Reverse-Genetic Characterization of Solasonine-Based Chemical Defense in Eggplant

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by

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Certificate

This is to certify that this dissertation entitled **“Reverse-Genetic Characterization of Solasonine-Based Chemical Defense in Eggplant”** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Archis Likhitkar at Indian Institute of Science Education and Research under the supervision of Dr. Sagar Pandit, Assistant Professor, Department of Biology, during the academic year 2025-2026.

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This thesis is dedicated to my parents, who raised me well to pursue my dreams.

Declaration

I hereby declare that the matter embodied in the report entitled **"Reverse-Genetic Characterization of Solasonine as Defense Compound Against *Spodoptera litura* in Eggplant"** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education & Research (IISER) Pune, under the supervision of Dr. Sagar Pandit, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions. I declare that no generative AI was used in this thesis.

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Index

List of Tables

List of Figures

List of Abbreviations

AD - Artificial diet

cDNA - Complementary DNA

CDS - Coding sequence

GAME - Glycoalkaloid metabolism

HPLC-ELSD - High-Performance Liquid Chromatography-Evaporative Light Scattering Detector

JKP - JK Purple Long

MES - Methyl ether sulphonate

PCR - Polymerase chain reaction

qPCR - Quantitative PCR

RE - Restriction enzyme

RNAi - RNA interference

SA - Steroidal alkaloid

SD - Solasodine

SGA - Steroidal glycoalkaloid

SGN - Sol Genomics Network

siRNA - Short interfering RNA

SM - Solamargine

SN - Solasonine

SOB - Super optimal broth

TCW - Tobacco cutworm (*Spodoptera litura*)

TRV - Tobacco rattle virus

UGT - UDP-dependent glycosyltransferase

VIGS - Virus-induced gene silencing

VOX - Virus-mediated overexpression

YEP - Yeast extract peptone

Abstract

Plants and insects interact with each other in the environment, and among these interactions, plant insect herbivore interactions are of considerable ecological and agricultural consequences. Plants have a rich repertoire of secondary metabolites which are species/family specific, which serve the purpose of combating various biotic and abiotic stressed, which includes herbivory. Steroidal alkaloids found in Solanaceae family is an example of class of such secondary metabolites. Umesh et al. 2021 using various insect assays in 5 eggplant varieties containing varying amounts of steroidal alkaloid contents demonstrated that steroidal alkaloid solasonine found in eggplant (*Solanum melongena*) negatively affects the preference and performance of generalist folivore tobacco cutworm (*Spodoptera litura*) thus indicating that solasonine might be involved in protection of eggplant from insect herbivores. Lucier et al. 2024 decoded the steroidal alkaloid biosynthesis pathway from cholesterol in eggplant and determined the genes involved, with UGT93N3 being the terminal gene in solasonine biosynthesis. Using this information, we hypothesized that high solasonine content is indeed responsible for lower preference and performance of high solasonine containing variety JK Purple Long. To test this hypothesis, we performed reverse genetic characterization of terminal solasonine biosynthesis gene UGT93N3 using virus-induced gene silencing (VIGS) and virus-mediated overexpression (VOX) to silence and overexpress UGT93N3 gene respectively for reducing and increasing solasonine content in eggplant variety JK Purple Long and performed insect preference (dual-choice assays) and performance (neonate mortality assays) assays to analyze the effect of solasonine content on preference and performance of tobacco cutworm.

1. Introduction

1.1. Plant-Insect Interactions

Plants and insects have coexisted and coevolved for at least 350 million years (Grimaldi & Engel, 2005). Plant-insect interactions show a staggering diversity which can be mutual like insects pollinating flowers, commensal like ants building their nests in crevices of tree bark and predatory like insect herbivores consuming various plant parts like leaves (folivores), roots (rhizovores), flowers (florivores) and fruits (frugivores). Insect herbivory is a key biotic stress on plants as insects have higher impact on primary productivity as compared to other herbivores in many ecosystems (Andersen & Lonsdale, 1990; Schowalter, 2016) and 401,000 insect species out of a million described insect species are herbivores (Grimaldi & Engel, 2005; Stork, 2018). Plants develop defenses to counter insect herbivory and in response insect herbivores (referred to as herbivores from now on) develop counteradaptations to counter these defenses, a phenomenon often referred to as an 'evolutionary arms race' (Nakadai, 2017).

Plant defenses can be classified into direct defenses, indirect defenses and tolerance. Also, based on their agents of defense and modes of expression as physical/chemical defenses and constitutive/induced defenses respectively (Kessler & Baldwin, 2002). Direct defenses are plant traits and mechanisms which reduce the fitness of herbivores directly without involving any other biotic agent thus inducing bottom-up control on herbivores, e.g. toxic secondary metabolites which reduce feeding performance and preference for plants. Indirect defenses are plant traits and mechanisms which reduce fitness of herbivores by attracting their natural enemies, namely their predators and/or parasitoids to increase their success and control the populations of herbivores, e.g. herbivory-induced plant volatiles (HIPVs) which are volatiles released by plants upon herbivory which attract natural enemies of herbivores (Kessler & Baldwin, 2002; Fürstenberg-Hägg et al., 2013). Tolerance refers to plant traits and mechanisms which decrease the negative consequences of herbivory for plants without necessarily affecting herbivore fitness, e.g. mechanisms like herbivory-induced compensatory growth, increased nutrient uptake and increased photosynthetic rates (Stowe et al., 2000; Kessler & Baldwin, 2002).

Based on modes of expression, plant defenses to insect herbivory are classified into constitutive defenses which are always active regardless of whether plant is undergoing herbivory or not and induced defenses which are activated only when plant senses it is undergoing herbivory through herbivory-specific damage and defense hormone induced-signaling pathways. There is a trade-off to whether a plant defense against herbivory should be constitutive or induced, as constitutive defenses are act instantaneously and often more effective for preventing herbivory but incur higher growth and developmental costs as plant's limiting resources are diverted in maintaining a defense system all the time whereas in induced defenses, chances are that plants can incur significant damage from herbivores between start of herbivory and mounting induced defense but at the same time more of plant's resources are diverted to its growth and development when it is not under herbivory. This is connected to the concept of 'growth-defense trade-off' in plants, where plant has to divert and manage its limiting resources in either high growth and development or defending against biotic and abiotic stresses, where constitutive defenses favour defense over growth and vice versa for induced defenses (Kessler & Baldwin, 2002; Huot et al., 2014). Based on agents of defense, plant defenses against insect herbivory are classified into physical and chemical defenses, where physical defenses are morpho-anatomical traits in plants like spines, prickles, thorns, trichomes, waxy cuticles, resin ducts, lactifers (latex ducts), raphides, etc. which reduce herbivores' foraging success by acing as mechanical barriers to herbivory and chemical defenses like defense proteins which include protease inhibitors (PIs), polyphenol oxidases (PPOs), amylase inhibitors, lectins, chitinases, etc. and a diverse array of secondary metabolites (discussed later in the section) which act as antifeedant, antidigestive, antinutritive and/or toxic to herbivores to reduce their fitness (Kessler & Baldwin, 2002; Fürstenberg-Hägg et al., 2013).

Plants contain a vast array of metabolites with estimates of 200,000 to over a million metabolites found in plants, out of which only 8,000 or so are believed to be primary metabolites, metabolites which are shared across species and are essential for basic functions of plants like metabolism, growth and development. Rest are secondary or 'specialized' metabolites which are taxa-specific and used for combating environment's biotic and abiotic stresses. Secondary metabolites are derived from simple precursors of primary metabolites which are diverted to different secondary metabolite biosynthesis pathways and modified to their final forms by enzyme classes like UDP-dependent glycosyltransferases (UGTs), cytochrome P450s (CYPs) and BAHD acyltransferases. Major classes of secondary metabolites include terpenoids, phenylpropanoids, alkaloids, cyanogenic glycosides, glucosinolates and fatty-acid derivatives (Huang & Dudareva, 2023). Many secondary metabolites are involved chemical defenses against herbivory. Nicotine is an alkaloid found primarily in genus *Nicotiana* and is the primary bioactive compound in tobacco plants *Nicotiana tabacum* and *N. rustica* is an example of secondary metabolite used for plant defense against herbivory. Nicotine is highly toxic to both vertebrate and insect herbivores as it binds to nicotinic acetylcholinergic receptors (nAChRs) causing overstimulation, paralysis and death. Thus, tobacco extracts and nicotine have been traditionally used as insecticides and insect repellents in agriculture (Ujváry, 1999).

1.2. Host Plant - Eggplant (*Solanum melongena*)

Eggplant (*Solanum melongena*) is an agronomically important solanaceous crop and is the fifth most economically important solanaceous crop after behind potato, tomato, pepper and tobacco. India is the second largest producer of eggplant after China at 12.7 million tons per year (Taher et al., 2017; Abrham & Shumbulo, 2024). Eggplant is often called 'king of vegetables' in South Asia due to its wide variety of shapes, sizes, colors and uses in different cuisines. Eggplant fruits are also low in calories and high in vitamins like niacin (B3), pantothenic acid (B5), pyridoxine (B6), folic acid (B9), ascorbic acid (C), and phylloquinone (K), minerals like potassium, magnesium, manganese, phosphorous and copper, dietary fiber, and antioxidants (Lalita et al., 2020). Farming eggplant is also advantageous for poor and middle income farmers in India because of its suitability to India's subtropical climate, easy and cheap maintenance and profitability in small-scale production (Sidhu et al., 2004; Abrham & Shumbulo, 2024).

Eggplant is infested by many arthropod pests, which include eggplant shoot and fruit borer (*Leucinodes orbonalis*) which is the most severe pest of eggplant and can cause up to 90% loss in marketable yield for eggplant (EFSA Panel on Plant Health (PLH) et al., 2021), leafhopper (*Amrasca devastans*), whitefly (*Bemisia tabaci*), thrips (*Thrips palmi*), aphids (*Aphis gossypii*), spotted beetles (*Epilachna dodecastigma* and *E. vigintioctopunctata*), leaf roller (*Eublemma olivacea*), ash weevil (*Mylocerus viridanus*), stem borer (*Euzophera perticella*), blister beetle (*Mylabris pustulata*) and red spider mite (*Tetranychus urticae*) (Srinivasan, 2009). Thus, it is important to devise pest management strategies to protect eggplant against such a wide variety of pests and reduce crop yield losses. These strategies include synthetic insecticides, screening and developing pest resistant eggplant varieties, biopesticides, pheromone traps, natural enemies and trap cropping among many others, and even a combination of some of the aforementioned techniques under integrated pest management (IPM) (Nayak et al., n.d., Srinivasan, 2008).

Eggplant has a large diversity of secondary metabolites which include phenolics, flavonoids, anthocyanins, terpenes, alkaloids, tannins and saponins among others (Contreras-Angulo et al., 2022). Extracts from many parts of eggplant including leaf, fruit, peel and roots have been shown to have antioxidant, antimicrobial, analgesic, antipyretic, anti-inflammatory, antidiabetic and anti-hypertensive activities, effects which are attributed primarily to secondary metabolites in eggplant (Solanke, 2019; Salamatullah et al., 2021). Rising use of synthetic pesticides and their harmful effects to both human health and non-human members of agroecosystem have alluded scientists to look for alternative ‘biopesticides’, metabolites with pesticidal activities naturally found in other organisms and believed to have lower negative effects on environment due to their biodegradability as compared to synthetic pesticides (Daraban et al., 2023). Spinosad, a biopesticide made of binary mixture of spinosyn A and D and extracted from actinomycete *Saccharopolyspora spinosa* acts via a mechanism similar to nicotine by allosterically binding and activating nAChRs and GABA receptors causing tremors, paralysis and death and has been successfully used as a biopesticide against a variety of Acaridan, Thysanopteran, Coleopteran, Lepidopteran and Dipteran pests and even control mosquitoes (Santos & Pereira, 2020). We suggest that due to its rich diversity of secondary metabolites and bioactive compounds, eggplant can be a potential source of many biopesticide candidates which need to be studied, evaluated and developed.

