

**Impact of inserting a thiol methyltransferase gene on the
competition between halide methylation and ethylene production in
tobacco.**

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Plants are exposed to a variety of environmental stresses, ranging from insects and phytopathogens known as biotic stressors, to edaphic stressors including drought and salinity - clustered under the group of abiotic stresses. Facing these pernicious risks, plants have adopted several strategies to survive in stressful environments.

With contemporary advances in technology and scientific research, it is possible to insert a specific gene or trait into a plant in order to confer salt tolerance. For example, a thiol methyltransferase enzyme in cabbage converts Cl^- and other halide ions into their corresponding methyl halides (CH_3X), which are released directly into the air. Cloning the *TMT1* gene and functionally expressing this gene into tobacco plants conferred salt tolerance on these plants. Although increased salt tolerance is a favourable and beneficial trait for plant productivity, the emission of methyl halides into the atmosphere can be noxious for the environment. Indeed, CH_3X are some of the factors contributing to climate change, widely believed to be the foremost environmental problem of the twenty-first century. Climate change is affecting all forms of life on earth and its impacts are often severe and irreversible. For that reason, it has been placed at the forefront of the international political agenda.

The present study focused on two main physiological traits of stress resistance in tobacco plants which have been genetically engineered with the cabbage *TMT1* gene: The production of CH_3X , particularly CH_3Cl , as a way to confer salt tolerance; and synthesis of the stress hormone, ethylene (C_2H_4). Both pathways share a common metabolite, S-adenosyl-L-methionine (SAM), for the synthesis of the final products. SAM, derived from the amino acid methionine (Met), is a universal methyl group donor. It plays essential roles in plant metabolism, by acting as a precursor for C_2H_4 , polyamines, and vitamin B1 synthesis, and as a source of atmospheric

dimethylsulphides. Therefore, it is important that the plant maintains the SAM pool at a level sufficient to serve all the different metabolic pathways.

The present work set out to determine whether addition of another SAM-dependent pathway through *TMT* insertion in tobacco plants would negatively affect ethylene synthesis by lessening the amount of SAM available for C₂H₄ biosynthesis. Detailed experimental studies were conducted, including *in vitro* and *in vivo* experiments, to examine the potential effect of *TMT* gene insertion on SAM availability for ethylene synthesis in tobacco plants. Three series of *in vitro* tests, differing by the number of leaf discs used, the final headspace volume in each flask, the incubation period in the different solutions and the time of introducing salt solutions, were carried out. Data obtained from two series of *in vitro* experiments revealed a decrease in ethylene emission when salt solutions were added at the beginning of the experiment and when leaves were incubated overnight in the presence of 0.1 M NaBr, coupled with the production of CH₃X, whereas the other series of *in vitro* tests as well as the *in vivo* results showed no alteration in ethylene accumulation because of *TMT* insertion when tobacco leaves were exposed to salt treatments. According to the results presented in this study, the amounts of available SAM in transformed tobacco are likely sufficient to serve both ethylene and methyl halides biosynthetic pathways. Besides, *in vivo* results indicate that inserting the *TMT1* gene that improves salt stress resistance in tobacco should not dramatically alter C₂H₄ synthesis, and therefore, would be unlikely to affect any physiological trait associated with C₂H₄ production if this gene is used to engineer salt tolerance in high-value crops.

Although *TMT* insertion does not alter C₂H₄ emission in transgenic plants and tends to be an efficient tool to confer salt tolerance in halo-intolerant crops, its environmental cost is of great

concern and, therefore, should be taken into consideration to mitigate the amounts of CH_3X emitted into the atmosphere because of this insertion.

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Dedication

I dedicate this work to my mom and dad who were my hope in despair, who showed me the way when I was lost, who were always there for me when I was on the verge of collapse, and who stood by me in times of hardship. I will always be grateful to you my dear parents

Moncef and Samira.

Fatma

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List of abbreviations

ACC:	1-Aminocyclopropane-1-carboxylic acid
ACO:	1-Aminocyclopropane-1-carboxylic acid oxidase
ACS:	1-Aminocyclopropane-1-carboxylic acid synthase
Ade:	Adenine
AOA:	Aminooxyacetic acid
At:	Astatine
ATP:	Adenosine 5'-triphosphate
AVG:	Aminoethoxyvinylglycine
CFCs:	Chlorofluorocarbons
CH ₃ Br:	Methyl bromide
CH ₃ Cl:	Methyl chloride
CH ₃ I:	Methyl iodide
CH ₃ X:	Methyl halides
CH ₄ :	Methane
DW:	Distilled water
FID:	Flame ionization detector.
GC:	Gas chromatography
GHGs:	Green house gases
Gt:	Gigatonnes
GWP:	Global warming potential

HBMT:	Halide/ bisulfide methyltransferase
HCN:	Cyanide
Hcy:	Homocysteine
KI:	Potassium iodide
KMB:	2-keto-4-methylthiobutyrate
MCT:	Methyl chloride transferase
Met:	Methionine
mM:	Millimolaire
MTA:	5'-methylthioadenosine
MTR:	5'-methylthioribose
N ₂ O:	Nitrous oxide
NaBr:	Sodium bromide
NaCl:	Sodium chloride
ODP:	Ozone depletion potential
ppb:	Parts per billion
ppm:	Parts per million
ppt:	Parts per trillion
SAH:	S- adenosyl- L-homocysteine
SAM:	S- adenosyl-L-methionine
SE:	Standard error
SMM:	S-methylmethionine
TMT:	Thiol methyl transferase

Chapter 1

Introduction

1.1 Methyl halides and ethylene:

Organohalogenes, including methyl halides, are ubiquitous compounds derived from both natural (Gribble, 2004) and anthropogenic sources (WMO, 2003). These chemicals can be anthropogenically produced by industries or human activities (WMO, 2003). Besides, nature is capable of producing a myriad of halogenated compounds, thus contributing to high amounts of such chemicals in the atmosphere (Gribble, 2004). For instance, methyl halides (CH_3X), particularly CH_3Cl , CH_3Br , and CH_3I , have been recognized to be produced by a variety of living organisms (Manley, 2002), ranging from bacteria, algae, and wood-rotting fungi to higher plants (Harper, 1993; Saini et al., 1995; Yokouchi et al., 2000; Yokouchi et al., 2007). The enzymatic reaction leading to methyl halides involves the use of S-adenosyl-L-methionine (SAM) as the methyl group donor. Besides its role in methylation reactions, SAM serves various other metabolic functions in plants (Moffat & Weretilnyk, 2001; Roje, 2006; Taiz & Zeiger, 2006), notably as a precursor in ethylene synthesis. Ethylene, a phytohormone, exists in a gaseous state under normal physiological conditions, and plays crucial roles in regulating many aspects of plant growth, senescence, and fruit ripening (Bleecker & Kende, 2000; Taiz & Zeiger, 2006).

Since the biosynthesis of ethylene and methyl halides share a common precursor, SAM, it is pertinent to inquire whether SAM availability for ethylene synthesis is affected when CH_3X are emitted.

1.2 Research objectives:

To date, scientific research revealing the impacts of *TMT* gene insertion on plant metabolism is still limited. The main goal of the present study was to determine whether inserting the *TMT1* gene –and thus the ability to produce CH_3X using SAM as a methyl group donor in tobacco plants – could put pressure on SAM availability for C_2H_4 synthesis, and thus disrupt ethylene emission. Hence, this work will attempt to give answers to the following two main questions:

- Would SAM be limiting for C_2H_4 synthesis when CH_3X are emitted?
- Should there be a concern that *TMT* insertion could cause unintended physiological and metabolic alterations in the transgenic plants, in addition to any environmental consequences of CH_3X emission?

1.3 Hypothesis and null hypothesis:

H1: *TMT* gene insertion in tobacco plants provokes a decrease in ethylene emission.

H0: Ethylene emission in tobacco plants is not altered by *TMT* gene insertion.

Chapter 2

Literature review

2.1 Climate change, GHGs and ozone depletion:

Earth's atmosphere is being changed at an unprecedented rate by pollutants resulting from human activities, inefficient and wasteful fossil fuel use, and the effects of rapid population growth all over the world (Dauncey & Mazza, 2001). According to IPCC (2007), Climate change refers to any change in climate or its properties over time whether due to natural variability or as a result of human activity. Global warming, one aspect of climate change, is widespread over the globe and is affecting the physical and biological systems of the earth (IPCC, 2007). According to data provided by the World Meteorological Organization (WMO), the year 2010 is ranked among the warmest years since 1850, with a global temperature estimated at about 0.55°C above the 1961-1990 annual average of 14°C . Furthermore, the period of 2001-2010 is considered to be the warmest ten year period on record, with a global temperature estimated at 0.43°C above the 1961-1990 average. The observed rise in sea levels, decrease in ice extent and snow cover, and the changes in oceans and lands, are all consistent with warming (IPCC, 2007). Estimates for future climate change are a warming of about 0.2°C per decade for the next two decades with a sea level rise expected to reach 0.3 to 0.8 m by 2300 (IPCC, 2007).

The atmosphere is composed of 77 % N_2 , 21 % O_2 , 0.9 % argon, 1 % of water vapour and 0.3 % of trace gases. For the past 10,000 years, trace gases mainly (CO_2 and nitrous oxide (N_2O) at 275 ppm and methane (CH_4) at 750 ppm), led to the earth's mean temperature of 15°C . These trace gases contribute to the historical greenhouse effect: in addition to the direct solar heating, trace gases trap the earth's heat as it is reflected back into the space, thus further heating the

earth. Without a greenhouse effect, the earth would plunge into an icebound state (Lacis et al., 2010).

The long wave radiation reemitted from the earth is mainly in the infrared region of the spectrum (Gebhardt, 2008). The main gases absorbing outgoing infrared radiation are water vapor (60%) and CO₂. However, the natural greenhouse effect can be accentuated by the presence of chemicals such as CH₃X, capable of absorbing the outgoing infrared radiation.

2.1.1 Drivers of climate change:

- CO₂: It is the major gas causing the change in earth's temperature. CO₂, considered as the single most important human-emitted GHG in the atmosphere, is responsible for 85% of the increase in radiative forcing over the last decade (WMO, 2009b), with an atmospheric lifetime of 100 years (IPCC, 2007). Radiative forcing measures the influence of a factor in altering the balance of incoming and outgoing energy in the earth-atmosphere system (IPCC, 2007). Besides, CO₂ has a global warming potential (GWP) of 1. GWP is defined as the ability of a GHG to trap atmospheric heat relative to that of CO₂ considered as a baseline for comparison (Gebhardt, 2008). The atmospheric abundance of CO₂ was 280 ppm in the preindustrial time (before 1750), reaching 385.2 ppm in 2008. It contributed 77 % of total anthropogenic GHG emissions in 2004 (IPCC, 2007). Agriculture contributes 10 to 12 % of the total anthropogenic greenhouse gas (GHG) emissions (Wreford et al., 2010), while the world's transportation sector is responsible for 33 % of the total CO₂ emissions. Since 1750, CO₂ levels increased by 38% because of emissions from fossil fuel combustion (8.62 Gt carbon in 2007), and from deforestation and land use change (0.5-2.5 Gt carbon/year over the 2000-2005 time period) (WMO, 2009b). Elevated CO₂ levels have led to the oceans' acidification which has negative impacts on marine shell-forming organisms and their

dependent species (IPCC, 2007). According to IPCC (2007), both natural and anthropogenic CO₂ emissions will contribute to warming for more than a millennium, due to the time required for its removal from the atmosphere. In addition to CO₂, other GHGs are capable of worsening the situation. A list of GHGs has been identified, including CH₄, N₂O, chlorofluorocarbons (CFCs), CH₃X, and many others.

- CH₄: Considered to be the second most important GHG after CO₂ (Lacis et al., 2010), CH₄ is derived from natural and anthropogenic sources. Natural origins, including termites and wetlands, account for 40 % of the total natural emissions of methane, whereas anthropogenic sources such as rice cultivation, fossil fuels, manure handling, and biomass burning, contribute the other 60 % to the total CH₄ atmospheric levels. CH₄ has a GWP of 72 for a period of 20 years. Since 1750, methane levels have increased by 157%, reaching 1,797 ppb in 2008, as shown in Table 1 (WMO, 2009b).
- N₂O: This is derived from both natural (soils, oceans) and anthropogenic sources (potash-based fertilizer use, and land management practices). N₂O has a GWP of 289 for a period of 20 years. In 2008, levels of N₂O reached 321.8 ppb, exceeding those in pre-industrial times by 19 % (WMO, 2009b).
- CFCs: These are mainly anthropogenic compounds historically used in refrigeration systems and air conditioners, before being phased out by the Montreal Protocol and replaced by hydrofluorocarbons (HFCs). This protocol was adopted in 1987 to protect the ozone, and has made large contributions towards reducing GHG emissions. Indeed, in 2010 the decrease in the annual amount of GHGs under the Montreal Protocol is estimated to have been about 10 Gt of avoided CO₂-equivalent (WMO & UNEP, 2010). The latter measure reflects the amount of CO₂ that would cause the same time-integrated radiative forcing as an emitted

quantity of GHGs (IPCC, 2007). This group of chemicals with CH_3X contribute as much as 12 % to the global radiative forcing.

