 10 days on *Na*HQT suppressed plants. Larval growth of *M. sexta* was negatively correlated with the foliar CGA. The larval mass of *M. sexta* was increased by 2-

2.5 folds when fed on *Na*HQT suppressed plants for 8 days (P>0.05). So, it gives a view on the negative effect of *N. attenuata*'s CGA on *M. sexta* larval mass.

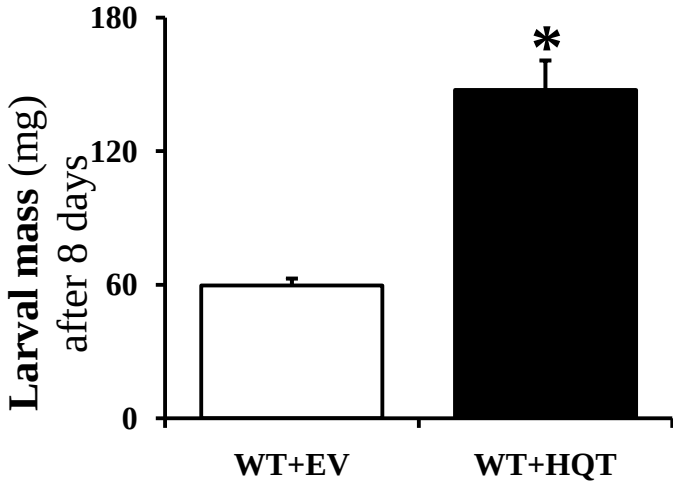

Figure 5. Effect of Chlorogenic acid on larval mass of *M. sexta*. Larval mass after 8 day feeding on *Na*HQT silenced plants was significantly increased. Neonates n= 30 on each line.

5.4 Plant mediated RNAi for *Ms*COE

We generated stable transgenic lines of *N.attenuata* plants transformed with pRESC8 vector harboring an inverted repeat (*ir*) of the selected 350kb COE cDNA fragment. Both *ir*COE and wild type plants are similar in morphology and development. These *ir*COE plants are also subjected to VIGS for the suppression of HQT (*ir*COE+HQT) and control plants of *ir*COE are VIGS with pTV00 (EV).

Neonates of *M. sexta* larvae were placed on control (*ir*COE+EV) and *ir*COE+HQT lines and allowed to feed for 10 days. COE transcript levels in midgut of 8 day old larvae feeding on *ir*COE plant lines was reduced by 92-95%, this is also similar in case of larvae fed on WT+HQT plants when compare to larvae fed wild type of *N. attenuate* plants. COE transcripts in larvae feeding on *ir*COE, WT+HQT lines with that of wild type plants (Fig.6) makes us an impression that the expression of COE in midgut of *M. sexta* larvae is based on CGA ingestion.