As mentioned previously, plant-herbivore interactions have great ecological, economical and agricultural consequences as many herbivores are destructive for plants in wild ecosystems and pests also cause tremendous losses for agriculture, making it important to develop model systems to study plant-herbivore interactions. Eggplant has a great potential to be a model system for studying plant-herbivore interactions due to many reasons like relatively short time to reach maturity (2-3 months), easy and cheap maintenance, high productivity, large germplasm collection, genetic resources like Sol Genomics Network (https://solgenomics.sgn.cornell.edu/organism/Solanum_melongena/genome) and Eggplant

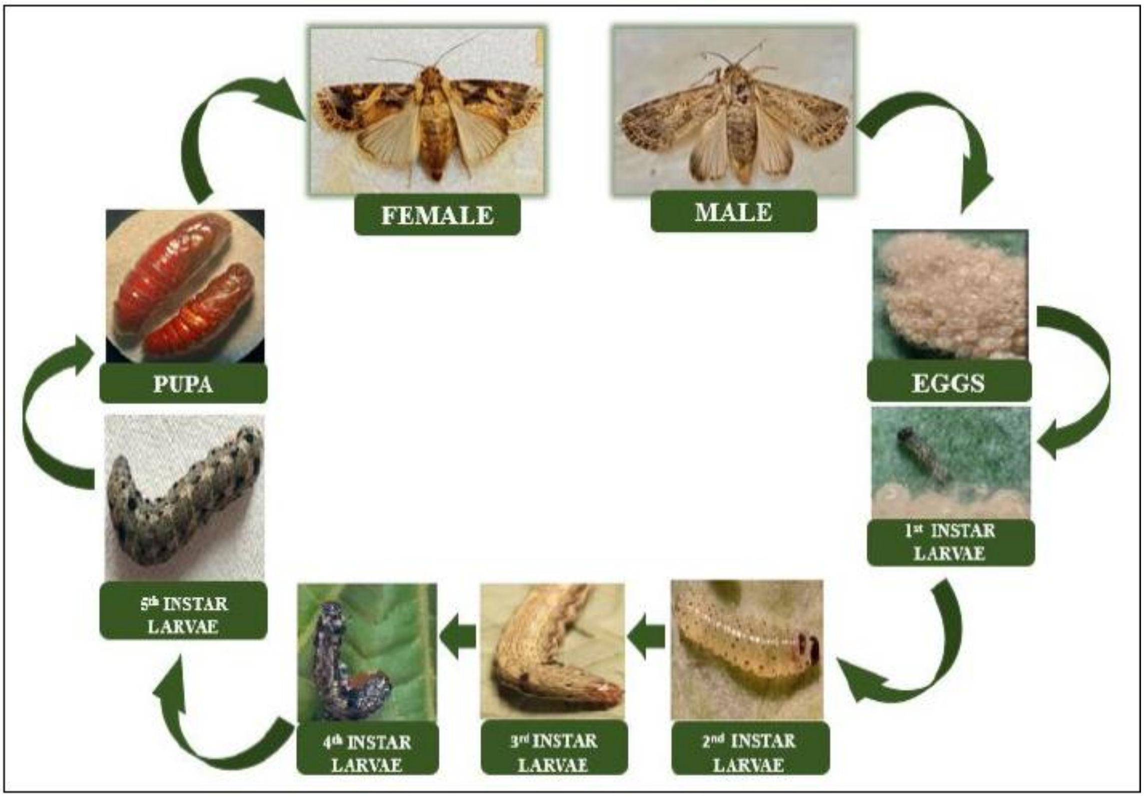
Genome Database (http://ddlab.dbt.univr.it/eggplant/) and wide variety of herbivores (insect and mite pests) feeding on eggplant (Srinivasan, 2009).

1.3. Insect Herbivore - Tobacco Cutworm (*Spodoptera litura*)

Tobacco cutworm (*Spodoptera litura*) (TCW from now on) is a highly polyphagous lepidopteran generalist folivore. It is widely present in South Asia, Southeast Asia, East Asia and Oceania in tropical to temperate climates with few reports from Middle East and Central Russia and has been found to feed on at least 180 plant species belonging to 40 different families, many of which are agronomically important crops where it can cause vigorous defoliation like tobacco, soyabean, castor, cotton, maize, tomato, cabbage and many more. Its larval stage is the most destructive stage of its life cycle for plants with larvae consuming leaves, buds, flowers and fruits resulting in decreased yield due to damage to photosynthetic plant parts and loss of marketable yield (Shekhawat et al., 2018; EFSA Panel on Plant Health (PLH) et al., 2019).

Life history of TCW is highly dependent on abiotic conditions like temperature with optimal temperature being between 25-30 °C for all stages and host plant with castor, cabbage and tobacco being the most commonly used plants for insect culture along with artificial diet due to their high fecundity, high growth rate and low mortality (Fand et al., 2015; Gupta et al., 2005; Munir Ahmad et al., 2013; Shekhawat et al., 2018). Gravid females lay yellowish-brown egg clusters each containing 250-300 eggs with eggs laid in 2-4 layers and covered in abdominal scales and these take 4-6 days to hatch into neonates. Larvae undergo 5-7 instars before pupation, going from pale yellow to dark greenish-black in color and develop their characteristic black spots, with each instar taking 2-4 days except the last one which takes 4-6 days growing from 1-2 mm in neonate stage to 33-38 mm in last instar. Pupae are initially pale-yellow in color which eventually changes to reddish-brown which are 13-17 mm in size and lasts for 7-11 days. Adults show light brown forewings with dark brown stripes and white hindwings, light brown abdomen with dorsal hair tufts and clusters and head covered with light and dark brown scales, body length ranging from 14-18 mm, wingspan 30-35 mm, lifespan ranging from 8-11 days and a female laying 850-900 eggs in its lifespan. Adults also show sexual dimorphism, with males being slightly shorter and short-lived (14-17 mm and 8-10 days) as compared to females (15-18 mm and 9-11 days) and male and female pupae showing their signature genital openings on 8th and 9th segments respectively (Chowdary et al., 2024; Sharma et al., 2025). Life cycle of TCW is demonstrated in Figure 1.

Figure 1 - Life cycle of Tobacco Cutworm (*Spodoptera litura*) as adapted from Sharma et al. 2025

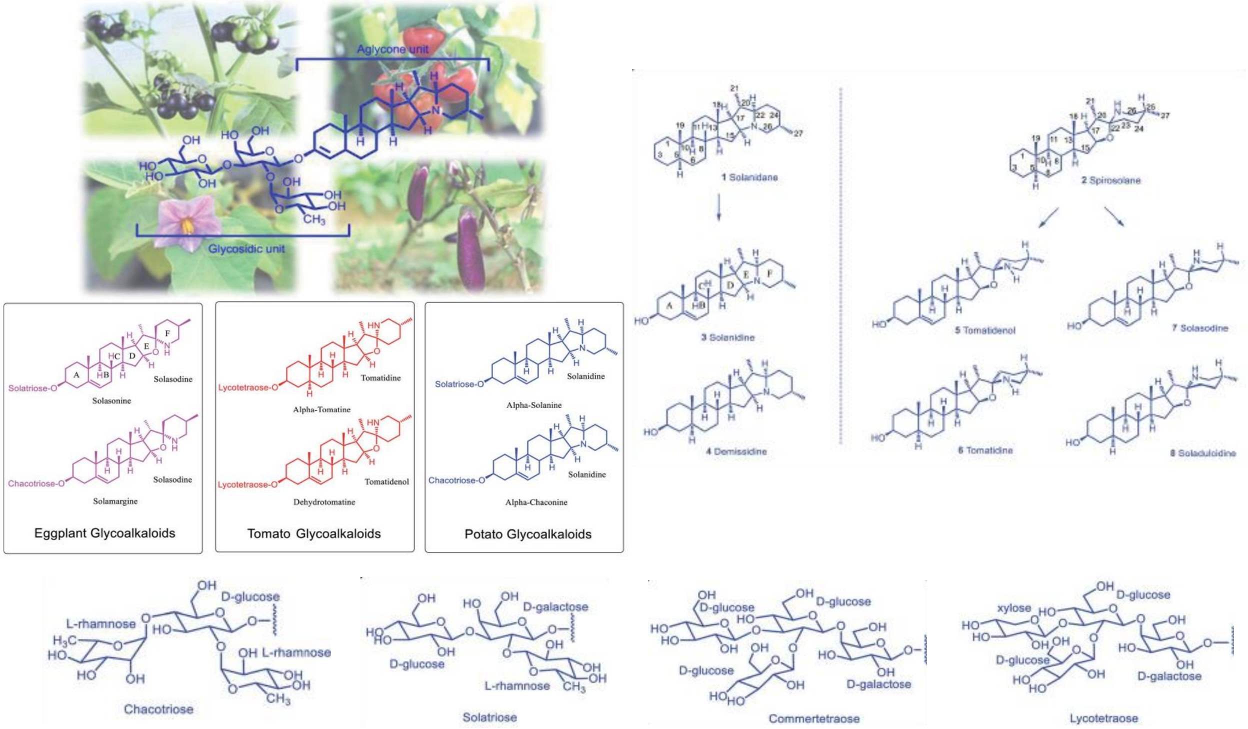


TCW exhibits many features which allow it to be a destructive agricultural pest, which include short life cycle, high fecundity, host plant-switching and mass-migration of both larvae and adult . These features also contribute to high adaptability of TCW, which has lead to development of pesticide resistance in TCW against many commonly used insecticides, which include various commonly used organochlorine and pyrethroid insecticides necessitating the need to develop better pest management strategies against TCW (Saleem et al., n.d.; Thakur et al., 2024). Currently used strategies for pest management of TCW include physical removal of larvae and egg masses, cultural practices of trap cropping, crop rotation and intercropping, .As mentioned previously, synthetic chemicals like organophosphates, pyrethroids, carbamates and benzenoid insecticides, botanical extracts like neem (*Azadirachta indica*), microbial biocontrol agents like insect pathogenic bacteria, viruses (Nuclear Polyhedrosis Virus or NPV), entomopathogenic fungi (EPF), entomopathogenic nematodes (EPNs) and natural enemies i.e., predators and parasitoids of TCW like assassin bugs (*Rhynocoris spp.*) and parasitoids *Trichogramma chilonis* (Saraswathi et al., 2023). As mentioned previously, biopesticides are currently being explored as viable alternatives to synthetic insecticides due to advantages like biodegradability and lower harm to environment. Eggplant being a both a rich reservoir of secondary metabolites and an inauspicious host of TCW suggests that eggplant might be a source of biopesticide candidates against TCW (Munir Ahmad et al., 2013; Contreras-Angulo et al., 2022).