- CH_3X : The group of interest in the present study is known for its high ozone-depleting potential. The ozone depletion potential (ODP) represents the extent of stratospheric ozone degradation expected from a chemical species relative to a reference potential level of 1 attributed to CFC-11 (WMO, 2003). The ODP of CH_3Cl and CH_3Br — considered as the main carriers of chloride and bromide to the stratosphere, where they catalyze ozone depletion— are 0.02 and 0.38 respectively (WMO, 2003).

Recent evidence states that water vapor accounts for 50% of the earth's greenhouse effect, clouds contribute by 25%, CO_2 by 20%, and CH_4 , N_2O , O_3 , and CFCs, along with other trace gases, account for the remaining 5% (Lacis et al., 2010).

Table1. Global abundances and changes of key GHGs, from the World Meteorological Organization (WMO)-Global Atmosphere Watch (GAW) global GHG monitoring network (WMO, 2009b). Assuming a pre-industrial mixing ratio of 280 ppm for CO₂, 700 ppb for CH₄, and 270 ppb for N₂O.

	CO ₂ (ppm)	CH ₄ (ppb)	N ₂ O (ppb)
Global abundance in 2008	385.2	1797	321.8
Increase since 1750	38%	157%	19%
2007-08 absolute increase	2.0	7	0.9
2007-08 relative increase	0.52%	0.39%	0.28%
Mean annual absolute increase during last 10 years	1.93	2.5	0.78

2.1.2 Ozone depletion:

Ninety % of the ozone (O₃) is located in the stratosphere, 10 to 40 km above the planet's surface, where it is spread thinly and in small amounts. Stratospheric O₃ is vital for much of the current biota's existence, as it absorbs some of the sun's biologically harmful ultraviolet-B or UV-B radiation (NASA, 2010; WMO, 2009a). However, the presence of human-created O₃ near the earth's surface can be harmful to humans (by increasing the frequency of cardio-respiratory diseases) (IPCC, 2007), animals, and plants (WMO, 2009a). For instance, atmospheric O₃, formed from pollutants, is considered to be a major phytotoxic air pollutant (Morgan & Drew, 1997). Ozone depletion is synonymous to the ozone hole, described for the area in which amounts of O₃ have dropped below the historical threshold of 230 Dobson units (NASA, 2010). The ozone hole has mainly been caused by pollutants—known as O₃ depleting substances—present in the atmosphere. Indeed, O₃ is mostly broken down by chemicals such as CFCs and N₂O. Decrease in stratospheric O₃ causes more UV-B radiation to diffuse to the earth's surface, thus damaging DNA in various living organisms, and causing severe human health problems such as skin cancer, cataracts, or body immune suppression (WHO, 2003).

2.2 Major environmental challenges and impacts of climate change:

Social-ecological systems are complex, and are made of various components strongly inter-related at many scales. These systems, subject to a multitude of variations and unpredictable stresses, are not static but changing in a perpetual way (Gibson et al., 2005).

Facing these changes, ecosystems can respond in five qualitatively different ways:

- The system can continue to operate as before, even though its operations may be initially or temporarily unsettled.

- The system can operate at a different level using the same structures it originally had (for example through a reduction or increase in species number).
- Some new structures can emerge in the system that replace or augment existing structures.
- A new system, made up of quite different structures, can emerge.
- A rare possibility: a total collapse of the system with no possible regeneration.
- The main challenge would be to live with these changes without compromising vital structures and functions within systems.

The failure of ecosystems is primarily due to the growing demand for, and overuse of, natural resources (water, land, biomass, energy), without taking into consideration their scarcity. Indeed, material demands are exceeding the planet's ecological capacity by 30 % (Gibson et al., 2005). Furthermore, the human population has increased nearly tenfold over the last three centuries, and by a factor of four in the last century. This human population explosion has severely impacted our relationship with natural-support systems, and deeply intensified our environmental impact (Steck, 2010).

The world is facing serious threats such as salinity, soil erosion, drought, desertification, hunger, and poverty (Godfray et al., 2010). These issues impose real challenges in terms of meeting the basic needs of a human population projected to reach 9 billion by 2050, in a world characterized by its finite natural resources. Recent studies reveal that a 70 to 100 % increase in food production is needed by 2050 (Godfray et al., 2010) . The main challenge will be to augment food production sustainably from the same current available land (Godfray et al., 2010). Climate change is a further threat, as it is affecting all forms of life on earth, and negatively impacting systems integrity and services all over the world - though to a different extent in different locations (IPCC, 2007). Africa, for example, is one of the most threatened locations in

the world among others by climate change, although the African contribution to the total carbon emission is atypically minor, and projected to continue to be marginal (Collier et al., 2008). Climate change is also considered to be a major cause of biodiversity loss. It symbolizes a menace for human security, as the food chain upon which humans depend is changing, while vital basic resources are receding or even disappearing. For instance, by 2020, significant loss of biodiversity is projected to occur in some ecologically rich sites in Australia, with up to 30 % of species at increasing risk of extinction (IPCC, 2007). Moreover, climate change is expected to alter food security, affecting access, stability, availability, and use of food (Schmidhuber & Tubiello, 2007). Climate change is also predicted to cause scarcity in water resources in some regions of the world, exposing hundreds of millions of people to water stress (Downing et al., 1997). Besides, because of climate change and sea level rise, coastal areas are at high risks of flooding and erosion, thus negatively affecting people living in the densely populated areas and low-lying mega deltas of Asia and Africa (IPCC, 2007). Furthermore, health impacts from climate change stem from altered temperatures, extremes of precipitation, air pollution, and infectious diseases (Patz & Kovats, 2002). Impacts on agriculture have been identified as critical in terms of both number of people affected, and the severity of impacts on those least able to cope (Wreford et al., 2010). Agriculture is a main socioeconomic activity, as it provides a variety of ecosystem services. Current evidence shows that agriculture is highly vulnerable to climate change, which leads to continuous disruption in ecosystem productivity. For instance, the 2003 heat wave in Europe resulted in a decrease in corn (*Zea mays* L) yields in Italy by 36 % (Wreford et al., 2010). Furthermore, El-Nino Southern Oscillation explains about 15 % to 35 % of yield variation in wheat (*Triticum aestivum* L) and oilseeds (Howden et al., 2007). However, these effects should be combined with the potentially benign carbon fertilization effect on plant growth

caused by elevated levels of CO₂, which tends to increase the rate of photosynthesis and reduce water use.

Since climate change stems from the presence of trace gases like CH₃X, it is crucial to have a good understanding of their physical properties, their anthropogenic and natural sources, and the factors influencing their atmospheric budget.

2.3 Methyl halides:

2.3.1 Physical characteristics:

The group of halides comprises five members: F, Cl, Br, I, and At (Gribble, 2004). Methyl halides have the following formula: CH₃X, where X is the halide ion. CH₃Cl and CH₃Br are both gases, whereas CH₃I is a rather low-boiling-point liquid at room temperature. A summary of the physical characteristics for these CH₃X is given in Table 2. The halomethane of interest for the present study is CH₃Cl, which has an atmospheric lifetime of about one year with GWP, for a period of 20 years, of about 45 (WMO, 2003). CH₃Cl is ubiquitous in the atmosphere, at a concentration of approximately 550 ppt (about 3-4 Tg in terms of global cycling). It is of particular interest to researchers because it is responsible for about 16 % of the destruction of the ozone (WMO, 2003).

Table 2. Physical characteristics of CH₃Cl, CH₃Br and CH₃I (ILO, 2008).

Compound	Molar mass (g/mol⁻¹)	Melting point (°C)	Boiling point (°C)
CH₃Cl	50.5	-97.6	-24.2
CH₃Br	94.9	-94	4
CH₃I	141.94	-66.5	42.5

2.3.2 Origins:

CH₃Cl originates from diverse sources: it is not only produced anthropogenically, but also naturally, through both biological and non biological processes. Anthropogenic CH₃Cl emissions, mainly from industries, incineration, and coal combustion, account for less than 10 % of the total natural CH₃Cl budget (WMO, 2003). Butler (2000) and Harper (1993) noted that the most abundant chlorine-containing compound in the atmosphere is CH₃Cl, being produced naturally at the rate of $2.5\text{-}5 \times 10^9$ kg/year, and anthropogenically at the rate of 30×10^6 kg/year (Ramussen et al., 1980). At first, oceans were thought to be the major global source of CH₃Cl emission; then intensive research on the atmospheric fluxes of this gas revealed that terrestrial biomes and plants are key emitters of CH₃Cl. Biomass burning is a key source of CH₃Cl, the latter being released as a product of forest fires. Moreover, the production of CH₃Cl was proven to be abiotically mediated using pectin as a methyl donor (Hamilton et al., 2003). It has been established that this process is likely to be triggered at high temperatures, such as during biomass burning, which then accounts for even further release of CH₃Cl (Gebhardt, 2008). Tropical and subtropical ecosystems are considered to be a major natural source of CH₃Cl. For instance, high emissions of CH₃Cl – reaching up to 1500 ppt – were recorded in tropical forests in Southeast Asia (Yokouchi et al., 2000; Yokouchi et al., 2007). As an additional significant source in the tropics and subtropics, an abiotic release from senescent and dead leaves was observed at ambient temperatures (Hamilton et al., 2003; Yokouchi et al., 2007). CH₃Cl is also emitted by volcanoes during both quiescent and eruptive phases (Gribble, 2004).

Several living organisms appear to be key emitters of CH₃Cl. The list includes polypore fungi, macroalgae and higher plants (Harper, 1993; Saini et al., 1995; Yokouchi et al., 2000; Yokouchi et al., 2007). The emission from wood-rotting fungi is estimated to be 160,000 tonnes/year; such

emission is prevalent in the *Hymenochaetaceae*. One member of this family, *Phellinus pomaceus*, is a key producer of CH₃Cl, capable of converting Cl⁻ into CH₃Cl with over 90 % efficiency, even at extremely low concentrations of the corresponding halide (Saxena et al., 1998; Walting & Harper, 1998). CH₃Cl production was also observed from algae. The giant kelp *Macrocystis pyrifera* was identified as a dominant producer in the Californian coastal waters (Harper, 1994). Further, a release of about 1.5 ng of CH₃Cl/day/g of wet tissue from the red alga *Endocladia muricata* was detected (Wuosmaa & Hager, 1990). Evergreen plants, potato tubers, and other plant families are capable of producing CH₃Cl (Gribble, 2004). Further, a survey of 118 herbaceous species, based on CH₃I emission by leaf discs, proved the capability of 87 species to methylate halides (Saini et al., 1995). The highest activities were associated with the order *Capparales* including 15 members of the *Barassicaceae* family and one of *Resedaceae*. These emissions were proven to be enzymatically mediated. The mechanism proceeds via a methyl group donor (SAM) and a halide ion methyltransferase to transfer the methyl group over to the halide ion. Wuosmaa and Hager (1990) demonstrated the involvement of a methyl chloride transferase (MCT) in the process of CH₃Cl synthesis, which was detected in a wood-rotting fungus (*Phellinus pomaceus*), a marine alga (*Endocladia muricata*), and a halophytic plant (*Mesembryanthemum crystallinum*).

Research has been conducted to demonstrate the relative affinity of MCT for halides (Saini et al., 1995):



An analogous activity has been reported in cabbage and other species, initially called H/BMT and later TMT (Attieh et al., 2000; Saini et al., 1995). It has been shown that thiol substrates from glucosinolate hydrolysis are preferred substrates of this enzyme in glucosinolates-

containing plants (Attieh et al., 2002; Attieh et al., 2000), which explains the nomenclature of TMT attributed to this enzyme. Remarkably, methyltransferases from the *Brassicaceae* family have the ability to methylate both halides and thiols through the same enzymatic reaction, using SAM as a methyl group donor.

Five TMT isoforms were reported in Cabbage (Attieh et al., 2002), whereas three Harmless to Ozone Layer (*HOL*) genes encoding for HOL proteins, which methylate glucosinolates' breakdown products and halides, were identified in *Arabidopsis thaliana* (Nagatoshi & Nakamura, 2007, 2009).

