1.1. Tobacco and its components

Tobacco is generally obtained from *Nicotiana tabacum* and *Nicotiana rustica*, both of them native from Peruvian and Ecuadorian Andes. The production of cigarettes was introduced at the end of the 19th century, by the British government within several of its colonies. Now, there are more than a billion smokers all over the world [1]. The cigarette fumes are known to contain more than 4,000 chemical compounds, among them nicotine, carbon monoxide, benzo [alpha] pyrene (a pre-carcinogen of the cigarette tar), respiratory irritants (as acrolein, formaldehyde, phenols, etc) are dominant. A xenobiotic compound to be especially considered is benzo [alpha] pyrene, a common environmental contaminant produced from the combustion of plant materials in tobacco, which is metabolized in animals, giving rise to benzo [alpha] pyrene-7, 8-dihydrodiol- 9, 10-epoxide, a potent carcinogen. However, most of the clinical studies show that, nicotine is the main agent responsible for the development of dependence on tobacco [2, 3].

1.2. Risks associated with tobacco use

Majority of the tobacco consuming population resides in developing countries. Half of Indian males use tobacco in some form and it is becoming more popular with younger people. It is alarming to note that 14.6% of children in India in the age group of 13 – 15 years of age use tobacco. Tobacco use causes more deaths each year than combined mortality of human immunodeficiency virus (HIV), illegal drug use, and alcohol. Smoking tobacco causes about 90%
of all lung cancer deaths in men and 80% of all lung cancer deaths in women. About 90% of all deaths from chronic obstructive pulmonary disease (COPD) are caused by smoking. Tobacco use also causes cardiovascular and respiratory diseases. It is estimated that, there are 1,100,000 world-wide lung cancer deaths per year, 85% of which caused by tobacco. The acute effects of nicotine on the cardiovascular system are as follows: peripheric vasoconstriction, increased systemic arterial pressure, and increased cardiac frequency.

1.3. Nicotine as the major addictive alkaloid in tobacco

More than 4000 chemicals are found in *Nicotiana tabacum* and tobacco smoke. Alkaloids and terpenoids form the major groups and about 60 of them are carcinogens. Nicotine (Fig 1.) is the major psychoactive alkaloid in tobacco that causes addiction in humans. It is a colorless, volatile basic alkaloid. It is estimated that 3-5 mg/day is a threshold level that can readily establish and sustain addiction among smokers. The concentration of nicotine varies from 0.6-9.0% in *Nicotiana tabacum* to 18.76% in *Nicotiana rustica* leaves.

![nicotine](image)

Fig 1.1. Structure of nicotine

1.4. Mechanism of addiction

Addiction is defined by the World Health Organization as “repeated use of a psychoactive substance or substances, to the extent that the user is periodically or chronically intoxicated, shows a compulsion to take the preferred substance(s), has great difficulty in voluntarily ceasing
or modifying substance use, exhibits determination to obtain psychoactive substances by almost any means, and tolerance is prominent and a withdrawal syndrome frequently occurs when substance use is interrupted.

1.4.1. Nicotinic Acetylcholine Receptors

Nicotine is one of the thousands of chemicals found in tobacco smoke. When inhaled, nicotine freely diffuses into the pulmonary blood and enters the systemic circulation. In the brain, nicotine binds to nicotinic acetylcholine receptors (nAChRs), which are allosteric membrane-bound protein channels that, when opened, allow the passage of cations like sodium, potassium, and calcium [4]. The flux of these cations depolarizes the cell, opening voltage-gated calcium channels that mediate neurotransmitter release from the presynaptic terminal [5]. Nicotinic acetylcholine receptors are composed of 5 protein subunits encoded by 17 genes. In the brain, 9 genes encode for alpha subunits and 3 genes code for beta subunits [6].