1.4. Steroidal Alkaloids and Glycoalkaloids

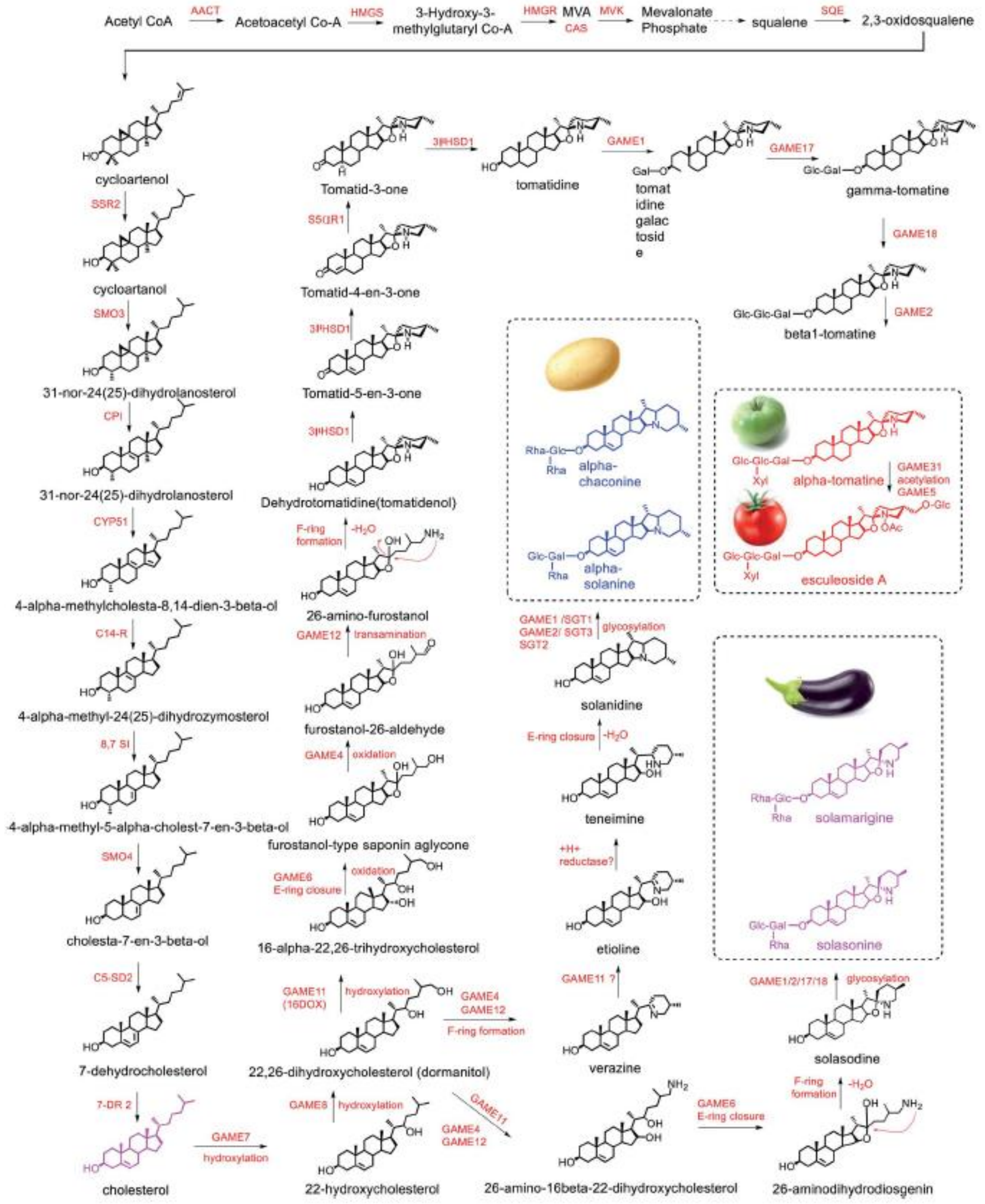
Steroidal alkaloids (SA) are secondary metabolites derived from triterpene biosynthesis pathway and found primarily in genus *Solanum spp*., with potato, tomato and eggplant SAs being the most commercially important and well-studied. SAs consist of (usually) 6-membered N-containing steroidal aglycone ring with many members containing a glycosidic unit at 3’-OH consisting of 1-4 monosaccharide containing sugar unit with such SAs also known as steroidal glycoalkaloids (SGAs). Two key types of aglycones form majority of known SAs, first is solanidane-type SAs with an indolizidine ring forming majority of potato SAs and second is spirosolane-type SAs with oxo-azaspirodecane system forming majority of tomato (22-α N type) and eggplant (22-β N type) SAs and these aglycone do not vary much in structure except for C5-C6 double bond. SGAs in addition to aglycone contain a 1-4 monosaccharide containing glycosidic unit at 3’-OH group, with most common glycosidic units being chacotriose (D-Gal-L-Rha-L-Rha), solatriose (D-Gal-L-Rha-D-Glu), lycotetraose (D-Gal-D-Glu-D-Glu-D-Xyl) and commertetraose (D-Gal-D-Glu-D-Glu-D-Glu) and based on number of monosaccharide units these are named as γ-, β- and α-compounds for trisaccharides with 1, 2 and 3 units and δ-, γ-, β-, and α-compounds for tetrasaccharides with 1, 2, 3 and 4 units respectively (Milner et al., 2011; Zhao et al., 2021). Structures of some important SAs and SGAs with their aglycones and glycosidic units are given in Figure 2.

Figure 2 - Structures of some important solanum SAs including their aglycones and glycosidic units, as adapted from Zhao et al. 2021.



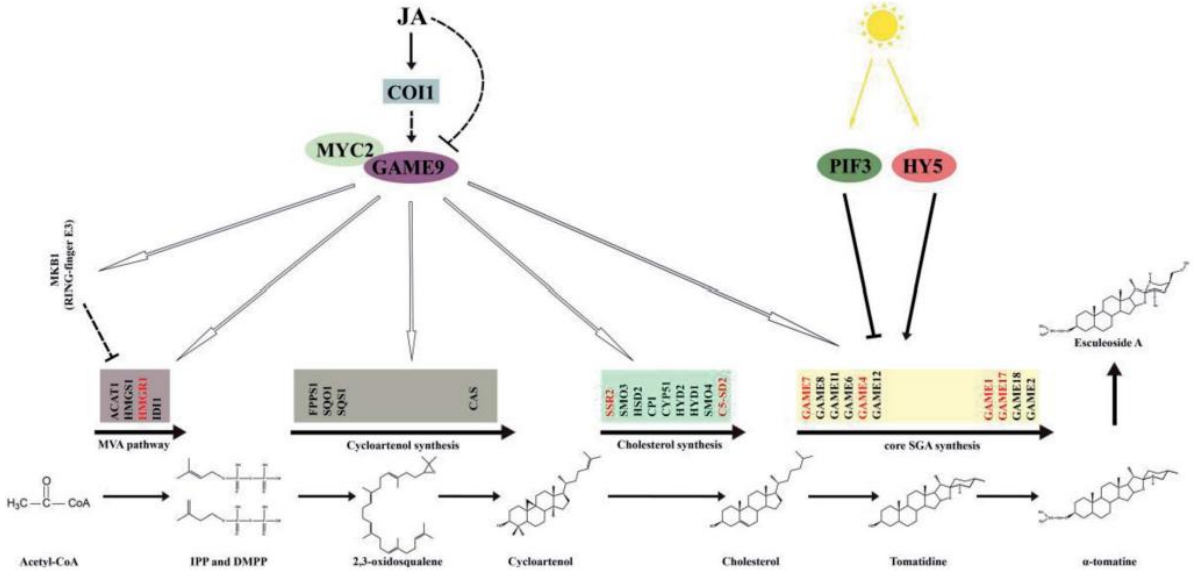
SA biosynthesis pathway starts with mevalonate pathway which leads to cycloartenol biosynthesis from acetyl-CoA, a ubiquitously found triterpenoid sterol in plants which is further converted to cholesterol. Then, cholesterol is converted to steroidal aglycones via a series of glycoalkaloid metabolism (GAME) enzymes which include a series of hydroxylation, amination and ring-closure steps. Finally, for addition of 3’ glycosidic unit in SGAs, a series of UDP-dependent glycosyltransferase or UGT enzymes catalyze sequential addition of sugar moieties one-at-a-time (Zhao et al., 2021). Recently, Jozwiak et al., 2014 discovered a new ER-localized protein cellulose synthase-like G (CSL-G) protein with cellulose glucuronidase activity and scaffold protein for other enzymes involved in SGA biosynthesis for better channelling of metabolic intermediates preventing their diffusion and ensuring higher enzymatic efficiency (an assembly known as ‘metabolon’) (Jozwiak et al., 2024). Detailed SA biosynthetic pathway starting from acetyl-CoA for potato, tomato and eggplant SAs is given

Figure 3 - Biosynthetic pathway of steroidal alkaloids in potato, tomato and eggplant as adapted from Zhao et al. 2021.



SA biosynthesis is regulated by many genetic, developmental and environmental signals; for example in potato, tissue types have varying SA content with flowers, leaves, tuber skin and sprouts having highest SA content in potatoes, genotype also determines potato SA levels with tuber SA content in potatoes varying from 20 to 220 mg/kg, light wavelength, intensity and photoperiod as tubers exposed to UV/IR light higher intensity and/or higher light period show higher SGA accumulation and mechanical damage and insect herbivory also lead to upregulation of SA levels in potato (Valkonen et al., 1996; Milner et al., 2011). SA biosynthesis is tightly regulated with many genes of SA biosynthesis pathway (GAME genes) being arranged in gene clusters for better regulation of SA biosynthesis pathways, for example, most SA biosynthesis genes in tomato are arranged in a ~200 kbp region in chromosome 7 which include CSL-G scaffold protein (GAME15), three UGTs (GAME2, GAME17 and GAME 18), a CYP450 (GAME6) and 2-oxoglutarate-dependent dioxygenase (2-OGD) enzyme (GAME11) (Itkin et al., 2013). A jasmonate-induced transcription factor (TF) JRE4/GAME9 belonging to group IX APETELA2/ERF family of TFs was found to upregulate many SA biosynthesis genes in tomato, which include hydroxymethyl-glutaryl reductase (HMGR), cycloartenol synthase (CAS), sterol side chain reductase 2 (SSR2), C5-sterol desaturase (C5-SD) and all GAME genes in both potato and tomato (Cárdenas et al., 2016; Abdelkareem et al., 2017; Nakayasu et al., 2018). GAME9 acts synergistically with MYC2 TF which recognizes G-box elements involved in jasmonate (JA) signaling pathway and signals inducing jasmonate-signaling which include wounding and insect herbivory also induce SA biosynthesis (Cárdenas et al., 2016). Light-signaling TFs ELONGATED HYPOCOTYL 5 (SlHY5) and PHYTOCHROME INTERACTING FACTOR 3 (SlPIF3) in tomato where shown to upregulate and downregulate GAME genes GAME1, GAME4 and GAME17 thus upregulating and downregulating SA levels in presence and absence of light respectively in tomato (Wang et al., 2018). Regulation of SA biosynthesis is shown in Figure 4.

Figure 4 - Regulation of SA biosynthetic pathway as adapted from Zhao et al. 2021.