2.3.3 Physiological significance of CH₃X emission:

Answers to the question of why living organisms produce CH₃X are still lacking. Nevertheless, several suggestions for the significance of this production have been made (Attieh et al., 2000; Saini et al., 1995; Itoh et al., 2009; Kaur, 2006). Since the methyltransferases from the *Brassicaceae* family have the ability to methylate both halides and thiols (with a higher affinity for thiols), it was stated that halide methylation could be a side reaction of sulfur metabolism for these plants (Attieh et al., 2000; Saini et al., 1995). Besides, halide methylation in algae contributes to the production of allelopathic chemicals (Itoh et al., 2009). Furthermore, Harper (1993) stated that some fungal species use CH₃Cl as a methyl group donor for the synthesis of methylesters and veratryl alcohols within their metabolism. Moreover, several explanations for CH₃Cl emission in plants were reported. It was stated that such emission could simply be a metabolic accident (Manley, 2002). Current evidence suggests that CH₃Cl emission can be considered as a possible mechanism of improving tolerance of high valuable crops against Cl⁻ toxicity under saline conditions (Kaur, 2006). Such proposition was verified by several experiments, conducted on a variety of organisms. For instance, to test the hypothesis that the

TMT gene may play a role in chloride detoxification and salt tolerance, a *TMT1* gene –isolated from red cabbage (Attieh et al., 2002) – was engineered into *E. coli*, potato, and tobacco roots and plants. Kaur (2006) demonstrated that *TMT1* can impart the ability to volatilize Cl⁻ and thus, increase salt tolerance of plants against NaCl toxicity when engineered into specific species –that naturally lacks the TMT activity and the gene encoding it– such as tobacco. Nevertheless, over expressing or silencing the *TMT1* gene in *Arabidopsis* did not change salinity resistance.

2.4 Roles of SAM in plants:

SAM is a compound found in living cells and organisms (Moffat & Weretilnyk, 2001; Roeder et al., 2009). Several studies have shown the involvement of SAM in numerous enzyme-catalyzed vital reactions in plants, ranging from the methylation of many cellular compounds and volatiles, to being a precursor and a substrate for the synthesis of ethylene, polyamines, biotine, and nicotianamine (Roeder et al., 2009; Roje, 2006). Thus, SAM is considered to be the major methyl group donor in plants, and is involved in methylation reactions that modify lipids, proteins, and nucleic acids (Wang et al., 2002). For that reason, it is crucial to have an insight into the mechanism of its synthesis and regeneration.

SAM cycle is a fundamental metabolic pathway supplying cells and tissues with SAM, methionine (Met) and various related compounds (Roeder et al., 2009). It consists of four reactions, each step being catalyzed by a different enzyme, as shown in Figure 1. The first step is a demethylation of SAM into *S*-adenosyl-L-homocysteine (SAH) by the action of methyltransferases. Secondly, SAH is hydrolyzed into homocysteine (Hcy) by homocysteine hydrolase. Thirdly, Hcy is methylated to Met by methionine synthase, followed by an adenylation of Met into SAM by *S*-adenosyl-L-methionine synthetase. In addition to being an essential building block of proteins, nearly 80% of cellular Met is converted into SAM by SAM

synthetase at the expense of Adenosine 5'-triphosphate (ATP) utilization (Wang et al., 2002). To understand the regulation of the SAM cycle, cellular localisation of SAM should be taken into consideration. Roeder (2009) stated that SAM is transported from the cytosol to the chloroplasts, where it is converted into Met via SAH and Hcy, in order to provide chloroplasts with this essential amino acid.

The following section will show the importance of SAM and its involvement in a myriad of reactions.

2.4.1 SAM as an intermediate in polyamines synthesis:

Polyamines –including spermidine, spermine, and putrescine– involved in plant stress responses are positively charged molecules at cellular pH levels, and thus chemically interact with DNA, RNA and some proteins (Roje, 2006). SAM serves as an aminopropyl group donor in the process of spermidine and spermine formation, essentially.

2.4.2 SAM as a precursor of nicotianamine and phytosiderophores:

Nicotianamine, a molecule frequently found in higher plants, plays a role of a strong chelator for iron and other metals. In non-graminaceous plants, nicotianamine binds to metal ions and participates in their trafficking inside the plant, while in graminaceous plants, it is the precursor of phytosiderophores synthesis, the latter being essential in acquiring metals, especially iron, from the soil (Roje, 2006). Three SAM molecules are required for the formation of one molecule of nicotianamine, with the release of three molecules of 5'-methylthioadenosine (MTA) through a reaction catalyzed by the nicotianamine synthase.

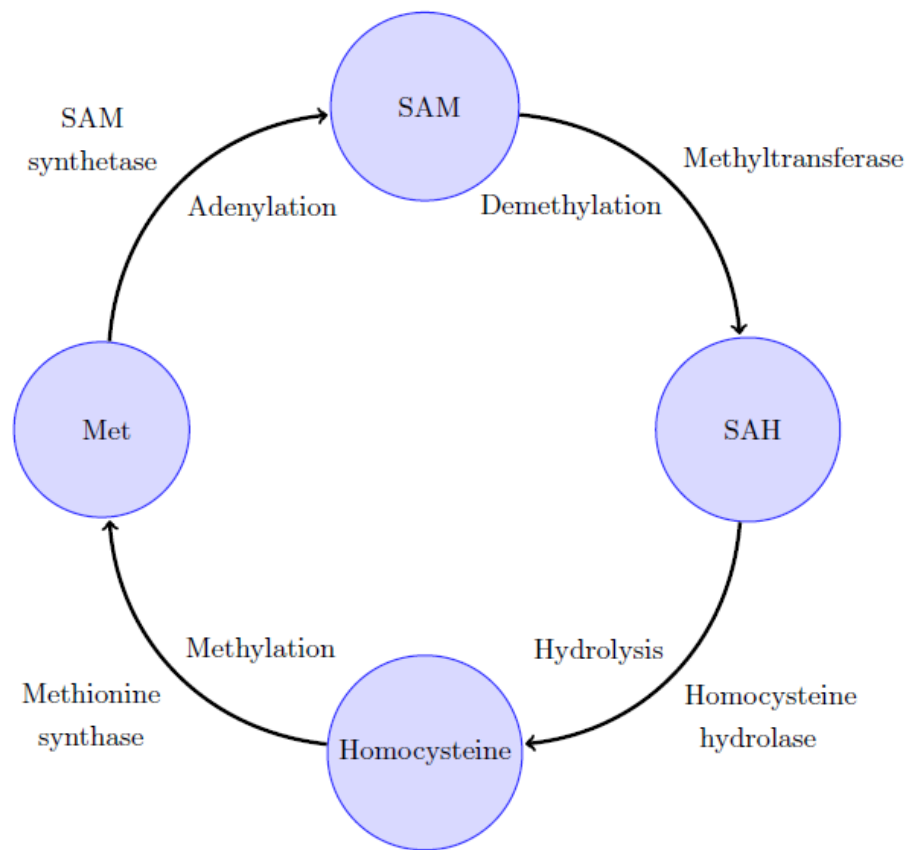


Figure 1. Simplified scheme of SAM cycle (Roeder et al., 2009). It consists of four reactions with each step being catalyzed by a different enzyme: The first step is a demethylation of SAM into *S*-adenosyl-L-homocysteine (SAH) by the action of methyltransferases. Secondly, SAH is hydrolyzed into homocysteine (Hcy) by homocysteine hydrolase. Thirdly, Hcy is methylated to Met by methionine synthase, followed by an adenylation of Met into SAM by *S*-adenosyl-L-methionine synthetase.

2.4.3 SAM as a precursor of ethylene biosynthesis:

Ethylene, the gaseous phytohormone, is involved in a diverse array of plant growth and developmental processes, as well as in response to biotic and abiotic stresses (Argueso et al., 2007; Bleecker & Kende, 2000; Taiz & Zeiger, 2006; Wang et al., 2002). Due to the simple chemical structure of ethylene, theoretically a variety of compounds can be converted into it through diverse chemical reactions. Such compounds include acrylic acid, ethionine, propanol, ethanol, Met, β -alanine, and acetic, linolenic, and fumaric acids (Yang, 1974). It has been long established that Met - but none of the compounds listed above - acts as an effective precursor of ethylene in higher plants (Yang & Hoffman, 1984).

Key advances in the understanding of the ethylene biosynthetic pathway were the evidence that SAM and 1-Aminocyclopropane-1-carboxylic acid (ACC) are the precursors of ethylene (Yang & Adams, 1979). The synthesis of this hormone from SAM involves two major steps: first, SAM is converted into ACC by 1-aminocyclopropane-1-carboxylic acid synthase (ACS), while the second step is catalyzed by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), the activity of which depends on membrane integrity (Bleecker & Kende, 2000; Kende, 1989; Yang & Hoffman, 1984). According to Yu and Yang (1979), only a small amount of SAM is used for ACC synthesis, implying that SAM would not limit ethylene synthesis.

The detailed mechanism of ethylene biosynthesis will be explained in the next section dealing with ethylene.

2.4.4 SAM as a ubiquitous methyl group donor:

SAM is the major methyl group donor for a myriad of reactions in plants. O-, N-, C- and S- are the main methyltransferase families in plants: O-methyltransferases act on hydroxyl and carboxyl moieties of small molecules; N-methyltransferases act on DNA, proteins, and

secondary metabolites; C-methyltransferases act on lipids; and S- methyltransferases act on various organic thiols such as thiocyanate or thiophenol (Moffat & Weretilnyk, 2001; Roje, 2006). Another group of methyltransferases acting on halides exists in plants (Saini et al., 1995) which are present in a variety of organisms as detailed in section 2.3.2.

The methyltransferase enzymes cited earlier are considered to be the most numerous among the SAM-utilizing enzymes in plants, because of the huge number of methylated products (Moffat & Weretilnyk, 2001).

Since the involvement of SAM in various plant physiological pathways is clearly identified, it is important to understand the competition for the use of SAM between different biosynthetic pathways. Therefore, the present investigation will try to elucidate the impact of *TMT* insertion on SAM availability for C₂H₄ synthesis in genetically engineered tobacco plants.

2.5 Ethylene synthesis in plants:

2.5.1 Discovery:

The capacity of certain gases to affect plant growth has been well known since the nineteenth century. In fact, as early as 1864, when streets were illuminated with coal gas, trees in the vicinity of street lamps defoliated more extensively than other trees (Salisbury & Ross, 1992). It was not until 1901, that Neljubow discovered that active defoliating component of the illuminating gas was ethylene, and that the gas affected plant growth. He determined, through experiments conducted on dark-pea seedlings grown in laboratory, that ethylene caused the so-called triple response:

- Reduction in stem elongation
- Increased lateral growth

➤ Abnormal horizontal growth

In addition to these symptoms, an inhibition in leaf expansion, as well as a delay in the normal opening of the epicotyl hook, was noticed. In contrast, placing these plants in fresh air permitted them to regain their normal growth.

2.5.2 Chemical properties of ethylene:

With its simple two-carbon structure, ethylene is the simplest alkene –with a molecular weight of 28– being lighter than air under physiological conditions, and being a flammable compound. This phytohormone is active at concentrations as low as 1 ppb (Taiz & Zeiger, 2006)

2.5.3 Mechanisms of ethylene synthesis:

Ethylene is produced enzymatically in most living organisms, including bacteria, several fungal species, lower plants, and the majority of higher plants (Taiz & Zeiger, 2006). Ethylene is synthesized from Met and ACC in essentially all plant tissues, including leaves, flowers, stems, roots, fruits and seedlings. Production of ethylene varies with the type of tissue, the plant species, and also the stage of development.

Another ethylene synthesis pathway exist in microbes which is different from the one used by plants: instead of ACC, microbial pathogens use 2-keto-4 methyl-thiobutyrate (KMB)–a transaminated derivative of Met– as ethylene precursor (Nagahama et al., 1992). KMB is then oxidized to ethylene through a non enzymatic process by oxidizing agents generated by a NADH: Fe (III) EDTA oxidoreductase.

2.5.3.1 Enzymes involved in ethylene synthesis:

The mechanism by which ethylene is produced from Met is a three-step process, involving three enzymes, as shown in Figure 2 (Salisbury & Ross, 1992). The first step in the ethylene

biosynthetic process consists of the conversion of Met into SAM by SAM synthetase. Yang and Hoffman (1984) proved that ethylene is derived from the amino acid Met by showing how labelled Met was efficiently converted into C₂H₄ by apple fruit, with ethylene being derived from the carbons 3 and 4 of Met. Secondly, SAM, under the action of ACS, is converted into ACC and MTA. Several studies have shown that this reaction is the rate-limiting step in C₂H₄ production. The last step is an aerobic reaction catalyzed by ACO, leading to the formation of C₂H₄, CO₂, H₂O, and HCN (cyanide). The latter, being lethal for the plant, is detoxified into β-cyanoalanine by the β-cyanoalanine synthase.