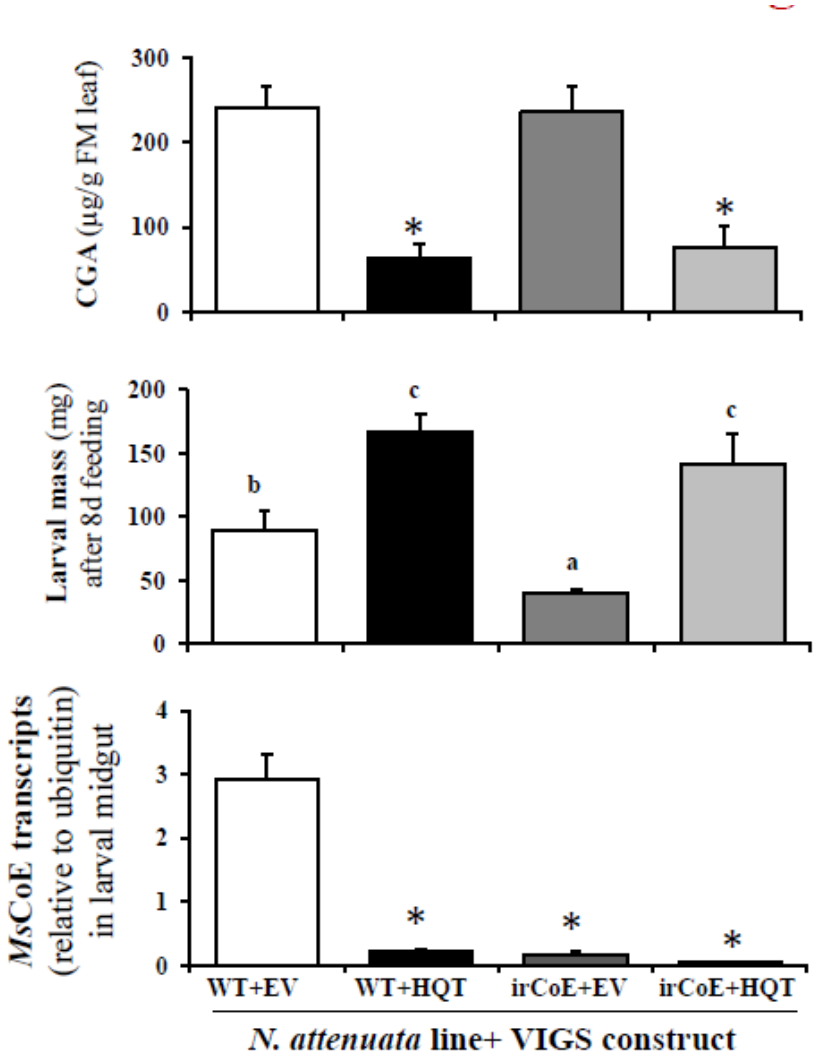

Figure 6: *Ms*COE reduces the effect of CGA

Also observed that chlorogenic acid reduction by *Na*HQT silencing reverts the negative effect of COE silencing in *M. sexta* from Fig. 6 In other words we can say that *Ms*COE reduces the negative effect of CGA.

5.5 Chlorogenic acid as UV absorbing screen:

Phenolics also involved in the minimization of hazardous effects of UV-B radiation. Phenolics protect the plants by absorbing harmful UV-B radiation. In our findings demonstrate that CGA play a role in plant growth under UV-B radiation. Our result describes that when *Na*HQT silenced plants were grown under UV-B radiation conditions has shown poor growth and development when compared to *Na*HQT silenced plants under normal conditions. The growth

and development of the *Na*HQT silenced plants growing under UV-B conditions were significantly varied from *Na*HQT silenced plants growing under normal conditions.