1.4.2. Nicotine and neurotransmitter release

The binding of nicotine to a nAChR mediates the release of neurotransmitters, either by promoting membrane depolarization and opening of voltage-activated calcium channels, or by the intrinsic calcium permeability of the nAChR [5,7]. One neurotransmitter of importance to nicotine addiction is dopamine, which is critical in the acute reward pathways associated with abuse of nicotine [8]. Nicotine stimulates dopaminergic transmission in the ventral tegmental area (VTA) of the midbrain, which in turn activates the nucleus accumbens, an area critical for nicotine-mediated physiological effects such as addiction, pleasure, and reward [5, 6, 8]. In addition to the nucleus accumbens, dopaminergic neurons of the VTA project to the prefrontal cortex (PFC), amygdala, habenulo-interpeduncular system, and hippocampus [6, 9]. Another function of nicotine is to facilitate the release of glutamate from the amygdala, which further activates the dopaminergic neurons of the VTA [8]. Chronic stimulation of nAChRs during nicotine addiction will desensitize the GABA-ergic neurons, which lose their inhibitory effect on dopamine release [5].
1.5. Nicotine lethality

A commercial grade cigarette of 10 to 15 mg nicotine yields on average 1 to 2 mg of nicotine that is absorbed in the bloodstream. The LD$_{50}$ of nicotine for humans and children has been estimated to be 30 to 60 mg (0.5-1.0 mg/Kg) and 10 mg respectively. A pack of 10 cigarettes yield 100 to 150 mg of nicotine with an absorbed yield of 20 to 25 mg. The statistics for bidis and other chewing type tobacco are exceedingly alarming. One cigarette with 10 mg nicotine yield nicotine of approximately 1 mg in blood stream. The optimum nicotine estimate for addiction is 3-5 mg and its effect on human physiology has a half life of 2 hours. Therefore, theoretically 5 cigarettes within 8 hours will make one addict.

1.6. Nicotine withdrawal symptoms

Withdrawal from nicotine causes severe symptoms, including anxiety, stress, irritability, malaise, loss of motivation, dysphoria, and emotional pain [10, 11]. Nicotine withdrawal increases levels of extrahypothalamic corticotrophin releasing factor (CRF) in the central nucleus of the amygdala and bed nucleus of the stria terminalis and increases binding of CRF to corticotropin-releasing factor 1 (CRF1) receptors in these areas [10, 12]. Whereas increased CRF levels cause anxiety, animal studies show that an inhibition of CRF1 receptors eliminates much of the anxiety associated with nicotine withdrawal [12, 13].

1.7. Genetics of Smoking

Studies have elucidated associations that show that there is a high level of heritability in cigarette smoking ($\geq$50 percent), as well as in factors such as level of dependence and the number of cigarettes smoked per day [14, 15]. There is also heritability in the negative effects of withdrawal in quitting smokers [16]. There have been many attempts to locate genes associated with nicotine addiction [15]. It is difficult to extract a causal relation between specific genes and nicotine addiction because of the interaction between numerous genes and the environment that determine complex behaviors. Genes that have been implicated as being relevant to nicotine
addiction include dopamine transporters, GABA receptors, opiate receptors, annabinoid receptors, and other kinds of receptors [17, 18]. One such study compared genomes of smokers who were dependent versus nondependent on nicotine, identifying 35 possible single nucleotide polymorphisms (SNPs) correlating to novel genes (e.g., Neurexin 1) and a known candidate gene (β3 cholinergic receptor) that have implications for developing a nicotine addiction [19]. Other studies have identified different genes that increase the risk for nicotine addiction, including the complete family of nAChR subunit genes; the most significant associations were shown in the α5/α3/β4 nAChR gene cluster on chromosome 15 [20, 21].

1.8. Complexity in tobacco use legislations

The tobacco use regulation and control is more complex a process because of diverse tobacco products and a large consequential burden of tobacco related disease and death. Comprehensive tobacco control legislation helps integrate disparate activities for tobacco control and overpower challenges in production, use and sale of tobacco. The Cigarettes and Other Tobacco Products (Prohibition of advertisement and Regulation of Trade and Commerce, Production, Supply and Distribution) Act2003 (COTPA) is the principal law regulating the use of tobacco. There are two primary concerns regarding labeling of tobacco product practiced, 1. FCTC art. 11 guidelines para. 44 specify that display of figures for emission yields of nicotine, tar and carbon monoxide should be prohibited as the numbers are misleading. Moreover COTPA Sec. 7 (5) requires that every package of cigarette or any other tobacco product indicate on its label the nicotine and tar content. 2. FCTC Art. 11 (1) (a) and FCTC Art. 11 guidelines para. 43 prohibits the use of misleading or deceptive terms which includes but is not limited to the use of words such as ‘light’, ‘ultra light’, ‘mild’, ‘low tar’, ‘smooth’, ‘slim’, ‘gold’ etc. The former would reflect on the amount of nicotine intake per cigarette and the latter is misleading for a safer product. The current economic social imbroglio needs a holistic approach towards tobacco control in any country. This includes state legislation, nicotine weaning programmes and modern breeding programs.
**1.9. Nicotine threshold levels in cigarettes to avert addiction**