- SAM synthetase: It ensures the conversion of Met into SAM (Kende, 1989; Taiz & Zeiger, 2006) with the utilization of one ATP molecule.
- ACS: This is a pyridoxal enzyme that requires pyridoxal phosphate (PLP) as a cofactor. ACS is encoded by multigene families in most plant species. It has been identified as the key enzyme in the C₂H₄ biosynthetic pathway. It catalyzes the conversion of SAM into ACC and 5'-MTA by a β-γ carbon elimination reaction (Argueso et al., 2007; Yang & Hoffman, 1984). ACS is an unstable cytosolic thermolabile enzyme, with an apparent half-life of about 0.5 hours (Yang & Hoffman, 1984). As it is a low-abundance enzyme, it was initially hard to purify for biochemical characterization.
- ACO: This catalyzes the conversion of ACC into C₂H₄, HCN, H₂O, and CO₂. The reaction consists of a modification of C2 and C3 of ACC, while C1 is converted into HCN, and C5 into CO₂ in the presence of O₂. It belongs to the Fe²⁺/ascorbate oxidase super family, thus requiring Fe²⁺ and ascorbate for activity (Argueso et al., 2007; Yang & Hoffman, 1984). This constitutive enzyme requires membrane integrity to function (Yang & Hoffman, 1984).

2.5.3.2 Inducers and inhibitors of ethylene synthesis:

Inducers:

Ethylene synthesis increases considerably during different stages of plant growth such as germination, fruit ripening, abscission and senescence of flowers and leaves (Taiz & Zeiger, 2006; Yang & Hoffman, 1984). Furthermore, a multitude of inducers enhance ethylene biosynthesis. For example, auxins induce ethylene production by activating ACS, and higher rates of C₂H₄ production are often associated with tissues rich in auxins. Another important group of stimuli includes environmental stresses such as drought, chilling, salinity, flooding, wounding, hypoxia, pathogen attack and toxic pollutants. Morgan (1997) stated that plants exposed to O₃, or other pollutants such as NO₂, emitted stress ethylene. Further studies proved that ethylene controls its own biosynthesis, either by increasing (auto stimulation) or decreasing (auto inhibition) its rate of production (Taiz & Zeiger, 2006). In plants in which fruits ripen in response to ethylene (called climacteric - for example apples), two systems of ethylene production operate. The first system acts in vegetative tissue in which ethylene inhibits its own biosynthesis. The second system- which occurs during fruit ripening or petal senescence in some species, and in which ethylene stimulates its own synthesis- is used to integrate ripening of the entire fruit once it has commenced (Taiz & Zeiger, 2006).

Inhibitors:

The following chemicals can inhibit the biosynthesis of ethylene at various steps:

- *Inhibitors of ACS:*

Aminoethoxyvinylglycine (AVG) and aminoxyacetic acid (AOA) are strong inhibitors of pyridoxal enzymes, and thus inhibit the ACS enzyme (Yang & Hoffman, 1984). *In vivo* and *in*

in vitro experiments both showed that AVG and AOA inhibit ethylene production by blocking the conversion of SAM into ACC (Adams & Yang, 1977).

- *Inhibitors of ACO:*

This category of inhibitors includes inorganic ions such as Co^{2+} or Ni^{2+} , chemicals such as 2, 4-dinitrophenol (DNP) or carbonyl-cyanide m-chlorophenylhydrazone (CCCP) and other membrane disruptive agents, free radical scavengers, and polyamines, as well as ACC analogs (Even-Chen et al., 1982). Such compounds inhibit ethylene production by blocking ACO. For example, Co^{2+} exerts its inhibitory effect by interacting with the sulfhydryl group of ACO, causing strong inhibition in ethylene synthesis at 5mM (Lau & Yang, 1976).

In addition to inhibitors of ethylene synthesis, a number of compounds inhibit ethylene action. Silver ions Ag^+ – applied as AgNO_3 or $\text{Ag}_2\text{S}_2\text{O}_3$ – and norbornadiene, are potent inhibitors of ethylene action. CO_2 at high concentrations (in the range of 5 to 10 %) also inhibits many effects of ethylene, although it is less efficient than Ag^+ . Several other compounds that bind irreversibly to ethylene receptors such as 1-methylcyclopropene (MCP) effectively block multiple ethylene responses.

2.5.4 Physiological roles of C_2H_4 in plants:

Ethylene controls a variety of plant responses, and is involved in many stages of plant growth and development (Buchanan et al., 2000; Salisbury & Ross, 1992; Taiz & Zeiger, 2006). It stimulates the release of seed dormancy and the abscission and senescence of leaves, petals and fruits, as well as shoot and root growth and differentiation. In addition to these effects, ethylene promotes adventitious root formation from tissues other than pre-existing roots, flower opening and germination of a number of species, and induces femaleness in dioecious flowers. Moreover, ethylene stimulates ripening of climacteric fruits such as bananas, apples, and tomatoes.

Ripening of these fruits is characterized by a sharp increase in ethylene synthesis at the mature green stage and by a concomitant rise in respiration (Bleecker & Kende, 2000). Furthermore, ethylene behaves as a stress hormone under biotic (pathogen attack) and abiotic (salinity, drought, chilling) stress conditions. For example, in the event of pathogen attack, ethylene helps to limit the spread of the pathogen by causing leaf abscission in cases where it exacerbates disease symptoms (Bleecker & Kende, 2000).

As both ethylene (natural defense mechanism) and CH_3Cl emission through TMT insertion (acquired salinity tolerance) compete for the use of SAM as a precursor, the present study will try to elucidate the potential effect of TMT insertion on SAM availability for the synthesis of the universal stress hormone ethylene.

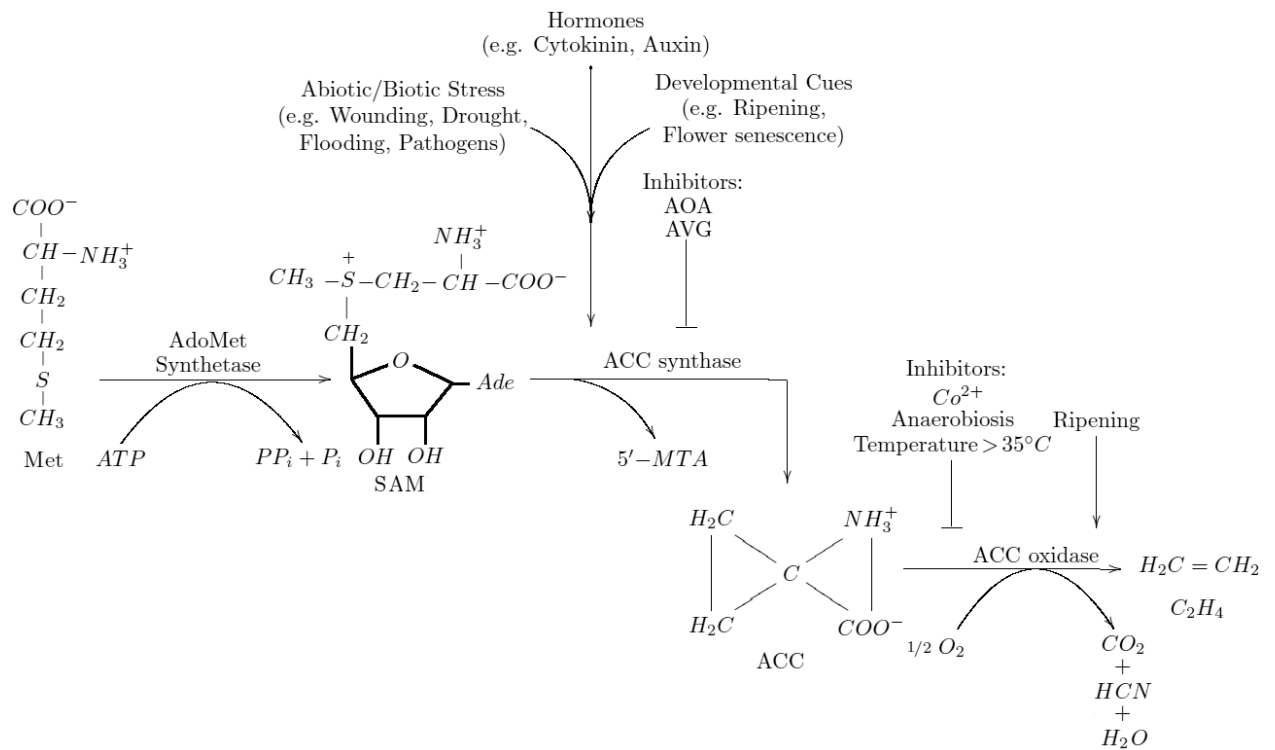


Figure 2. Ethylene biosynthetic pathway (Taiz & Zeiger, 2006). A three-step process in which each step is catalyzed by a specific enzyme presented above the arrows. A group of inducers and inhibitors of ethylene synthesis are shown in the Figure by ↓ and ⊥ respectively.

Chapter 3

Material and methods

The present study was conducted to determine whether *TMT1* gene insertion in tobacco plants would negatively affect ethylene emission by limiting SAM availability for ethylene synthesis.

3.1 Plant material:

Transgenic tobacco (*Nicotiana tabacum*) plants, produced in Dr Saini's laboratory (Kaur, 2006), were used in this study. The transformation consisted of transferring the *TMT1* gene isolated from red cabbage (Attieh et al., 2002) into tobacco plants, which naturally do not contain TMT or any other enzymatic activity capable of methylating chloride and other halides. Seeds collected from mature plants were sown directly into pots containing a commercial soil mix (Sunshine number four). Transformed tobacco plants were grown and maintained in the greenhouse located in the Department of Biology at the University of Waterloo (Canada) under temperature set at 20°C and light conditions consisting of 8 hours darkness and 16 hours light. Transformed tobacco plants, confirmed by southern blot analysis to have one *TMT* gene copy, were exclusively used for this study.

3.2 Chemicals:

CoCl₂, ACC, NaBr and KI were purchased from Sigma-Aldrich (USA), whereas NaCl was obtained from EMD Chemicals (USA). CH₃Br, CH₃I and C₂H₄ were procured from Sigma-Aldrich (USA), while CH₃Cl was purchased from Boc gases (Mississauga, On). Helium (He) and nitrogen gases were both obtained from Paraxair (Kitchener, On).

3.3 Experimental protocol:

Experiments were conducted in four different ways, under *in vitro* and *in vivo* conditions. Each treatment for each experimental method was given in two or three replicates. Moreover, each experiment was repeated four times, and in each time, the obtained results followed similar trend. Therefore, the results of one typical set of experiments are reported in this thesis. Because of the time needed for the gas chromatography (GC) to detect the different gaseous products emitted by plant material, and in order to maintain the same physiological state of tissue in each experiment, it was not possible to run more than three replicates at once.

In all experiments, 6 superficial radial cuts were made on the lower surface of tobacco leaf disks to facilitate diffusion of solutions into the leaf tissue.

Three series of tests were carried out using 10 mm diameter tobacco leaf discs. Although the treatments and number of leaf discs used varied from one experiment to another, these tests share a common purpose: revealing the effect of *TMT* gene insertion on ethylene emission.

3.3.1 *In vitro* experiments:

❖ Measurement of *in vitro* C₂H₄, CH₃Cl and CH₃Br emissions from 22 tobacco leaf discs in 50 ml flasks:

Twenty-two leaf discs from transformed tobacco plants were collected from nine-week-old plants. In order to ensure that similar material was used for all treatments, leaves were placed in a stack and the disks punched out with a cork borer. Consequently, a mixture of leaf discs from young and old leaf samples was present in all 50 ml flasks. Preliminary work was conducted in order to define the adequate number of leaf discs to maximize the emission of the measured gases, prepare the GC standards, and develop a thermal GC program capable of detecting the different products emitted by leaves in the same headspace sample. A smaller number of leaf

disks, for example 11, gave erratic results because the amount of ethylene detected was too low for reliable comparisons among treatments. Doubling the number of disks overcame this problem. Therefore, the potential effect of *TMT* insertion on ethylene emission was tested by placing 22 leaf discs from transformed tobacco plants at the bottom of 50 ml flasks, each lined with one layer of 42.5 mm diameter filter paper as shown in Figure 3. Each flask contained 4 ml of 0.1 M NaCl or NaBr solution, which would induce the synthesis of the corresponding methyl halide. The flasks were incubated overnight at 25°C. A control consisting of leaf discs in the presence of 4 ml distilled water (DW) was included to assess ethylene emission. To ascertain that the gas being detected was ethylene, 4 ml of 2 mM ACC solution was added to the flasks. This treatment, as is previously known (Adams & Yang, 1979), boosted ethylene production, proving that ethylene was the correct gas detected.

In order to evaluate the chloride methylating activity and reveal the likely effect of CH₃Cl release on SAM availability for ethylene emission, 0.1 M solution of NaCl was added to leaf discs (Saini et al., 1995). CH₃Br was quantified using the same methodology, replacing NaCl with 0.1 M NaBr solution. As the rate of bromide methylation by the TMT enzyme is much greater than that of chloride methylation, the purpose of this treatment was to test the limits of SAM availability by boosting SAM use to a level beyond what would be needed for chloride methylation.

Further treatment using 5 mM CoCl₂, an inhibitor of C₂H₄ synthesis, was also included. The rationale behind this was to reveal whether inhibiting C₂H₄ biosynthesis would enhance CH₃X synthesis, by augmenting SAM amounts used for halides methylation. The flasks were closed with rubber stoppers for an overnight incubation at 25°C. After the overnight incubation, one ml

headspace samples were drawn every hour through the rubber septum with a syringe into the GC for the analysis of gases in the flask for a period of seven hours.