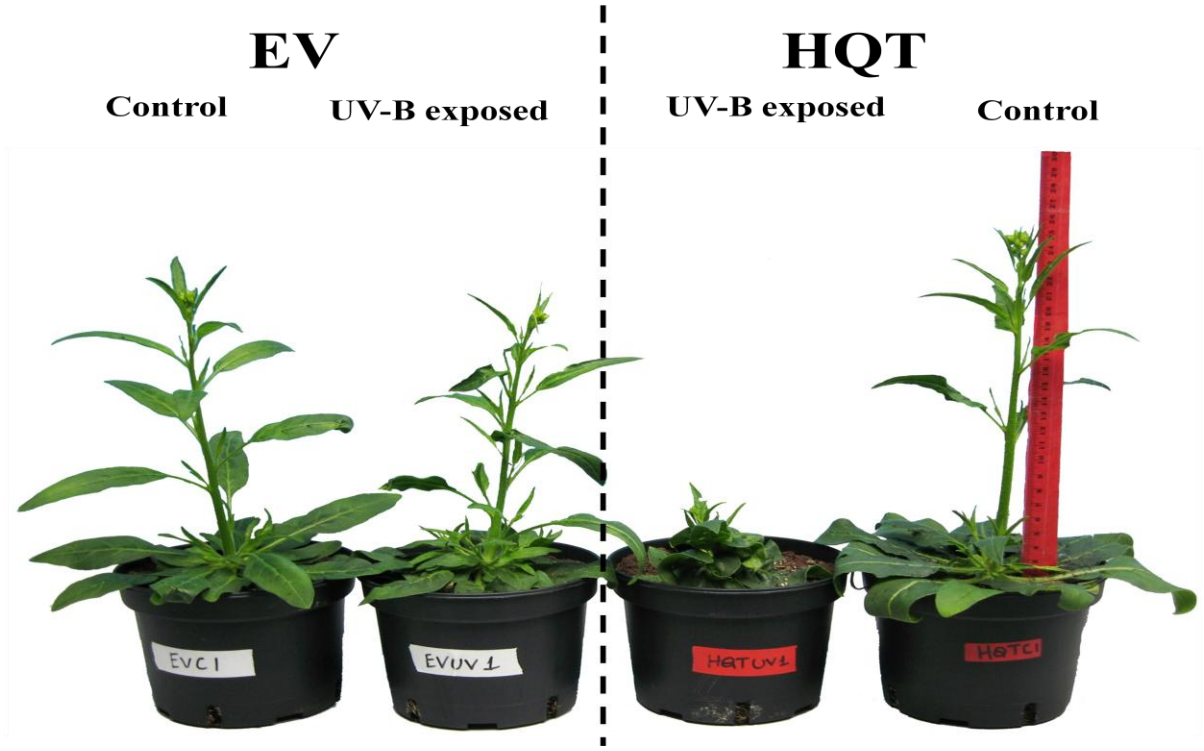

Figure 7: UV-B effect on *N.attenuata* plant growth and development. *Na*HQT suppressed plants shown phenotypical difference under UV-B conditions.

6. Discussion

The common phenolic compound of plant phenylpropanoid pathway is CGA, which can influence insect herbivore host-plant selection (Harborne, 1984). When CGA is ingested through artificial diet, it exerts negative effects on growth and development of insect species (Isman & Puffey, 1982; Wisemann et al., 1990; Stevenson et al., 1993). Several literatures have shown how CGA is involved in the poor growth of *M. sexta* when ingested through artificial diet and under different environmental conditions on tomato plants (stamp et al., 1994; stamp & yang, 1996; Jasen et al., 1997). The effect of CGA on *M. sexta* larvae may depend on how it is ingested. The negative effect of CGA is more when it is ingested through artificial diet than its

ingestion through plants. It is due to the absence of antioxidants and availability of more free transitional elements in artificial diet when compared to plant system. The artificial diet increases the effect of CGA on insect herbivores. The increase or decrease of CGA due to over or under expressing PAL does not influence the larval mass of M. sexta (Bi et al., 1997). The midgut of *M. sexta* is alkaline in nature (Felton & Duffey, 1991).In oxidizing environment at alkaline pH phenolic acids converts to quinones and superoxide anions in the presence of peroxidases. These superoxide anions induces the oxidative stress in the midgut, which results in protein digestibility (pierpoint, 1985) and reduces the bioavailability of amino acids (Felton et al., 1982). The decrease in bioavailability of nutritive elements results in the loss of larval mass. In contrast, CGA acts as antioxidant (Niggeweg et al., 2004) and strong alkalinity and detergency of *M. sexta* midgut lowers the antinutritive effects of oxidized phenolics (Felton & Duffey, 1991). we generated *N.attenuata* lines with silenced HQT, which is involved in the biosynthesis of CGA. In our results we found there is a significant increase in larval mass when fed on *Na*HQT silenced plants. This result suggests that *N.attenuata* CGA is harmful to *M. sexta* larval growth. From the previous results of our group suggests that *Ms*COE is upregulated when CGA is ingested to *M. sexta* larvae. The correlation between *Ms*COE and CGA is observed in our results, when larvae fed on *ir*COE and WT+HQT lines the transcript levels of *Ms*COE is significantly reduced. This makes a note that *Ms*COE minimizes the effect of CGA on larval growth of *M. sexta*. *Ms*COE is involved in the metabolism of CGA. CGA not only acts as defense metabolite against insect herbivores and also protects *N.attenuata* from UV-B radiation. Generally chlorophyll producing plants will synthesize their food using sunlight. But a small fraction of solar radiation i,e., UV-B radiation may cause harmful effects to the plants. Plants have evolved to protect themselves by producing secondary metabolites as defensive chemicals against UV-B radiation. These defense metabolites acts as UV-B protecting or absorbing screens and protects the plants from destructive effects of harmful solar radiation. Our observations supports that CGA is involved in the development and growth of the *N.attenuata* under UV-B radiation. In the absence or reduced levels of CGA *N.attenuata* plants undergo ailing development and growth.

7. CONCLUSION

In summary, following conclusions can be drawn from the above results. A) COE is specifically induced by CGA. B) COE cleaves CGA into CFA and QA. C) Silencing of HQT reduces leaf's CGA content. D) *N. attenuata*'s CGA exerts negative effect on *M. sexta*'s larval mass.

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Jena, 31st March 2014 Bharath Ramraj

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