A ten per cent reduction has happened in the levels of nicotine and tar recently. According to the Central Tobacco Research Institute (CTRI), Rajahmundry, Andhra Pradesh, the average tar content in a 66-mm long cigarette is down to 16 mg from 17 to 24 mg and nicotine has been lowered to about 1.5 mg from 2 mg. In a regular size filter cigarette of 69 mm length the tar content now ranges between 15 and 17 mg and nicotine between one and 1.4 mg (Godfrey Phillips). Earlier, the nicotine content varied from 3.4 mg/g (chewing tobacco) to 26.9 mg/g in bidis. The filtered and unfiltered cigarettes contain nicotine 14.5 and 15.6 mg/g respectively (Sujatha and shaik, 2008). Assuming that the estimated target daily dose of nicotine should be 3-5 mg or less to avert addiction, a person smoking up to 20 cigarettes per day, one can conclude that a maximal available dose of 0.1 to 0.2 mg of nicotine per cigarette is the threshold level for a less addictive cigarette. Assuming a bioavailability of 10 percent, an absolute nicotine content of 1 mg to 2 mg per cigarette should be adequate to prevent addiction in people. At the same time, it may provide enough nicotine for taste and sensory stimulation.

**1.10. FDA approved nicotine weaning methods**

A couple of nicotine replacement therapy is commercially available. The use of FDA approved nicotine patch, gum, nasal spray, inhalers and lozenges have their own shortcomings in the form of sleeplessness, nausea, skin irritations, withdrawal symptoms, and more. CHANTIX is a prescription medicine Varenicline, works by blocking nicotine receptors in the brain. Chantix is usually prescribed for a 12-week period, with the option of another 12-week maintenance course. About 33% of smokers who use the drug successfully quit. Varenicline is a partial agonist of the α4β2 subtype of the nicotinic acetylcholine receptor. As a partial agonist it both reduces cravings for and decreases the pleasurable effects of cigarettes and other tobacco products. Chantix costs about $3.00 per pill (INR. 180.00).

The most common side effects of CHANTIX include nausea (30%), sleep problems, constipation, gas and/or vomiting. CHANTIX in some people had serious allergic or skin
reaction. These can include rash, swelling, redness, and peeling of the skin. In November 2007, the FDA announced post-marketing reports that patients using varenicline for smoking cessation had experienced several serious side-effects, including neuropsychotic events. On February 1, 2008 the FDA issued an alert to further clarify its findings, noting that "it appears increasingly likely that there is an association between Chantix and serious neuropsychiatric symptoms". On June 16, 2011, the FDA issued a safety announcement that Chantix may be associated with "a small, increased risk of certain cardiovascular adverse events in patients who have cardiovascular disease.

1.11. Genetic engineering for low nicotine tobacco

The objective of the study was to produce low nicotine content tobacco plants. Breeding for low nicotine content in tobacco is difficult as it has a narrow genetic base. The diversity among tobacco species is considerably low to breed for low nicotine. Moreover, reducing nicotine content in tobacco leaves by post harvest processing is not cost effective. There are various physical and chemical and microbial methods of leaf processing. These leaves are constrained by the use of various chemicals which can oxidize upon combustion. Therefore, genetic engineering for low nicotine is cost effective option to produce low nicotine tobacco. Manipulation of a single gene would produce plants isogenic to the wild type except for the character manipulated. The different methods of gene silencing are co-suppression, antisense and RNA interference. RNAi is a post transcriptional gene silencing tool that can be used in crop improvement and value addition. RNAi works on the regulation of gene expression at post transcription by degrading mRNA and inhibiting translation. RNAi is stable, inheritable method of gene silencing in plants. Here we have attempted to silence the gene putrescine N-methyl transferase2 (PMT2) to produce low nicotine in Nicotiana tabacum cv. Petit Havana.