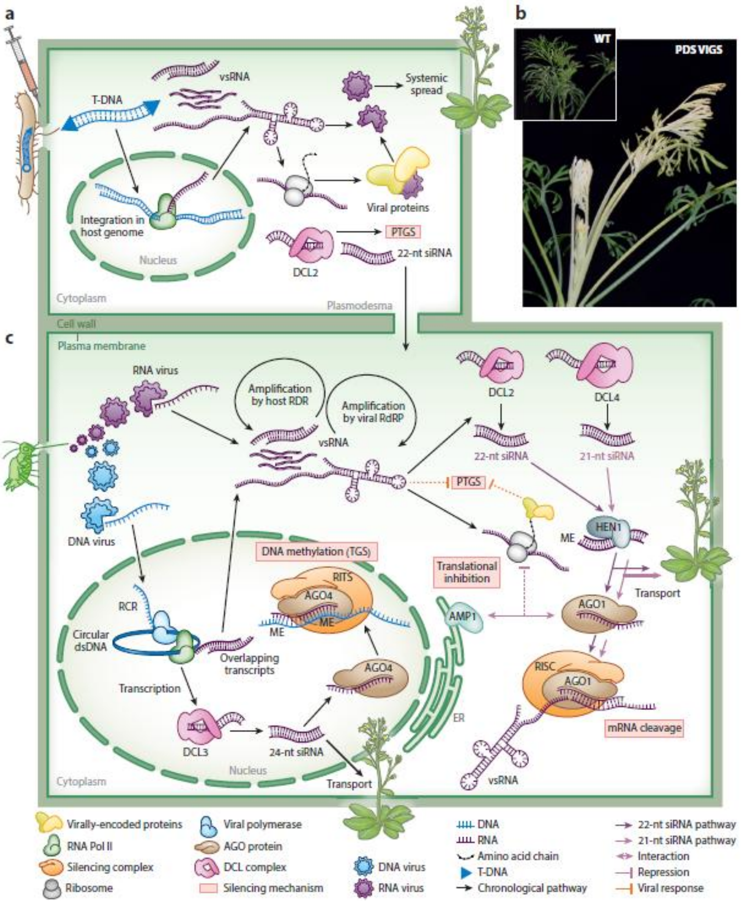
SAs have been shown to have insect/nematode/mollusc deterrent and insecticidal/molluscicidal activities on many pests which include Colorado potato beetle (*Leptinotarsa decemlineata*), potato leafhopper (*Empoasca fabae*), green peach aphid (*Myzus persicae*), tobacco hornworm (*Manduca sexta*), golden potato cyst nematode (*Globodera rostochiensis*) and snails *Helix aspersa* and *Lymnea cubensis*, and also showed embryotoxicity in many vertebrates like mouse (*Mus musculus*), frog (*Xenopus laevis*) and cow (*Bos taurus*), with frog embryo teratogenesis assay (FETAX) commonly being used to assess embryotoxicity of SAs. SA levels are also a concern in commercial solanaceous vegetables like potato, tomato and eggplant because they taste bitter and excess SA levels (>20mg/100g fresh weight in potato) can cause burning sensation in mouth and throat, which lead to selection, domestication and development of varieties which had reduced content of SAs (Sinden et al., 1984; Johns & Keen, 1986). Aside from negative effects, positive effects of SAs have also been well-studied and documented, which include anticancer, anti-inflammatory, anticholesterol and antimicrobial activities, with cream Curaderm^{BEC5}, a preparation with α-solasonine and α-solamargine SAs available for treating skin cancers and many other related SA products undergoing clinical trials, especially as anticancer drugs as SAs many SAs have been shown to have preferential cytotoxicity, anti-proliferative, antimetastatic and apoptotic activities against many cancer cell lines. Most plants contain not one but two or more major SGAs, with α-solanine and α-chaconine in potato, α-tomatine and esculeoside A in tomato and α-solasonine and α-solamargine in eggplant, which is often attributed different individual activities of SAs and increased synergistic effects of their mixtures. Mechanisms of bioactivity of SAs have also been studied and demonstrated, with three key properties involved - first, inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) involved in functioning of nervous system; second, membrane disruption and loss of integrity caused by complexation of aglycone moieties of SGAs with membrane sterols, interactions of glycosidic units of SGAs to form rigid glycoalkaloid-sterol complexes and finally membrane budding; and third, disruption of membrane potential and Na^+ transport. Structure-activity relation studies have also revealed that glycosidic units are essential and very potent for many bioactive properties of SGAs with corresponding aglycone SAs showing little to no bioactivity (with a few exceptions) and aglycone also playing an important role in bioactivity with features like presence of N-atom and C5-C6 saturation playing key roles in determining bioactivity of many SAs (Milner et al., 2011).

1.5. Virus-Induced Gene Silencing (VIGS) and Virus-Mediated Overexpression (VOX)

Virus-induced gene silencing (VIGS) is a technique in plant biology used for transient silencing of targeted gene(s) plants using viral vectors and RNA interference (RNAi) mechanism. In VIGS, a 100-500 bp long fragment of gene of interest (GOI) (called VIGS fragment) is cloned in a viral vector (plasmids containing parts of plant-infecting viruses), transformed in *Agrobacterium tumefaciens* and agro-infiltrated in young plant parts like leaf and fruit, from where the VIGS fragment is expressed, amplified and used to silence GOI using RNAi mechanism. Upon agro-infiltration, viral genome elements in viral vectors are transformed in plant cells and transcribe parts of viral genome producing double-stranded viral short interfering RNAs (vsRNAs) via RNA Pol II for DNA viruses or both host and virally encoded RNA-dependent RNA polymerases (RdRPs) for RNA viruses, with *Arabidopsis thaliana* having six RdRPs (RdRP1-6) each with its own functions and some redundancy with other

RdRPs. vsRNAs are then recognized by DICER-LIKE (DCL) proteins which cleave them into primary short interfering RNAs (siRNAs), with *A. thaliana* having four DCLs (DCL1-4) producing primary siRNAs of different length which are subsequently methylated at 3’ end by HUA ENHANCER 1 (HEN1) to enhance their stability. The most common mechanism for RNAi using siRNA in plants is post-transcriptional gene silencing (PTGS) via cleavage of target RNA from GOI thereby silencing it, which is achieved by ARGONAUTE (AGO) nucleases which bind to siRNA, convert it to single-stranded siRNAs, assemble with other cofactors to form RNA-induced silencing complex (RISC), use sequence complementarity of bound siRNA to find target RNA from GOI and cleave target RNA using RNAse H-like activity, with *A. thaliana* having ten AGOs (AGO1-10) with each AGO targeting siRNAs with different sizes, sequences, locations and viral origins. Other mechanisms for gene silencing by RNAi via siRNAs include translational inhibition as siRNA-RISC complex binds to mRNA and prevents progress and promotes release of translational machinery from mRNA, transcriptional gene silencing (TGS) which includes RNA-directed DNA methylation (RdDM) pathway which uses AGO4/siRNA complex (often called RNA induced transcriptional silencing or RITS complex) to recruit DNA methyltransferases to methylate cytosines at GOI inducing hypermethylation and thus silencing, and chromatin modification by deposition of repressive H3K9me2 histone marks at GOI for chromatin compaction and further silencing. These vsRNAs are then transcribed and expressed to generate both viral particles and siRNAs, which are spread from infected plant parts to other plant parts first by intercellular spreading using plasmodesmata, and then long-distance (often systemic) spreading through the phloem thus achieving long-distance (systemic) silencing in plants using VIGS (Rössner et al., 2022). Mechanism of RNAi and VIGS is demonstrated in Figure 5.

Figure 5 - Mechanism of RNAi and VIGS as adapted from Rössner et al. 2022



Many viruses which cause diseases in plants have been used for designing VIGS viral vectors, some of which include tobacco mosaic virus (TMV), potato virus X (PVX), barley stripe mosaic virus (BSMV), pea early browning virus (PEBV) and foxtail mosaic virus (FoMV).Of thgese viral vectors, tobacco rattle virus (TRV) vectors made from TRV virus which is a non-enveloped, rod shaped positive-stranded ssRNA in *Tobravirus* genus, has a bipartite genome (TRV1 and TRV2), known to cause diseases in 500 species of plants belonging to 40 families with varying symptoms like mottling, chlorotic lesions and systemic necrosis and is transmitted by stubby root nematodes (*Trichodorus* spp.). TRV1 genome (SYM strain) consists of 134K and 194K replicases for replication of vital genome, a 29K protein involved in cell-to-cell movement of viral particles (movement protein or MP) and a 16K protein involved in weak suppression of RNAi and entry in meristem for viral particles (Hamilton et al., 1987; Ziegler-Graff & Guilford, 1991; Martín-Hernández & Baulcombe, 2008). TRV2 genome (PPK20 isolate transmitted by *Paratrichodorus pachydermus* nematodes) consists of coat protein which makes up the viral envelope, a 29.4K protein involved in transmission of virus from nematode vector to plant and a 32.8K protein with no well-established function (Hernandez et al., 1995; Brown et al., 1997; Visser & Bol, 1999). As the 29.4K and 32.8K proteins are not required for viral replication and their systemic infection, TRV viral vectors omit these sequences in their TRV2 sequence, replacing it with sequences used for efficient cloning and expression of viral vector, for example, Liu et al. 2002a produced the most commonly used TRV vectors pYL192 (pTRV1) and pYL156 (pTRV2) obtained from RNA1 and RNA2 genomes of PPK20 strain, with removal of 29.4K and 32.8K sequences and addition of double 35S promoter, self-cleaving ribozyme (Rz) before nopaline synthase terminator (NOSt) and multiple cloning site (MCS) for restriction-ligation cloning (Liu, Schiff, & Dinesh-Kumar, 2002; Liu, Schiff, Marathe, et al., 2002). Many factors affect efficiency of TRV-VIGS, which include *A. tumefaciens* strain, age and state (developmental and physiological) of plants, temperature (low temperatures of 16-22 °C optimal) and inoculation method and duration (leaf injection via syringe most commonly used) (Shi et al., 2021). Although TRV vectors have been developed and most commonly used for VIGS in Solanaceae plants like tobacco, potato, tomato and eggplant, they have also been successfully used for plants from other families like dicot families Malvaceae in cotton and Brassicaceae like *A. thaliana* and monocot families like Gramineae in wheat and maize and Orchidaceae in *Phalaenopsis spp.* (Shi et al., 2021). Key advantages of using TRV vectors for VIGS include wide choice of vectors for different applications, mild disease symptoms, high silencing efficiency, wide variety of host plants and most importantly, silencing in meristematic tissues (which is not possible with most viral vectors) which leads to systemic gene silencing and in many cases, even VIGS inheritance, where VIGS silencing is carried over to future generations (Senthil-Kumar & Mysore, 2011; Shi et al., 2021).

Many other methods of gene silencing, overexpression and modification which are related to VIGS in using either viral vectors and/or RNAi mechanism are also being used in plant biology and biotechnology, three of the examples being virus-induced overexpression (VOX), virus-induced genome editing (VIGE) and host-induced gene silencing (HIGS). Virus-mediated overexpression (VOX) consists of inserting CDS of GOI instead of VIGS fragment in viral vectors either upstream of a constitutive promoter like 35S CaMV promoter for DNA viruses, fused to highly expressed viral protein and cleaved by autoproteolytic peptide like 2A or cloned ahead of a sub-genomic promoter like PeBV promoter in RNA viruses and agro-infiltrating them in plants where these are transformed and highly expressed. Virus-induced genome editing (VIGE) consists of delivering single guide RNAs (sgRNAs) to *Cas9* transgenic plants using viral vectors, which often results in a higher efficiency for delivering sgRNAs due to systemic nature of viral particles and using mobile sgRNAs fused with fragment FLOWERING LOCUS T mRNA which makes sgRNAs more mobile. Host-induced gene silencing (HIGS) (also called plant-mediated RNAi or PMRi) utilizes siRNAs which can travel from plants to organisms from other kingdoms like fungi (pathogens), nematodes and insects (animals) and silence their genes (cross-kingdom siRNA transfer). Here, plant is agro-infiltrated with VIGS vectors containing VIGS fragments corresponding to host's genes expressing their siRNAs, and then host is made to feed upon the transformed plants either by pathogen infestation (for fungi and nematodes) or herbivory (insects) where siRNAs expressed in transformed plants are transferred to other hosts and perform gene silencing in them. These RNAi-assisted technologies are have found tremendous applications in both plant biology in functional genomics of genes involved in plant growth, development, primary and secondary metabolism, abiotic stress and plant-pathogen and plant-herbivore interactions and agriculture for generating transgenics with desirable qualities like increased yield, better quality, better abiotic stress tolerance and higher resistance to pests and pathogens (Rössner et al., 2022).