❖ **Measurement of *in vitro* C₂H₄, CH₃Cl, CH₃Br and CH₃I emissions from 6 tobacco leaf discs in small containers:**

6 leaf discs were placed at the bottom of 9.5 ml vials as shown in Figure 4. Initially, 0.5 ml DW was added to the vials to assess ethylene emission (Control). Then, after 2 hours of incubation in DW, 0.5 ml of salt solution (0.1 M NaCl, NaBr or KI) were added to the vials to compare the ethylene emission profile in the presence or absence of CH₃X biosynthesis. As Br⁻ and I⁻ are methylated by TMT at much higher rates than is Cl⁻ (Saini et al., 1995), NaBr and KI treatments were included to determine the effect of boosting SAM use beyond what would be needed for chloride methylation. In another set of experiments, the three salt solutions at 0.1, 0.02 and 0.04 M, respectively, were added at the beginning of the experiment concomitantly with the start of ethylene measurements. In both types of experiments, vials were closed with rubber stoppers and gently shaken continuously on a rotary shaker to maintain leaf discs in direct contact with the solution. After one hour of incubation, 1 ml headspace samples, taken every hour, were injected into the GC to quantify the amounts of C₂H₄, CH₃Cl, CH₃Br and CH₃I emitted.

❖ **Estimation of the amounts of SAM required for ethylene and methyl halides emissions:**

The rates of ethylene and methyl halides production from leaves of transgenic tobacco were calculated using data collected from this study. Considering that one molecule of SAM is used to produce one molecule of ethylene and one molecule of CH₃X, the rate of production reflects the amounts of SAM consumed to produce ethylene or a specific methyl halide per hour. The rate of

production was calculated for the period of 3 to 5 hours, as it coincides with the linear production phase.

The data on C_2H_4 , CH_3X emissions were converted to n/cm^2 of leaf surface, and the SAM levels ($pmol/cm^2$ leaf surface/h).

3.3.2 *In vivo* experiments:

CH_3Cl and CH_3Br emissions were measured after dipping transgenic tobacco leaves in solutions of 0.1 M NaCl or NaBr for 3 minutes and then monitoring gas emission for a period of 3 hours. A control was included in parallel to this test by dipping leaves in DW only. A bag enclosure method was used to collect foliar emissions of CH_3X and C_2H_4 . This method consisted of placing a young and healthy leaf in a 12.7X10.16 cm Tedlar bag with a volume of 400 ml (Qubit Systems Inc. Kingston, Ontario, Canada), and the top of each bag was closed carefully with a clamp as shown in Figure 5. After 20 min of incubation, 1 ml headspace samples were analyzed by GC for their C_2H_4 , CH_3Cl and CH_3Br content.

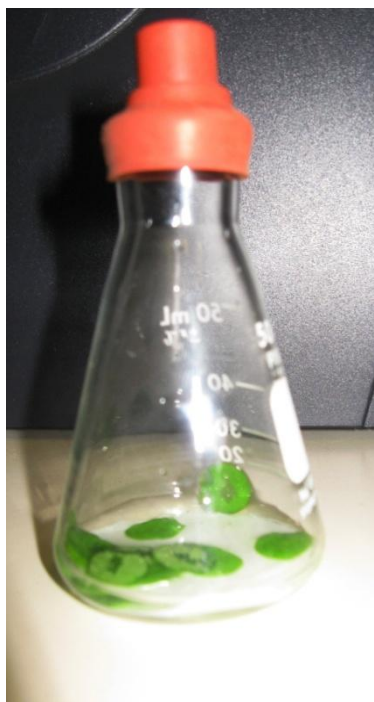


Figure 3. Photograph of 50 ml flask closed with a rubber stopper. A mixture of 22 leaf discs derived from young and old leaf samples from transformed tobacco plants were placed in 50 ml flasks in the presence of different solutions.



Figure 4. Photograph of a 9.5 ml vial closed by a rubber stopper. 6 leaf discs were incubated in 9.5 ml vials in the presence of 0.5 ml of DW or 0.1 M NaCl, NaBr or KI.



Figure 5. A young transgenic tobacco leaf in a Tedler bag, with the top of the bag closed with a clamp. The bag enclosure method was employed to collect foliar *in vivo* emissions of C₂H₄, CH₃Cl, and CH₃Br.

3.4 Gas chromatography:

In vitro emissions of CH₃Cl, CH₃Br, CH₃I and C₂H₄ from tobacco leaf disks were analyzed by gas chromatography for a period of 7 hours from the beginning of the experiment. One ml headspace samples were injected into a Hewlett-Packard 5890 series II gas chromatograph equipped with an 80-100 mesh Porapak-Q (Supelco, Canada) packed column (0.3 cm x 210 cm) and a flame ionisation detector (FID). The flow rate of the carrier gas, He, was 40 ml min⁻¹ while the column temperature was programmed in two steps: initially 80°C for 2 minutes, followed by an increase at the rate of 40 ml min⁻¹ to 130°C and holding at that temperature for 16 minutes. This temperature program was used to detect C₂H₄, CH₃Cl, CH₃Br and CH₃I emitted, by comparison of their retention times with those of corresponding standards that were used to calibrate the instrument. The concentration of C₂H₄ standard was 1.06 nl ml⁻¹, whereas that of CH₃Cl, CH₃Br, and CH₃I was 10.6 nl ml⁻¹. The column was purged by heating to 200°C between injections to remove any trace of contaminants.

3.5 Statistical analysis:

All data presented in this study were statistically analyzed using two-tailed t-test with an error rate (α) of 0.05. Means from various treatments were compared with control values to show whether these means were different or equal, which enable to accept or refute the null hypothesis (H₀) against the alternative hypothesis (H₁). The analysis was based on comparison of p-value calculated with α .

H₀: Means are the same: The difference is not significant (p-value>0.05)

H₁: Means are different: The difference is statistically significant (p-value<0.05).

Chapter 4

Results

The main goal of the present study was to determine whether SAM would be limiting for ethylene synthesis because of its utilization by the competing halide-methylation reaction in transgenic plants containing the *TMT1* gene.

Several *in vitro* and *in vivo* experiments were conducted, as detailed in the previous section, to confirm or reject this hypothesis. *In vitro* conditions differed from one treatment to another mainly by the number of leaf discs used, final air volume in each flask, incubation period in the different solutions, and the time of introducing the salt solution.

The profiles of emissions of C_2H_4 , CH_3Cl , CH_3Br and CH_3I from transformed tobacco plants, showing the retention times of each product, are presented in Figure 6.

4.1 Investigating the effect of TMT insertion on SAM availability for ethylene emission from leaves of transgenic tobacco:

4.1.1 Measurement of ethylene emission when salt solutions were added at the beginning of the experiment:

Figures 7 and 8 show an accumulation of ethylene over time in 50 ml flasks containing leaf disks incubated in distilled water and salt solutions. Statistical analysis show that ethylene accumulation was not altered when leaf disks were incubated in 0.1 M NaCl ($p=0.356$) (Table 3) whereas in the presence on 0.1 M NaBr, ethylene emission was less pronounced ($p=0.008$) (Table 5) compared to that observed in DW. The fact that Br^- is methylated at a higher rate by TMT than is Cl^- , would have triggered additional use of SAM via CH_3Br production route which probably explains the decrease in ethylene emission observed when leaves were incubated in the

presence of NaBr. CH₃Cl and CH₃Br were produced at rates matching the relative preference for Br⁻ over Cl⁻ by TMT. Conversely, ethylene production, and thus SAM use by this pathway, was blocked by inhibiting ethylene biosynthesis by adding CoCl₂. Based on statistical analysis, the accumulation of CH₃Cl increased substantially (p=0.008) (Table 4) over that observed in the absence of ethylene inhibition. Hence, ethylene and methyl halides compete for the use of SAM as a substrate.

Another set of experiments, using 9.5 ml vials, was conducted to determine the effect of various salt concentrations (0.02, 0.04 and 0.1 M NaCl or NaBr) on ethylene emission. The reason behind switching from 50 ml flasks to 9.5 ml vials is to determine whether the change in volume (a lower dilution factor) yields the same results obtained with 50 ml flasks. Different salt concentrations were used in this part of the study in order to reveal whether increasing salt concentrations would lead to increased amounts of SAM drained for CH₃X synthesis, thus negatively affecting C₂H₄ emission. Ethylene accumulation was only altered at 0.1 M NaCl (p=0.0003) whereas 0.02 and 0.04 M of NaCl had no negative effect on ethylene emission (Table 6, Figure 9). On the contrary, a decrease in ethylene emission, along with increasing NaBr concentrations, was observed (Table 7, Figure 10) presumably because increased bromide methylation in the presence of increasing NaBr concentrations diverted proportionately greater amounts of SAM for the synthesis of CH₃Br.

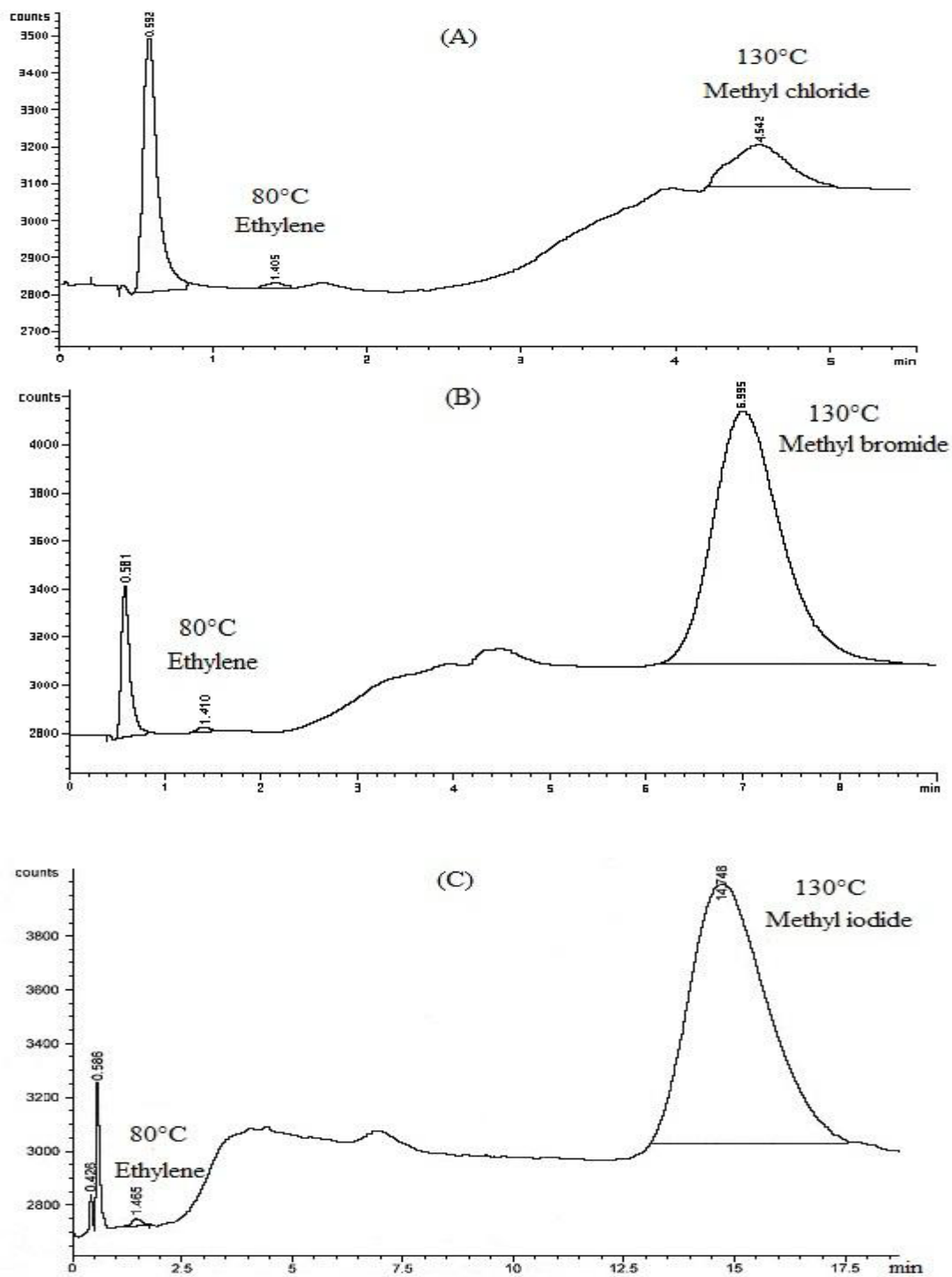


Figure 6. Gas chromatograms showing the retention times of selected gaseous products emitted by tobacco leaves: Ethylene and methyl chloride (A), ethylene and methyl bromide (B) and ethylene and methyl iodide (C).