1.6. Motivation

Previous work done in Umesh et al., 2022 (Umesh et al., 2022) studied the differential preference of TCW on 5 eggplant varieties and tried to pinpoint the potential metabolites present in these eggplant varieties which are responsible for this differential preference. They employed UPLC-ESI-QTOF-based metabolomics and various insect preference and performance assays to study this differential preference and made three key findings -

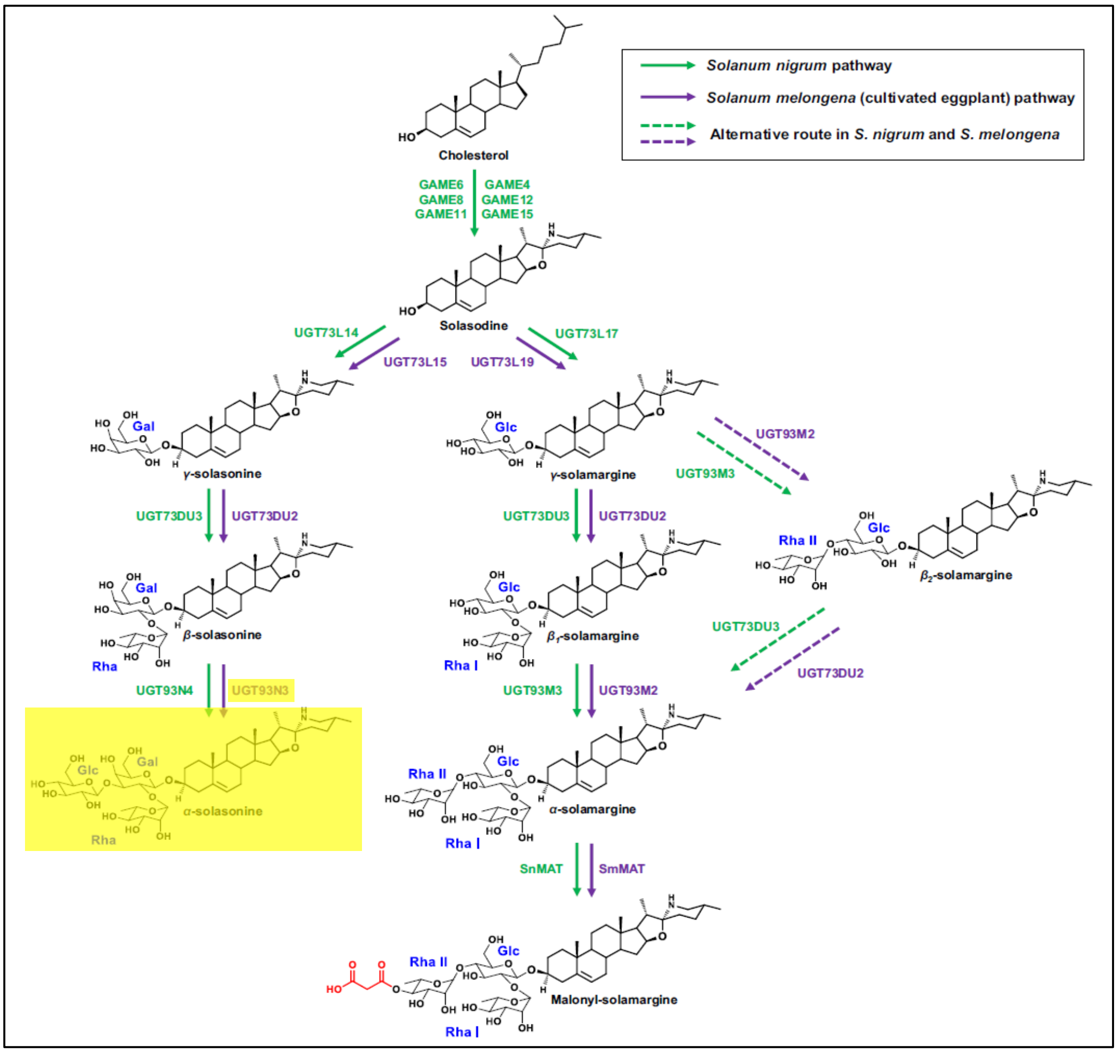
1. TCW shows reduced preference and performance on eggplant variety JK Purple Long (JKP from now on).

2. JKP shows highest content of eggplant steroidal alkaloid solasodine (SD from now on) and steroidal glycoalkaloids solasonine and solamargine (SN and SM from now on respectively).

3. SN and not SD and SM reduce TCW's preference and performance in artificial diet-based feeding assays.

In another paper Lucier et al., 2024 (Lucier et al., 2024), biosynthetic pathway of SD, SM and SN and the various enzymes involved in it was elucidated in both black nightshade (*Solanum nigrum*) and eggplant. They used a combination of comparative transcriptomics and metabolomics, combinatorial heterologous expression in *N. benthamiana* and enzyme assays to elucidate this biosynthetic pathway. This biosynthetic pathway is very similar to potato and tomato SGA biosynthesis pathway with the first stage involving formation of aglycone SD from cholesterol using six GAME genes and a series of six UGTs adding sugar moieties to steroidal scaffold to prepare SM and SN with three UGTs involved in each. For SN biosynthesis in eggplant, UGT73L15 adds galactose to 3' hydroxyl of SD to make γ-solasonine, then UGT73DU2 adds rhamnose to γ-solasonine at 2’ hydroxyl of galactose moiety to make β-solasonine and finally UGT93N3 adds glucose to 3’ hydroxyl of galactose moiety to make α-solasonine or SD. SA biosynthetic pathway in eggplant as given in Lucier et al. 2024 is given in Figure 6.

Figure 6 - SA biosynthesis pathway in eggplant (*Solanum melongena*) and black nightshade (*S. nigrum*) as adapted from Lucier et al. 2024. Metabolite α-solasonine and its terminal biosynthesis gene in eggplant UGT93N3 which are the targets of this study are highlighted.



1.7. Hypothesis

Based on previous work done in Umesh et al., 2022, I summarized three key findings of this paper which are -

1. TCW has lower preference and performance on eggplant variety JK Purple Long (JKP from now on).

2. JKP has higher contents of eggplant SAs solasodine (SD), solasonine (SN) and solamargine (SM) in it as compared to other assayed varieties.

3. SN, and not SD and SM negatively impacts preference and performance of TCW when used in artificial diet (AD) based assays.

Based on these three key findings, I formulated a hypothesis which connects all these three key findings which is -

Hypothesis -

Higher SN content in eggplant is responsible for lower preference and performance of TCW.

To test this hypothesis, we performed reverse-genetic characterization of SN for antifeedant activity against TCW through transient silencing and overexpression of terminal SN biosynthesis gene in eggplant, namely UGT93N3. I further hypothesize that transient silencing and overexpression of SN will increase and decrease the preference and performance of TCW on eggplant respectively.

1.8. Aim and Objectives

Aim -

To preform reverse-genetic characterization of gene UGT93N3 involved in SN biosynthesis for antifeedant activity against TCW using transient silencing and overexpression of UGT93N3 using VIGS and VOX respectively.

The objectives of this project are -

1. To clone UGT93N3 gene's VIGS fragment and CDS in pTRV2 VIGS and pSPDK VOX vector respectively.

2. To perform and verify VIGS and VOX of UGT93N3 gene in JKP using qPCRs for gene expression and HPLC-ELSD-based metabolomics for SN metabolite levels.

3. To perform insect preference and performance assays, namely dual-choice assay and neonate mortality assay on UGT93N3 VIGS and VOX plants to assess the effects of reduced and increased SN on TCW's preference and performance.

2. Materials and Methods

2.1. Cloning of UGT93N3 VIGS Fragment and Coding Sequence (CDS) in pTRV2 Vectors

pTV00 (pTRV2) and pSPDK3876 (pSPDK) vectors were chosen for cloning VIGS fragment and CDS of UGT93N3 gene for VIGS and VOX respectively (Ratcliff et al., 2001; Ellison et al., 2020). 1425 bp long sequence of UGT93N3 was retrieved from Supplementary Material of Lucier et al. 2024 and used as query for BLAST on Eggplant Genome V4.1 Transcripts on Sol Genomics Network (https://solgenomics.net/tools/blast/?db_id=325) at word_size 11 and e_value 10^{-100} and three sequences, SMEL4.1_11g019060.1.01 (query sequence, 100% match), SMEL4.1_11g019100.1.01 (9100 from now on) (87.35% match) and SMEL4.1_11g019070.1.01 (9070 from now on) (87.22% match) were obtained as close matches (> 85%) to the query. For designing VIGS fragment, one needs to clone it in antisense orientation and select a 100-500 bp region in the CDS near 3' end or 3' untranslated region (UTR) in the gene's transcript which does not have any similar stretch of greater than 18 bp for some other transcript. Using SGN VIGS Tool (https://vigs.solgenomics.net/), a 192 bp long stretch from 1100 to 1291 was chosen which satisfies the above criteria for VIGS fragment. Primers were also designed for UGT93N3 VIGS fragment and full-length CDS with and without RE sites for cloning (BamHI and SalI for VIGS fragment, BamHI and SacI for full-length CDS) and qPCR primers for UGT93N3 for assessing silencing and overexpression in VIGS and VOX respectively, and 9100 and 9070 for assessing off-target silencing in VIGS.