Table 3. A summary of the data analysis of C₂H₄ emission (nl/cm²leaf surface) from leaf discs of transgenic tobacco incubated overnight in DW and 0.1 M NaCl solution (n=3).

t-Test: Two-Sample Assuming Unequal Variances		
	α	0.05
Equal Sample Sizes		
Ethylene	<i>DW</i>	<i>0.1 M NaCl</i>
Mean	0.177571	0.162143
Variance	0.000938	0.000867
Observations	7	7
Hypothesized Mean Difference	0	
Df	12	
t Stat	0.961	
P(T<=t) two-tail	0.356	
T Critical Two-tail	2.179	

Table 4. A summary of the data analysis of CH₃Cl emission (nl/cm² leaf surface) from leaf discs of transgenic tobacco incubated overnight in 0.1 M NaCl and 5 mM CoCl₂ (n=3).

t-Test: Two-Sample Assuming Unequal Variances		
	α	0.05
Equal Sample Sizes		
	<i>0.1 M NaCl</i>	<i>5 mM CoCl₂</i>
Mean	6.022429	8.317571
Variance	0.094461	2.315865
Observations	7	7
Hypothesized Mean Difference	0	
Df	6	
t Stat	-3.911	
P(T<=t) two-tail	0.008	
T Critical Two-tail	2.447	

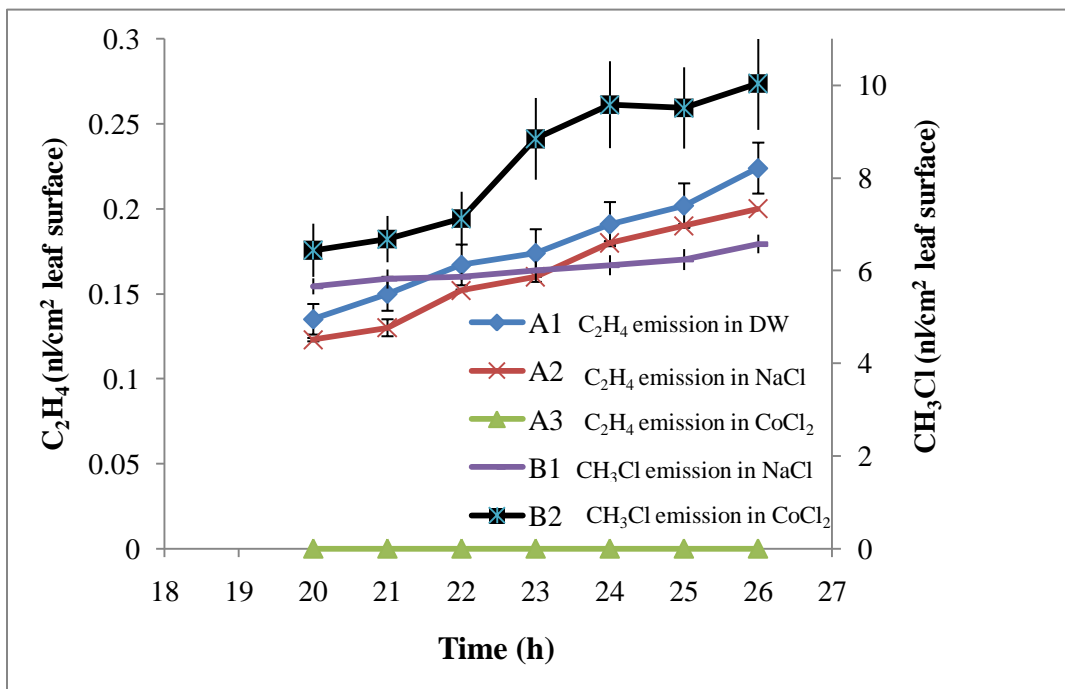


Figure7. Emission of C₂H₄ and CH₃Cl over time from leaf discs of transgenic tobacco incubated overnight in 50 ml flasks in the presence of 4 ml of different solutions. C₂H₄ and CH₃Cl emissions were measured after 20 hours of incubation in DW, NaCl and CoCl₂ at room temperature. Values are the mean ± SE (n = 3).

Table 5. A summary of the data analysis of C₂H₄ emission (nl/cm²leaf surface) from leaf discs of transgenic tobacco incubated overnight in DW and 0.1 M NaBr solution.

t-Test: Two-Sample Assuming Unequal Variances		
	α	0.05
Equal Sample Sizes		
Ethylene	DW	0.1 M NaBr
Mean	0.17757	0.13071
Variance	0.00094	0.00053
Observations	7	7
Hypothesized Mean Difference	0	
Df	11	
t Stat	3.232	
P(T<=t) two-tail	0.008	
T Critical Two-tail	2.201	

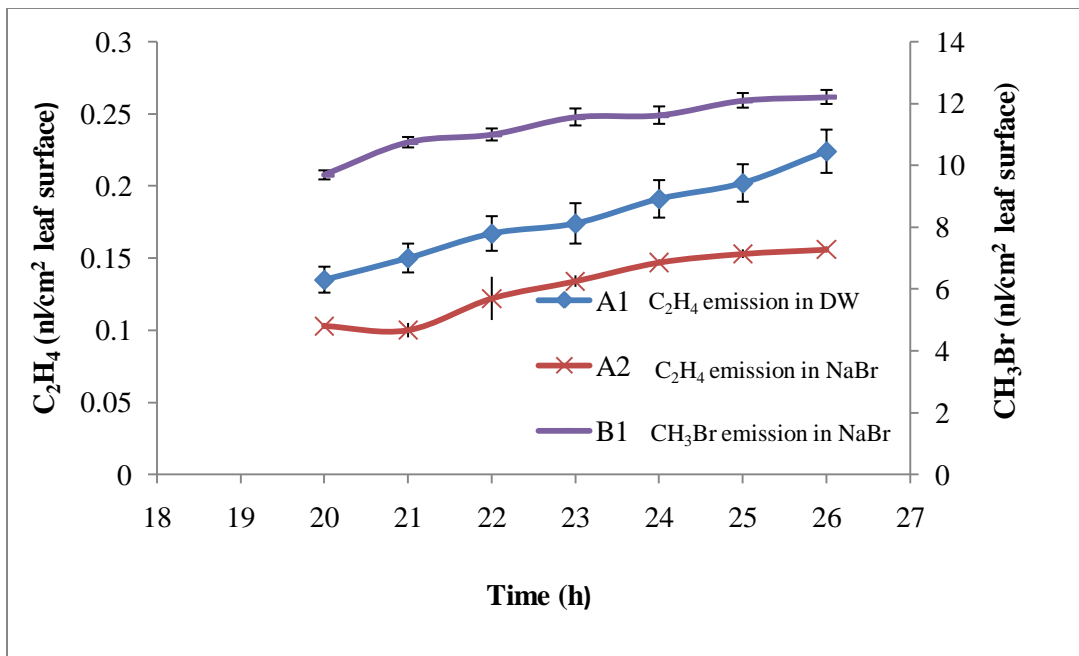


Figure 8. Emission of C₂H₄ and CH₃Br over time from leaf discs of transgenic tobacco incubated overnight in 50 ml flasks in the presence of 4 ml of different solutions. C₂H₄ and CH₃Br emissions were measured after 20 hours of incubation in DW and NaBr at room temperature. Values are the mean± SE (n = 3).

Table 6. A summary of the data analysis of C₂H₄ emission (nl/cm²leaf surface) from leaf discs of transgenic tobacco incubated in DW and 0.02, 0.04 and 0.1 M NaCl solution.

t-Test: Two-Sample Assuming Unequal Variances

<i>Ethylene</i>	<i>DW</i>	<i>0.02 M NaCl</i>
Mean	0.8088	0.6033
Variance	0.13706	0.050839
Observations	10	10
Hypothesized Mean Difference	0	
Df	15	
t Stat	1.499166	
P(T<=t) two-tail	0.154581	
t Critical two-tail	2.13145	

<i>Ethylene</i>	<i>DW</i>	<i>0.04 M NaCl</i>
Mean	0.8088	0.5259
Variance	0.13706	0.042111
Observations	10	10
Hypothesized Mean Difference	0	
Df	14	
t Stat	2.113485	
P(T<=t) two-tail	0.052993	
t Critical two-tail	2.144787	

<i>Ethylene</i>	<i>DW</i>	<i>0.1 M NaCl</i>
Mean	0.8088	0.1606
Variance	0.13706	0.010052711
Observations	10	10
Hypothesized Mean Difference	0	
Df	10	
t Stat	5.344212	
P(T<=t) two-tail	0.000326	
t Critical two-tail	2.228139	

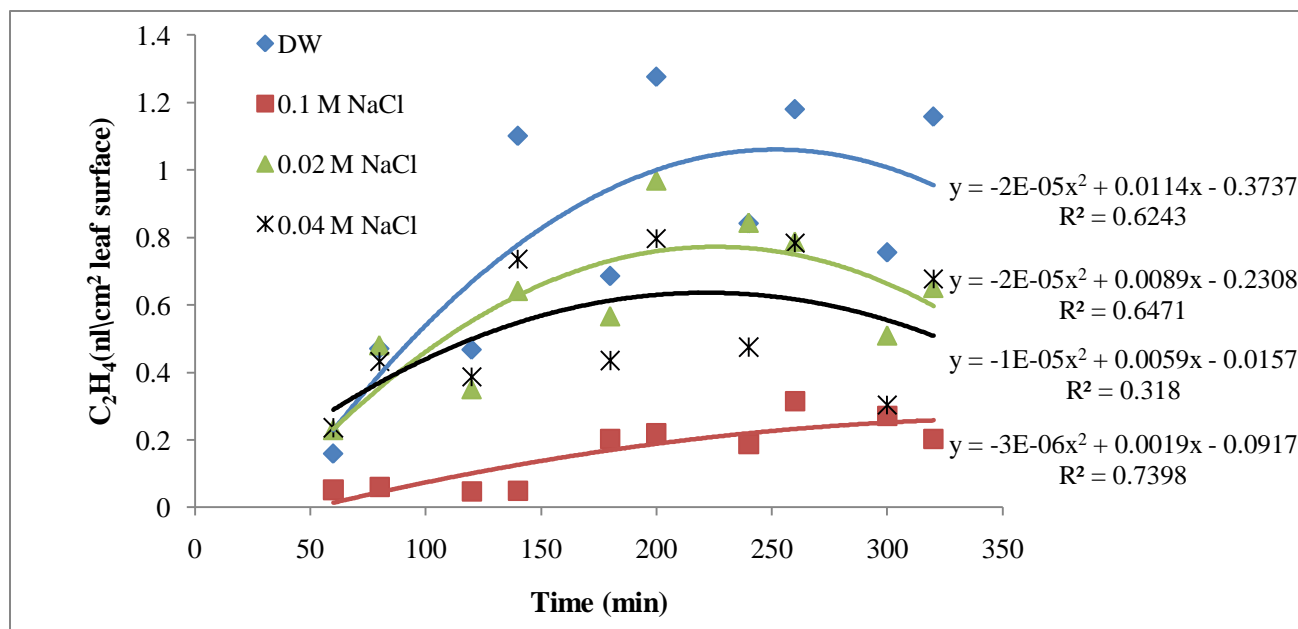


Figure 9. Effect of various NaCl concentrations on ethylene emission from tobacco leaf discs. The disks were incubated in 9.5 ml vials containing 0.5 ml of NaCl solutions as indicated in the legend. Salt solutions were added at the beginning of the experiment, which corresponds to time zero. C₂H₄ emissions from these leaf discs were measured after 1 hour of incubation in various solutions. The lines on the scatter-graph were fitted using Excel polynomial regression of order 2. The equation of each line along with the corresponding squared R (R²), which reflects the strength of the model fit, are presented in the Figure.