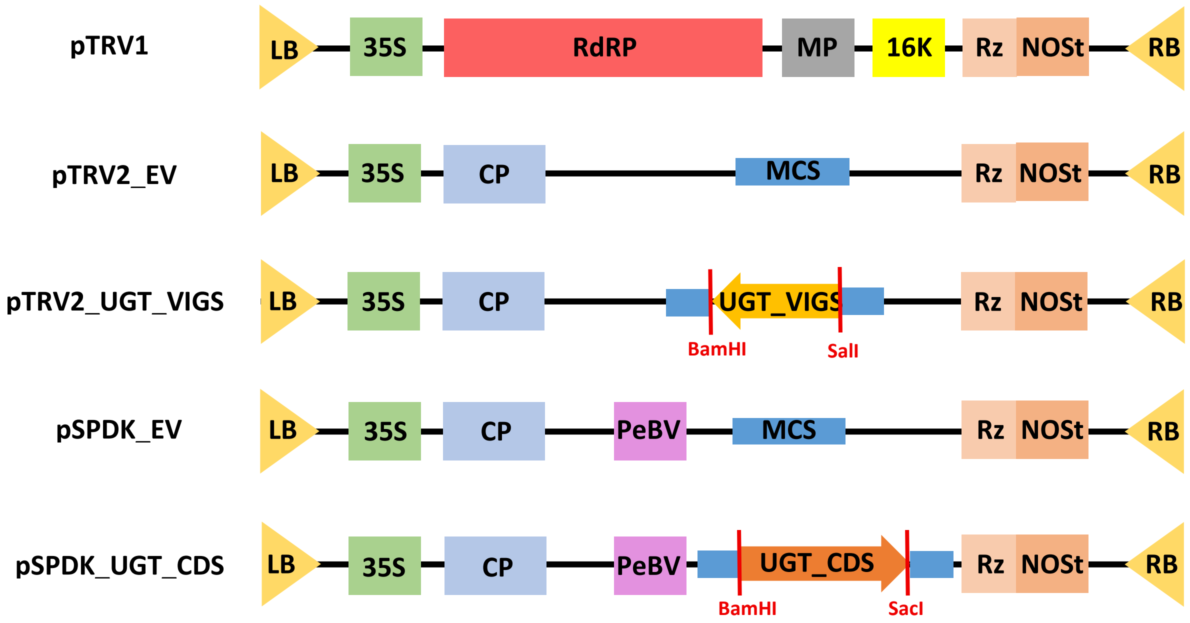
JKP plants were grown to maturity for 2 months and healthy and fully expanded leaves were collected, flash-frozen in liquid N_2 and finely homogenized using mortar and pestle. 1 ml RNAiso Plus™ reagent (TaKaRa®) was added to ~100 mg homogenized tissue and RNA was isolated according to manufacturer's protocol and was used for cDNA synthesis using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa®). cDNA was used as template to amplify VIGS fragment using primers without RE sites using Ex Taq DNA Polymerase (TaKaRa®) at thermocycling conditions 95 °C for 10 minutes, 45 cycles of 95 °C for 30 seconds, 45 cycles of 60 °C for 30 seconds, 45 cycles of 72 °C for 40 seconds and 72 °C for 10 minutes, purified using NucleoSpin™ Gel and PCR Clean-up, Mini kit (MACHEREY-NAGEL®), used as template for PCR for RE site-containing primers with same thermocycling conditions. RE-site containing VIGS fragment was then digested along with pTRV2 using BamHI-HF® and SalI-HF® (New England Biolabs®) and ligated using T4 DNA Ligase (Promega®). Ligated pTRV2_UGT93N3_VIGS construct was transformed into homemade chemicompetent TOP10 (*E. coli*) cells and plasmids were isolated using HiPurA® Plasmid DNA Miniprep Purification Kit (HiMedia®). Isolated plasmids were given for sequencing to verify the fidelity of VIGS fragment and transformed into homemade chemicompetent GV3101 (*A. tumefaciens*) cells. Same steps were followed for cloning of UGT93N3 CDS for making except thermocycling conditions were for amplifying without and with RE site containing CDS fragment were 95 °C for 10 minutes, 45 cycles of 95 °C for 30 seconds, 45 cycles of 58 °C for 30 seconds, 45 cycles of 72 °C for 1 minute and 72 °C for 10 minutes and BamHI-HF® and

SacI-HF® were used for restriction digestion of both plasmid and insert. Details of constructs are given in Figure 7. List of primers used in this study is given in Table 1.

Table 1 - List of primers used in this study

Gene Name	Purpose of Primers	Primer Code	Primer Sequence
UGT93N3	VIGS fragment	UGT_F	GTCATTGCGGATGGAACTCC
		UGT_R	TCTTGACAGATTTTTCGATCGAC
UGT93N3	RE site addition to VIGS fragment	UGT_VF	GCGGCGGTCGACGTCATTGCGGATGGAACTCC
		UGT_VR	GCGGCGGGATCCTCTTGACAGATTTTTCGATCGAC
UGT93N3	CDS fragment	UGT_FLF	ATGGCTTCTATCAACAATTCAATAA
		UGT_FLR	TTATTTGGTAATATATGATATGAAAGATTC
UGT93N3	RE site addition to CDS fragment	UGT_VFLF	GCGGCGGGATCCATGGCTTCTATCAACAATTCAATAA
		UGT_VFLR	GCGGCGGAGCTCTTATTTGGTAATATATGATATGAAAGATTC
UGT93N3	qPCR	UGT_qF	GCCTTAAAGATGAATGTTTTGAGG
		UGT_qR	CAACAACTCTATAATCGACAGCAG
SMEL4.1_11g019100.1.01	qPCR	9100_qF	CTTGTATCGAGAGCATTAGCACG
		9100_qR	CTGATGTCACTAACTCATCCTTGC
SMEL4.1_11g019070.1.01	qPCR	9070_qF	AGTGATCGTGTATCTTCAATCCAC
		9070_qR	GTGTCGAAGTAAAGAATAGCGGC

Figure 7 - Details of vector constructs used in this study. LB - Left Border, RB - Right Border, 35S - 35S Promoter, RdRP - Viral RNA-dependent RNA Polymerase, MP - Movement Protein, 16K - 16 kDa Protein, Rz - Ribozyme, NOSt - NOS Terminator, CP - Coat Protein, MCS - Multiple Cloning Site, PeBV - Pea Early Browning Virus Promoter



2.2. Plants

For isolating cDNA for cloning JKP seeds stored at -20 °C were planted in seed trays with cocopeat mixed with cow dung manure and Soilrite™ mixture, supplemented with Hoagland No. 2 Basal Salt Mixture (HiMedia®), wrapped in an autoclave bag and allowed to germinate for 2 weeks. After germination, seedlings were supplemented with water every day and Hoagland's solution twice a week for a month. Double autoclaved soil mixed with urea, cow dung manure and NPK was supplemented with turmeric and asafoetida, transferred to clean pots and plants were transferred to these pots. Plants were grown for two more weeks in pots and mature leaves were collected for cDNA synthesis. For VIGS and VOX, 4-5 leaf stage plants of variety PanchaGanga® Hybrid F1 Gaurav plants were potted using same procedure as mentioned above. Plants were then transferred indoor in growth cabinets and supplemented with 0.1 g/l urea, Hoagland's solution and water and grown at relative humidity 70%, photoperiod 16h light-8h dark and temperature 22 °C and used for performing VIGS and VOX.

2.3. Insects

TCW larvae were procured as egg masses and larvae from ICAR-Central Institute for Cotton Research (ICAR-CICR) and ICAR-Indian Institute of Horticultural Research (ICAR-IIHR). Upon hatching, larvae were fed a mixture of healthy castor (*Ricinus communis*) and cabbage (*Brassica oleraceae var. capitata*) leaves which were washed with water, then soaked in 2%

bleach solution and then washed again and changed every day and also given sunlight and UV-A light every day for 15-30 minutes till they became pupae after 18-22 days. Pupae were transferred gently using forceps into small containers filled with autoclaved red soil and kept till they eclosed into adult moths after 8-10 days. Eclosed moths were transferred to cages with castor leaves kept in water for oviposition and cotton soaked in 10% sucrose solution for feeding moths till they died for 8-10 days. Egg patches deposited on castor leaves were collected and transferred to a container till they hatched to neonates after 4-6 days and transferred to fresh castor leaves to continue the insect culture. All the insects and their stages were maintained in an insect culture room with temperature 27 °C and 12h light-12h dark photoperiod.

2.4. VIGS and VOX

Plants were maintained in indoor growth cabinets for VIGS and VOX with relative humidity 70%, photoperiod 16h light-8h dark and temperature 22 °C at 4-5 leaf stage for VIGS and VOX. GV3101 glycerol stocks of pTRV1, pTRV2_EV (empty vector) and pTRV1, pTRV2_UGT93N3_VIGS for VIGS and pSPDK_EV and pSPDK_UGT93N3_CDS were inoculated in YEP medium containing 25 µg/ml rifampicin and 50 µg/ml kanamycin and grown at 28 °C at 200 rpm for 24 hours to make primary cultures. Secondary cultures were made by inoculating 1 ml primary medium in 100 ml YEP medium containing 10 mM MES (2-(4-morpholine)-ethane sulphonic acid), 20 µM acetosyringone, 25 µg/ml rifampicin and 50 µg/ml kanamycin and grown at 28 °C at 200 rpm until OD_{600} reached 2.0. Cells were collected by centrifugation at room temperature (RT) at 4000 rpm for 10 minutes, culture media was discarded and cell pellets were resuspended in same volume of infiltration buffer consisting of 10 mM $MgCl_2$, 10 mM MES and 200 µM acetosyringone suspended in Milli-Q® water adjusted to pH 5.6. Just before infiltration, pTRV1 and respective pTRV2_EV, pTRV2_UGT_VIGS, pSPDK_EV and pSPDK_UGT_CDS cultures were mixed in 1:1 ratio and infiltrated in leaves using needleless syringes. Plants were infiltrated and kept in dark for 48 hours to enhance efficiency of VIGS and VOX. The subsequent set of leaves which arose after infiltration after 8-10 days were used for verification of VIGS and VOX respectively using qPCRs and metabolite analysis and used for insect assays. Thirty (n = 30) plants were taken for each pTRV2_EV, pTRV2_UGT_VIGS, pSPDK_EV and pSPDK_UGT_CDS inoculations and thirty plants which were uninfiltrated were designated as wild-type (WT).

To assess gene-silencing and overexpression in VIGS and VOX plants and to assess any off-target silencing effects in VIGS, qPCRs of UGT93N3, 9100 and 9070 were conducted in VIGS plants and only UGT93N3 were conducted in VOX plants and housekeeping gene CyclophilinA was used as internal reference standard for analyzing relative transcript levels using $2^{-\Delta\Delta Ct}$ method. Leaves were collected, flash-frozen in liquid N_2 and finely homogenized using mortar and pestle. 1 ml RNAiso Plus™ reagent (TaKaRa®) was added to ~100 mg homogenized tissue, RNA was isolated according to manufacturer's protocol and was used for cDNA synthesis using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa®). 1 µl cDNA was used for performing qPCRs using qRT-PCR cocktail made from TB Green® Premix Ex Taq II™ (Tli RNase H Plus) (TaKaRa®). and qPCRs were run on CFX96™ Touch Real-Time

PCR Detection System (BioRad®) and run with thermocycling conditions of 95 °C for 1 minute and 40 cycles of 95 °C for 45 seconds, 60 °C for 15 seconds and 72 °C for 20 seconds.

To assess whether gene-silencing and overexpression in VIGS and VOX plants have reduced and elevated the levels of SN respectively, levels of SD, SM and SN in leaves of WT, pTRV2_EV, pTRV2_UGT_VIGS, pSPDK_EV and pSPDK_UGT_CDS were analyzed using HPLC-ELSD detection on Agilent® 1200 Infinity HPLC system coupled with 1260 Infinity ELSD detector using Phenomenex Gemini® C18 column (50 x 4.6 mm, 5 µm, 110 Å) with attached guard column. 200 mg leaf sample which was finely crushed in liquid nitrogen using mortar and pestle. Metabolite extraction was performed by vortexing crushed tissue in LC-MS grade methanol mixed with 50 µg/ml adonitol as internal standard, centrifuging at 10000 RPM at RT for 10 min, decanting the supernatant, centrifuging again at 15000 RPM at RT for 20 min, decanting the supernatant, incubating samples overnight at -80 °C, centrifuging at 15000 RPM at 4 °C for 20 min and collecting top layer of supernatant in LC vials. Standards of SD, SM and SN were first dissolved in minimal amount of DMSO and then LC-MS grade methanol and prepared in serial dilutions of 1 mg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml and 15.625 µg/ml and run initially on HPLC-ELSD along with 3 blanks before and after standards and then samples were run with one blank after each 10 samples. Mobile phase constituted of water with 0.1% formic acid (v/v) as component 'A' and methanol with 0.1% formic acid (v/v) as component 'B'. With the flow rate of 0.5 ml min^{-1}, a gradient of 'B' with 5% (0 min), 5% (1.5 min), 95% (10 min), 95% (11 min), 5% (12 min) and 5% (15 min) was used. ELSD parameters used were nebulizer temperature of 35 °C, evaporator temperature of 70 °C and gas flow rate of 1.4 SLM (standard litres per minute).

2.5. Dual-Choice Assays

Dual-choice assays were conducted as prescribed by Umesh et al. 2021. Six types of dual choice setups were used using detached leaves from VIGS and VOX plants - pTRV2_EV vs. WT, pTRV2_EV vs. pTRV2_UGT_VIGS, pTRV2_UGT_VIGS vs. pTRV2_UGT_VIGS+Solvent, pTRV2_UGT_VIGS+Solvent vs. pTRV2_UGT_VIGS+SN, pSPDK_EV vs. WT and pSPDK_EV vs. pSPDK_UGT_CDS where solvent represents 0.0002% DMSO used to make SN stocks and 'SN' represents 0.0002% DMSO solution in which SN is added in physiological concentrations as observed in leaves of WT JKP using HPLC-UV Vis-based metabolomics and both were complemented 500 µl per leaf using a pipette. One freshly eclosed 4th instar TCW larva was placed between two freshly detached leaves in each setup and after 24 hours, the plants were inspected for presence or absence of larvae, where the presence of larva was scored as '1' and absence was scored as '0'. Ten (n = 10) such assays were performed for each combination and by considering the initial larval number on each host as 100%, the percentages of larvae remaining on each leaf were calculated.

2.6. Neonate Mortality Assays

Neonate mortality assays were conducted as prescribed by Umesh et al. 2021. Twenty freshly hatched neonates were placed on freshly detached leaves of WT, pTRV2_EV, pTRV2_UGT_VIGS, pTRV2_UGT_VIGS+Sol, pTRV2_UGT_VIGS+SN, pSPDK_EV and pSPDK_UGT_CDS placed in circular polypropylene containers. After 48 h, the number of dead neonates were counted and by considering initial larval number of 20 as 100%, neonate mortality percentage was calculated. Five (n = 5) such setups were used for each treatment.

2.7. Statistical Analyses

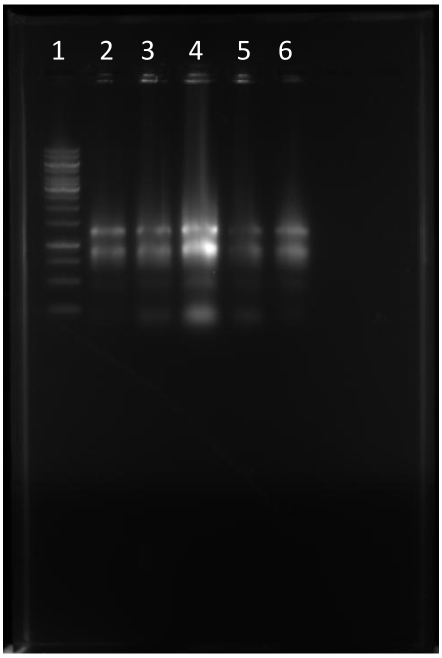
All quantitative data (mean ± SE) was subjected to homogeneity tests and non-homogeneous data was subjected to normality tests to determine the use of appropriate statistical tests to use for each set of experimental data. Levene's test was used to analyze homogeneity and Shapiro-Wilk test was used to analyze normality in non-homogeneous data. One-way ANOVA was used to analyze neonate mortality data with statistical significance determined by Tukey's *post hoc* test ($p \leq 0.05$). Statistical significance of binary outcomes of dual-choice assays was determined using Fisher's exact test ($p \leq 0.05$).

3. Results

3.1. Cloning of UGT93N3 VIGS Fragment and Coding Sequence (CDS) in pTRV2 Vectors

RNA was isolated successfully from JKP plants and verified by running on 1% agarose gel which gave two major visible bands corresponding to 28S and 18S rRNA (Figure 8).

Figure 8 - 1% agarose gel of RNA isolated from crushed tissue samples. 1 - 1 kb ladder; 2 - RNA sample 1; 3 - RNA sample 2; 4 - RNA sample 3; 5 - RNA sample 4; and 6 - RNA sample 5



Isolated RNA was successfully made into cDNA and two PCRs of 50 µl reaction volume each were performed using first UGT93N3 F/R primers with cDNA as template to amplify VIGS fragment and VF/VR primers using purified F/R PCR reaction to attach restriction sites to PCR product and then purified. NanoDrop™ readings and gels corresponding to PCR products were performed to analyze the integrity of PCR products and attachment of RE sites to VIGS fragment are given in Figures 9 and 10 respectively.

Figure 9 - NanoDrop™ readings of PCR purified products. 1 - F/R reaction; 2. F/R reaction 2; VF/VR reaction 1; and 4 - VF/VR reaction 2

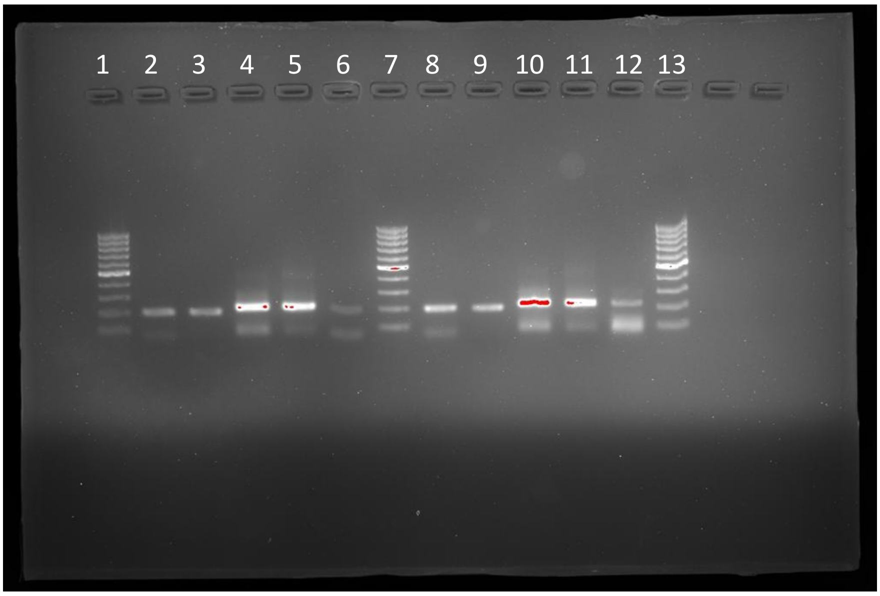
#0890 FR_Pur_1
24.9 ng/µL
A260: 0.498
A260/A280: 1.69
A260/A230: 1.51

#0891 FR_Pur_2
24.5 ng/µL
A260: 0.490
A260/A280: 1.62
A260/A230: 1.47

#0892 VFVR_Pur_1
66.3 ng/µL
A260: 1.326
A260/A280: 1.82
A260/A230: 1.89

#0893 VFVR_Pur_2
65.6 ng/µL
A260: 1.312
A260/A280: 1.82
A260/A230: 1.86

Figure 10 - 2% agarose gels of PCR and PCR purified products for F/R and VF/VR PCRs. F/R fragment size (without RE sites) = 192 bp and VF/VR fragment size (with RE sites) = 216 bp. 1 - 100 bp ladder; 2 - F/R reaction 1; 3 - F/R reaction 1 purified; 4 - VF/VR reaction 1; 5 - VF/VR reaction 1 purified; 6 - F/R negative (PCR reaction without template) 7 - 100 bp ladder; 8 - F/R reaction 2; 9 - F/R reaction 2 purified; 10 - VF/VR reaction 2; 11 - VF/VR reaction 2 purified; 12 - VF/VR negative (PCR reaction without template); and 13 - 100 bp ladder.



1 µg of both insert (RE-site containing UGT93N3 VIGS fragment) and plasmid (pTRV2 empty vector) were double digested using BamHI-HF® and SalI-HF® enzymes and then purified. NanoDrop™ readings were taken to verify the quality of digested product given in Figure 11.

Figure 11 - NanoDrop™ readings of BamHI-HF® and SalI-HF® double digested insert and plasmid. 1 - Digested UGT93N3 insert; and 2 - Digested pTRV2 empty vector

dsDNA
#0943 pTRV2_RED_Pur_1
26.1 ng/µL
A260: 0.522
A260/A280: 1.76
A260/A230: 0.40

dsDNA
#0945 UGTVIGS_RED_P_1
33.8 ng/µL
A260: 0.677
A260/A280: 1.80
A260/A230: 0.96

Digested insert and vector were then ligated in 10:1 insert:vector ratio in a 10 µl reaction and this reaction was transformed in homemade *E. coli* TOP10 chemicompetent cells using Inoue transformation method. Transformed cells were plated on three SOB agar plates with 50 µg/ml concentration of each streptomycin (for strain selection) and kanamycin (for transformant selection). Fourteen colonies total were obtained on 4 plates and colony PCRs were performed on the obtained colonies, of which 3 colonies were positive. Results of chemical transformation on *E. coli* in plates and colony PCRs are given in Figures 12 and 13 respectively.

Figure 12 - Plates for transformation of pTRV2_UGT93N3_VIGS transformation in TOP10. 1 - Plate 1 vs -ve control plate (6 colonies); 2 - Plate 2 vs. -ve control plate (2 colonies); and 3 - Plate 3 ns. -ve control plate (4 and 2 colonies respectively)