Table 7. A summary of the data analysis of C₂H₄ emission (nl/cm²leaf surface) from leaf discs of transgenic tobacco incubated in DW and 0.02, 0.04 and 0.1 M NaBr solution.

t-Test: Two-Sample Assuming Unequal Variances

<i>Ethylene</i>	<i>DW</i>	<i>0.02 M NaBr</i>
Mean	0.830714	0.341429
Variance	0.068884	0.01589
Observations	14	14
Hypothesized Mean Difference	0	
df	19	
t Stat	6.287743	
P(T<=t) two-tail	4.9E-06	
t Critical two-tail	2.093024	

<i>Ethylene</i>	<i>DW</i>	<i>0.04 M NaBr</i>
Mean	0.830714	0.261429
Variance	0.068884	0.001952
Observations	14	14
Hypothesized Mean Difference	0	
df	14	
t Stat	8.003283	
P(T<=t) two-tail	1.36E-06	
t Critical two-tail	2.144787	

<i>Ethylene</i>	<i>DW</i>	<i>0.1 M NaBr</i>
Mean	0.830714	0.472143
Variance	0.068884	0.00668
Observations	14	14
Hypothesized Mean Difference	0	
df	15	
t Stat	4.88071	
P(T<=t) two-tail	0.0002	
t Critical two-tail	2.13145	

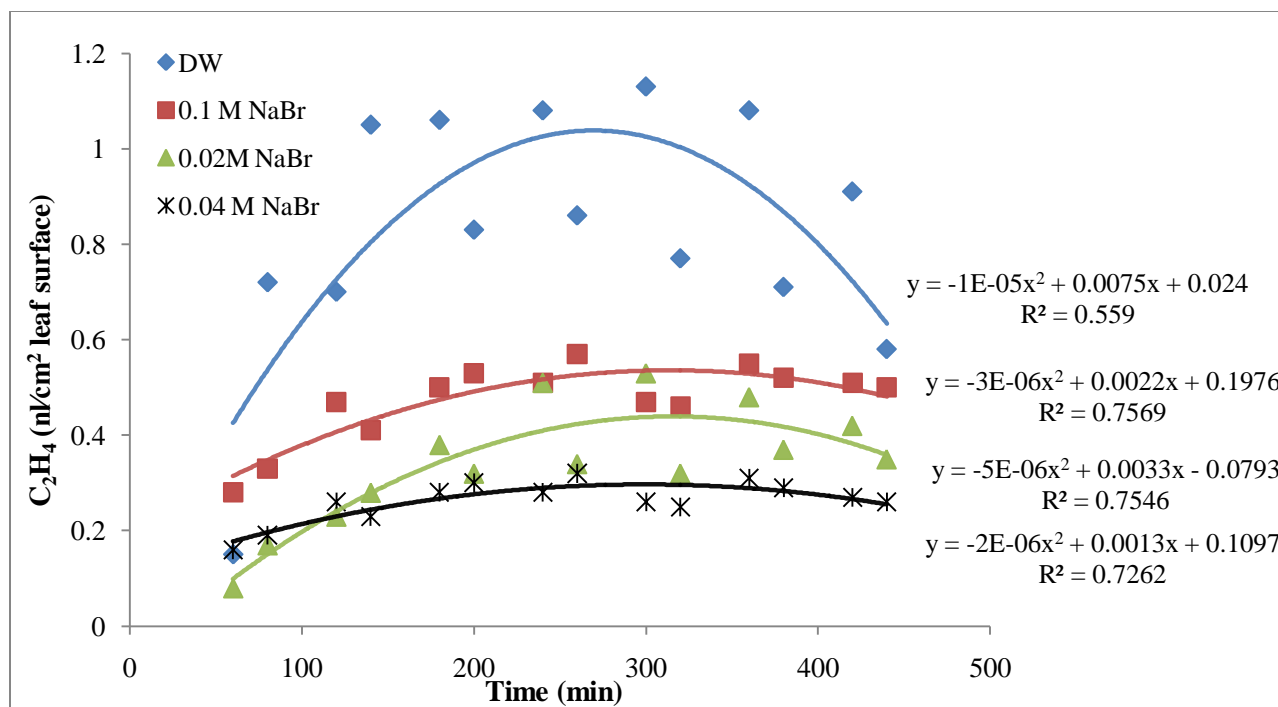


Figure 10. Effect of various NaBr concentrations on ethylene emission from tobacco leaf discs.

The disks were incubated in 9.5 ml vials containing 0.5 ml of NaBr solutions as indicated in the legend. Salt solutions were added at the beginning of the experiment, which corresponds to time zero. C₂H₄ emissions from these leaf discs were measured after 1 hour of incubation in various solutions. The lines on the scatter-graph were fitted using Excel polynomial regression of order 2. The lines on the scatter-graph were fitted using Excel polynomial regression of order 2. The equation of each line along with the corresponding squared R (R²), which reflects the strength of the model fit, are presented in the Figure.

4.1.2 Evaluation of ethylene emission when salt solutions were added after 2 hours from the beginning of incubation:

In response to all salt treatments, ethylene emission was not altered compared to the DW controls (Tables 8, 9 and 10), parallel with the emission of the corresponding methyl halide (Figures 11,12 and 13) at rates matching the relative affinity of TMT for halides: $I^- > Br^- > Cl^-$ (Saini et al., 1995). These results support the hypothesis that the amount of SAM available in the plant is probably sufficient to serve both ethylene synthesis and halide methylation concomitantly. Therefore, introduction of halide methylation capability in a plant is unlikely to affect its ability to produce ethylene at a normal rate.

4.1.3 *In vivo* measurement of ethylene emission from leaves of transgenic tobacco:

Statistical analysis shows no alteration in the *in vivo* ethylene accumulation (Tables 11 and 12) when leaves were dipped in NaCl or NaBr solutions compared to that observed in DW (Figures 14 and 15). The *in vivo* results indicate that TMT insertion is unlikely to alter ethylene synthesis in tobacco plants.

Table 8. A summary of the data analysis of C₂H₄ emission (nl/cm² leaf surface) from leaf discs of transgenic tobacco leaves incubated in DW and 0.1 M NaCl added after 2 hours from the beginning of the experiment (n=3)

t-Test: Two-Sample Assuming Unequal Variances		
	α	0.05
Equal Sample Sizes		
Ethylene	DW	0.1 M NaCl
Mean	0.435	0.369571
Variance	0.025705	0.022132
Observations	7	7
Hypothesized Mean Difference	0	
Df	12	
t Stat	0.791	
P(T<=t) two-tail	0.444	
T Critical Two-tail	2.179	

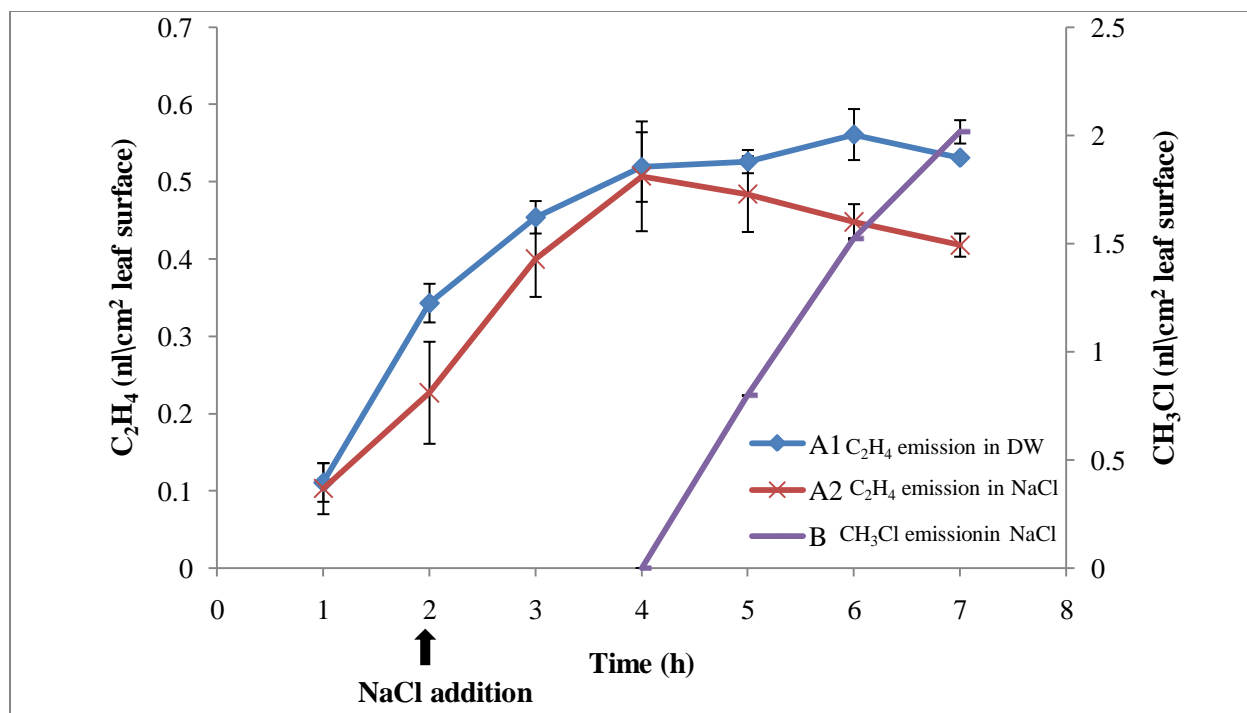


Figure 11. Emission of C₂H₄ and CH₃Cl from tobacco leaf discs incubated in the absence or presence of 0.1 M NaCl. Two sets of matching leaf discs were placed in 9.5 ml vials in DW, and after 2 hours of incubation (indicated by the arrow), 0.5 ml of NaCl solution at a final concentration of 0.1 M was added to one set while the other was maintained in DW. In both treatments, ethylene emission was measured periodically after 1 hour of incubation whereas CH₃Cl measurements were commenced after 3 hour of incubation in NaCl. No CH₃Cl was detected in the presence of DW. Values represent the mean ± SE (n=3).

Table 9. A summary of the data analysis of C₂H₄ emission (nl/cm² leaf surface) from leaf discs of transgenic tobacco leaves incubated in DW and 0.1 M NaBr added after 2 hours from the beginning of the experiment (n=3)

t-Test: Two-Sample Assuming Unequal Variances		α	0.05
Equal Sample Sizes			
Ethylene	<i>DW</i>	<i>0.1 M NaBr</i>	
Mean	0.345286	0.403571	
Variance	0.026583	0.0415	
Observations	7	7	
Hypothesized Mean Difference	0		
Df	11		
t Stat	-0.591		
P(T<=t) two-tail	0.566		
T Critical Two-tail	2.201		

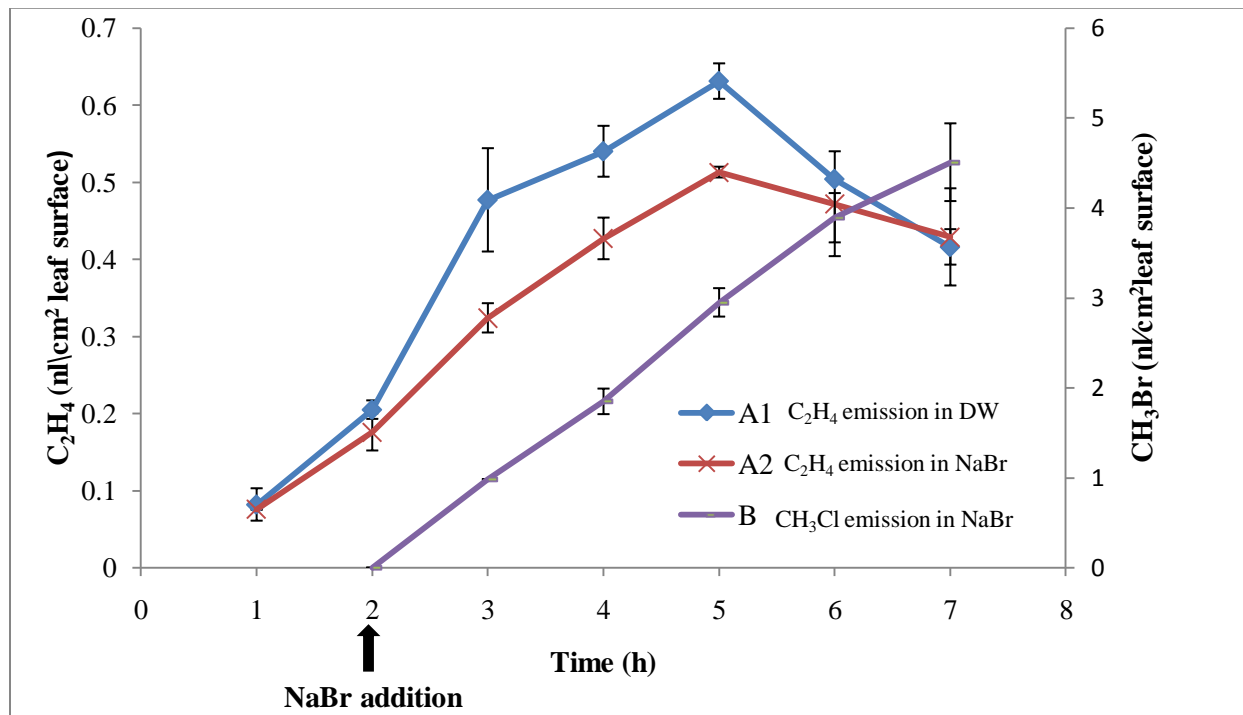


Figure 12. Emission of C₂H₄ and CH₃Br from tobacco leaf discs incubated in the absence or presence of 0.1 M NaBr. Two sets of matching leaf discs were placed in 9.5 ml vials in DW, and after 2 hours of incubation (indicated by the arrow), 0.5 ml of NaBr at a final concentration of 0.1 M was added to one set while the other was maintained in DW. In both treatments, ethylene emission was measured periodically after 1 hour of incubation, whereas CH₃Br measurements were commenced after 1 hour of incubation in NaBr. No CH₃Br was detected in the presence of DW. Values represent the mean ± SE (n=3).