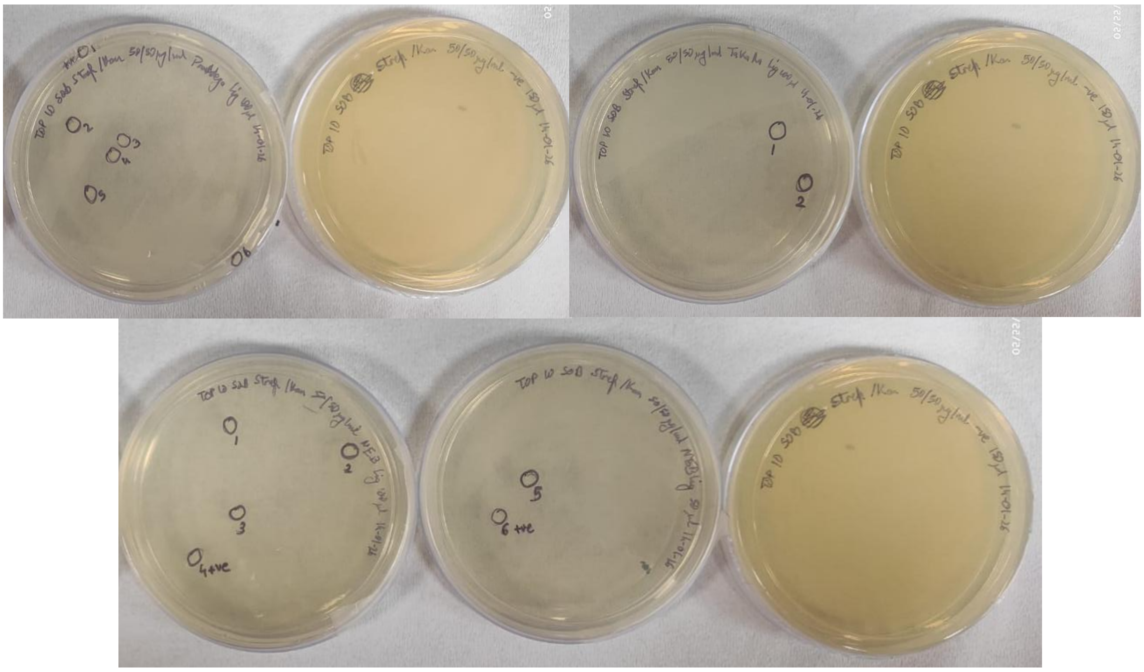
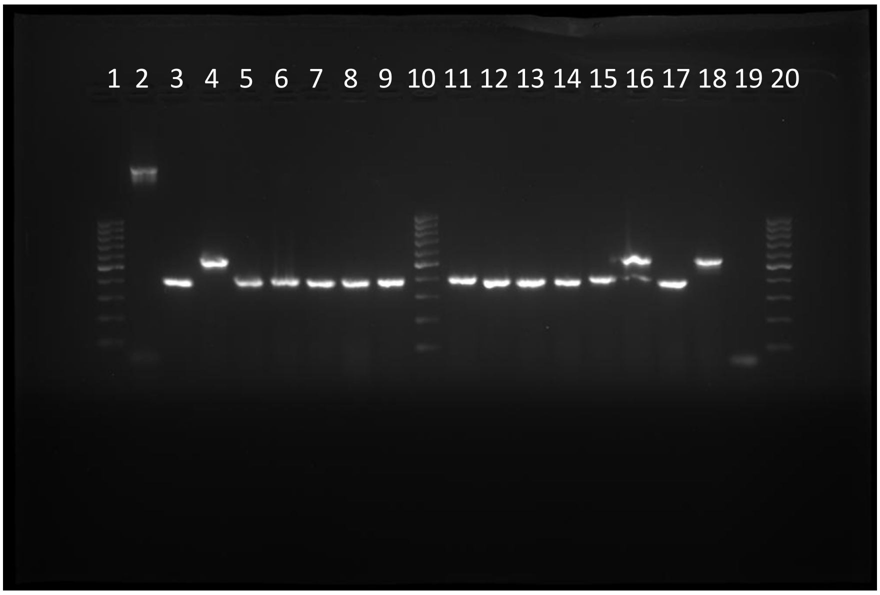


Figure 13 - 2% agarose gel of colony PCRs for pTRV2_UGT93N3_VIGS transformation in TOP10. Non-recombinant fragment size = 376 bp and recombinant fragment size = 529 bp. 1 - 100 bp ladder; 2 - Transformation +ve control colony 1; 3 - Transformation +ve control colony 2; 4 - Colony 1 (+ve); 5 - Colony 2; 6 - Colony 3; 7 - Colony 4; 8 - Colony 5; 9 - Colony 6; 10 - 100 bp ladder; 11 - Colony 7; 12 - Colony 8; 13 - Colony 9; 14 - Colony 10; 15 - Colony 11; 16 - Colony 12 (+ve); 17 - Colony 13; 18 - Colony 14 (+ve); 19 - PCR -ve control; and 20 - 100 bp ladder



Positive colonies were inoculated in 7 ml LB broth with 50 µg/ml concentration of each streptomycin (for strain selection) and kanamycin (for transformant selection) and incubated at 37 °C and 200 rpm overnight. Glycerol stocks were made from 2 ml culture and rest of 5 ml culture was used for plasmid isolation. Images of inoculated bacterial culture and NanoDrop values of isolated plasmids are given in Figure 14. Plasmids were also sent for sequencing to verify the sequence identity of inserts (UGT93N3 VIGS fragment).

Figure 14 - Images of inoculated cultures of positive colonies after incubation and NanoDrop™ values after plasmid isolation. 1, 2 and 3 - Inoculated cultures of positive colonies (left) as compared to uninoculated broth (right) for positive colonies 1,2 and 3 respectively; and 4, 5 and 6 - NanoDrop™ values for isolated plasmids for positive colonies 1, 2 and 3 respectively.

```
#1213 pTRV2_VIGS_P1
98.4 ng/µL
A260: 1.969
A260/A280: 1.84
A260/A230: 2.14
```

```
#1214 pTRV2_VIGS_N4
103.0 ng/µL
A260: 2.061
A260/A280: 1.89
A260/A230: 2.27
```

```
#1215 pTRV2_VIGS_N6
95.2 ng/µL
A260: 1.903
A260/A280: 1.87
A260/A230: 2.25
```

1.5 µg plasmid of all positive colonies were transformed in *A. tumefaciens* GV3101 chemicompetent cells and plated on YEP agar plates containing 25 µg/ml rifampicin (for strain selection) and 50 µg/ml kanamycin (for transformant selection) which were incubated at 28 °C for 2 days. Five colonies were obtained (3 for positive colony 1, none for positive colony 2 and 2 for positive colony 3) which were verified to be recombinant using colony PCR and then these colonies were inoculated in 2 ml YEP broth containing 25 µg/ml rifampicin (for strain selection) and 50 µg/ml kanamycin (for transformant selection) and incubated at 28 °C at 250 rpm for a day to prepare glycerol stocks. Images of plates for GV3101 transformation and colony PCR are given in Figures 15 and 16 respectively.

Figure 15 - Plates for transformation of pTRV2_UGT93N3_VIGS transformation in GV3101. From left to right - positive colony1, positive colony 2, positive colony 3 and negative control for transformation.

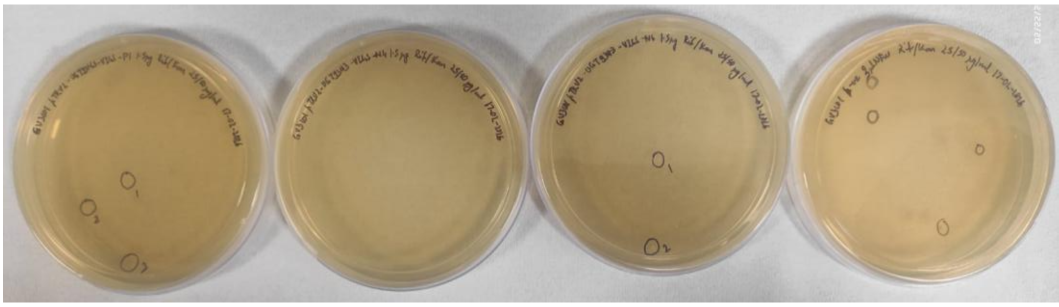
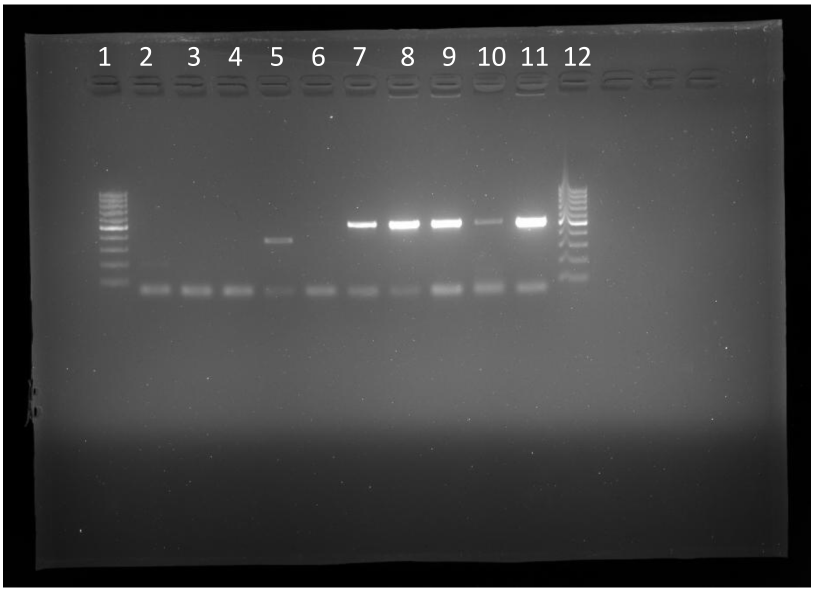


Figure 16 - 2% agarose gel of colony PCRs for pTRV2_UGT93N3_VIGS transformation in GV3101. Non-recombinant fragment size = 376 bp and recombinant fragment size = 529 bp. 1 - 100 bp ladder; 2 - PCR +ve control; 3 - PCR -ve; 4 - Transformation +ve colony 1; 5 - Transformation +ve colony 2; 6 - Colony PCR -ve; 7 - Colony 1; 8 - Colony 2; 9 - Colony 3; 10 - Colony 4; 11 - Colony 5; and 12 - 100 bp ladder.



Similar results were obtained for successful cloning of UGT93N3 CDS fragment in overexpression vector pSPDK (results not mentioned).

3.2. VIGS and VOX

All the plants have been infiltrated and kept in dark for 48 hours and currently in incubation period where new leaves are growing.

3.3. Dual-Choice Assays

Dual choice assays will be performed after VIGS and VOX are completed and verified.

3.4. Neonate Mortality Assays

Neonate mortality assays will be performed after VIGS and VOX are completed and verified.

4. Discussion

In this thesis we attempted to explore the hypothesis of whether silencing or overexpressing a defense metabolite gene (SN in eggplant in this study) leads to differential effects on preference and performance of an insect herbivore (TCW in our case) using the transient silencing and overexpression methods of VIGS and VOX respectively. We were successfully able to complete the first objective of this project, which is cloning of VIGS fragment and CDS of UGT93N3 gene of eggplants in VIGS and VOX silencing and overexpression vectors of pTRV2 and pSPDK respectively. VIGS and VOX experiments are on the way and their data is expected to come soon. After VIGS and VOX experiments, neonate mortality and dual-choice assays will be performed to assess the differential changes in preference and performance on silencing and overexpression of SN biosynthesis gene UGT93N3. Generation of stable transgenic plants is a very tedious, resource intensive and time-consuming process with the inherent risk of whether the expected results from gene manipulation will be reflected in phenotype or not. Compared to making stable transgenics, VIGS and VOX are transient gene-manipulation methods which are much less tedious and quicker and can be used to quickly assess phenotype effects of gene-manipulation and make the decision whether to invest in creation of stable transgenics or not. For example, in this thesis we tried to do VOX to see whether overexpression of a defense metabolite biosynthesis gene UGT93N3 for SN provides enhanced protection to plant against an insect herbivore TCW which can further be used to decide whether is it worth investing in creating eggplant transgenic varieties with increased SN content for better protection against insect pests. We thus advocate VIGS and VOX as approaches for transient silencing and expression in both plant science and agriculture.

Although there has been tremendous research on bioactive effects of steroidal alkaloids, their activity on many insect pests is still not well characterized given the fact that primary role of steroidal alkaloids in plants seems to be to provide protection against insect pests. One future prospective of this project includes expanding this project to more insect pests which commonly feed on eggplant which include ash weevil (*Myllocerus* spp.), eggplant shoot and fruit borer (*Leucinodes orbonalis*), whiteflies (*Bemisia* spp.) and many more, i.e. the pest complex of eggplant. Another future prospective is to observe whether the defense response of steroidal alkaloids is inducible in eggplant via herbivory or not. Previous research has shown that in eggplant, JA-mediated hormone signaling pathway mediates SA levels in eggplant, so another target in future studies to manipulate SA levels in eggplant and study its effects on pest complex of eggplant might be JA-mediated hormone signaling pathway, especially the COI1 inhibitor which is a key inhibitor of JA-mediated genes and JRE/ERF4 transcription factor which is a key transcription factor for SA biosynthesis in many Solanaceae plants like potato, tomato and eggplant.

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