Table 10. A summary of the data analysis of C₂H₄ emission (nl/cm² leaf surface) from leaf discs of transgenic tobacco leaves incubated in DW and 0.1 M KI added after 2 hours from the beginning of the experiment (n=2).

t-Test: Two-Sample Assuming Unequal Variances		α	0.05
Equal Sample Sizes			
Ethylene	<i>DW</i>	<i>0.1 M KI</i>	
Mean	0.671429	0.636714	
Variance	0.16718	0.108841	
Observations		7	7
Hypothesized Mean Difference		0	
Df		11	
t Stat		0.175	
P(T<=t) two-tail		0.864	
T Critical Two-tail		2.201	

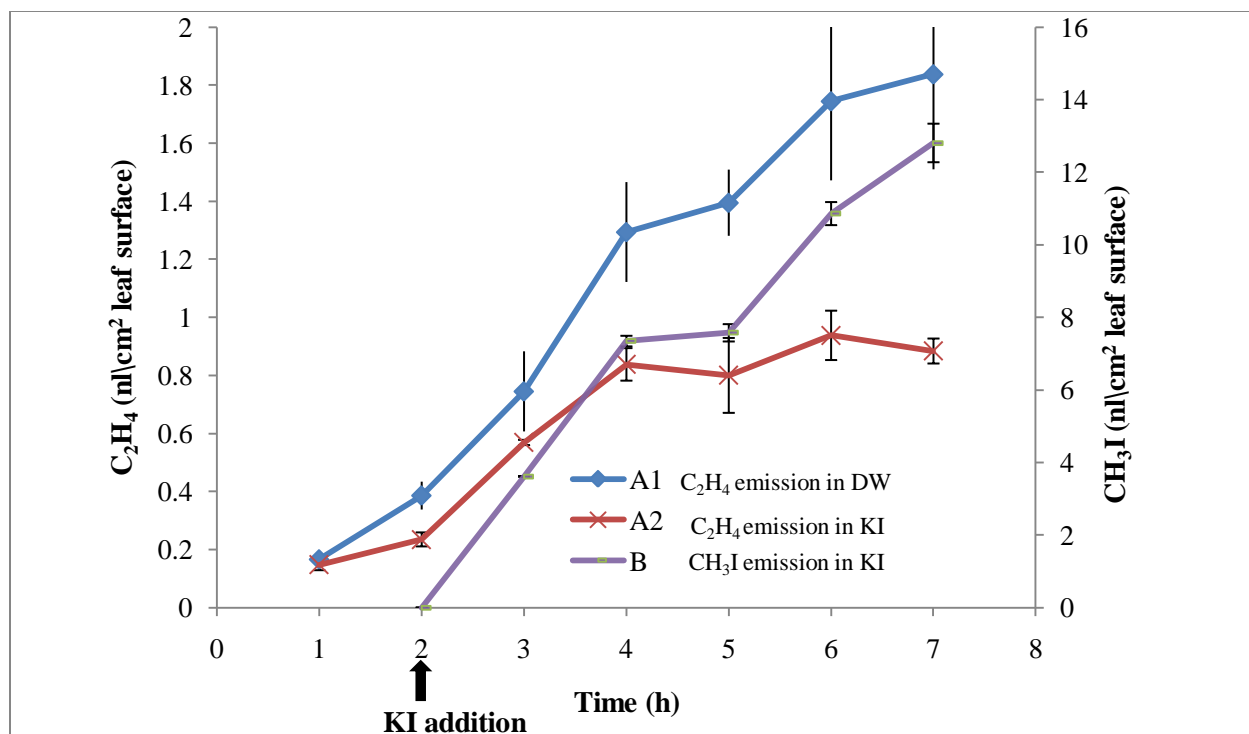


Figure 13. Emission of C₂H₄ and CH₃I from tobacco leaf discs incubated in the absence or presence of 0.1 M KI. Two sets of matching leaf discs were placed in 9.5 ml vials in DW, and after 2 hours of incubation (indicated by the arrow), 0.5 ml of KI at a final concentration of 0.1 M was added to one set while the other was maintained in DW. In both treatments, ethylene emission was measured periodically after 1 hour of incubation whereas CH₃I measurements were detected after 1 hour of incubation in KI. No CH₃I was detected in the presence of DW. Values represent the mean ± SE (n=2).

Table 11. A summary of the data analysis of *in vivo* C₂H₄ emission (nl/cm² leaf surface) from intact leaves of transgenic tobacco treated by DW and 0.1 M NaCl (n=2)

t-Test 1-sample			
Test Mean	0.184		
Confidence Level	0.95		
N	2		
Average	0.2195	Test Stdev	p 1-sample Stdev
Stdev	0.050205	0.0502046	0.635
SE Mean	0.0355		
T	-1.000		
TINV	6.313752		
p - two sided	0.5	Accept Null Hypothesis because p > 0.05 (Means are the same)	

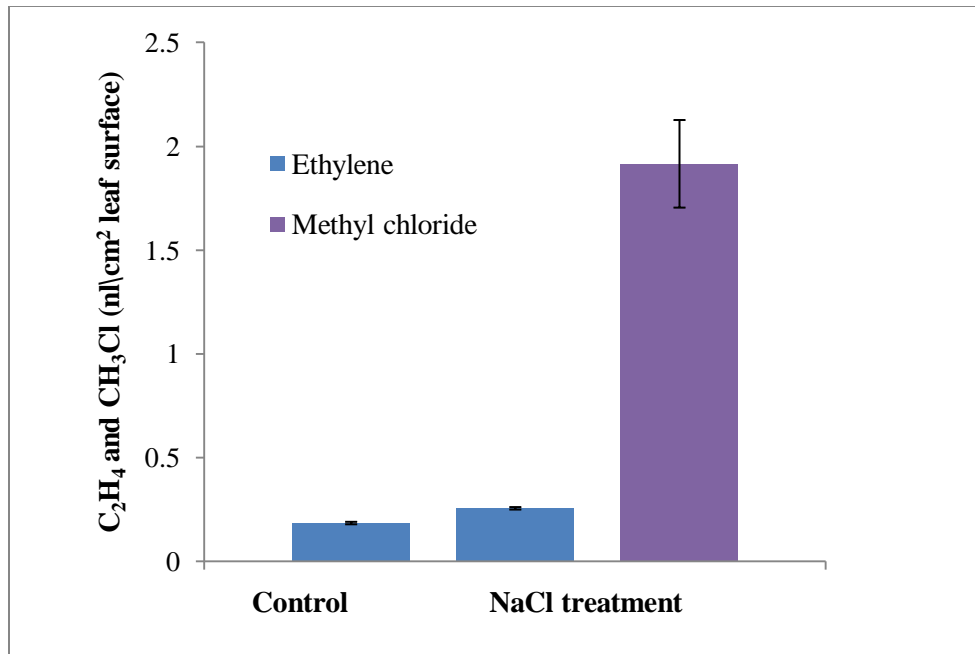


Figure 14. Effect of NaCl treatment of intact tobacco leaves on their ethylene emission. A leaf was dipped for 3 minutes in DW (control) or 0.1 M NaCl solution, enclosed in a Tedler bag (see Materials & Methods), and the emissions of ethylene and methyl chloride were measured *in vivo* after 3 hours. No CH₃Cl emission was detected from the control leaves. The values are the mean ± SE (n=2).

Table 12. A summary of the data analysis of *in vivo* C₂H₄ emission (nl/cm² leaf surface) from intact leaves of transgenic tobacco treated by DW and 0.1 M NaBr (n=2).

t-Test 1-sample			
Test Mean	0.189		
Confidence Level	0.95		
N	2		
Average	0.182	Test Stdev	p 1-sample Stdev
Stdev	0.009899	0.009899	0.635
SE Mean	0.007		
T	1.000		
TINV	6.313752		
p - two sided	0.5	Accept Null Hypothesis because p > 0.05 (Means are the same)	

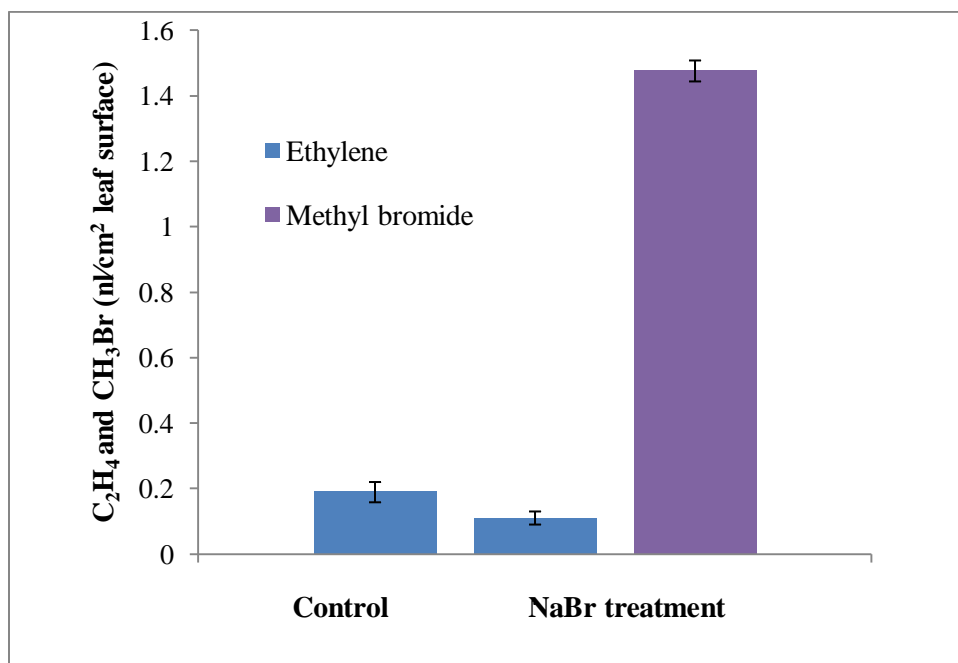


Figure 15. Effect of NaBr treatment of intact tobacco leaves on their ethylene emission. A leaf was dipped for 3 minutes in DW (control) or 0.1 M NaBr solution, enclosed in a Tedler bag (see Materials & Methods), and the emissions of ethylene and methyl bromide were measured *in vivo* after 3 hours. No methyl bromide emission was detected from the control leaves. The values are the mean \pm SE (n=2).

4.2 Estimation of the amounts of SAM required for ethylene and methyl halide emissions:

For all salt treatments, high amounts of SAM – 16, 80.7 and 82.5 pmol/cm² leaf surface/h – were utilized in the presence of NaCl, NaBr and KI respectively to produce the corresponding methyl halide consistent with the increasing order of the rate of methylation of three halides by TMT (Table 13). On the contrary, only small amounts of SAM – 2, 3 and 5 pmol/cm² leaf surface/h – were used for ethylene emission in the presence of NaCl, NaBr and KI respectively (Table 13). The data presented in this table suggest that tobacco plants have a great deal of flexibility to increase the amounts of SAM needed to serve the increase in demand as halide methylation is added to the basal ethylene's biosynthetic pathway.

Table 13. Estimation of the amounts of SAM used for ethylene and methyl halide synthesis.

Leaf discs of transgenic tobacco were incubated in the presence of DW and 0.1 M of NaCl, NaBr and KI. Salt solutions were added after 2 hours of incubation in DW. Amounts of ethylene and methyl halides detected per unit leaf area reflect the amounts of SAM used to produce both of them.

Reaction product	SAM (pmol/cm ² leaf surface/h) under the treatments:					
	DW	NaCl	DW	NaBr	DW	KI
C ₂ H ₄	3.4	2	7.8	3	11.5	5
CH ₃ X	0	16	0	80.7	0	82.5
Total	3.4	18	7.8	83.7	11.5	87.5

Chapter 5

Discussion

Plants, as sessile organisms, are exposed to a variety of environmental stresses which represent a real threat for plant productivity and the world food supply. With the human population increasing at an alarming rate, and projected to reach 9 billion people by 2050 (Steck, 2010), the problem of food insecurity is expected to expand. Thus, the major challenge for modern agriculture would be to maintain crop yields under unfavorable environmental conditions in order to meet the needs of an increasing human population, while competing with urban development for premium arable land.

Facing stresses such as salinity, drought, flooding, atmospheric pollutants, UV light, and phyto-pathogens, plants have developed efficient and complex resistance mechanisms to survive. C_2H_4 , for example, is considered to be a universal stress hormone, as many types of environmental stress and wounding are capable of inducing its synthesis (Yang & Hoffman, 1984). C_2H_4 is involved in plant stress responses by causing abscission, senescence, wound-healing and increased disease resistance (Bleecker & Kende, 2000).

In addition to natural plant defense strategies, it is possible to introduce stress resistance by inserting a specific gene conferring that resistance into high-value sensitive crops. For instance, Kaur (2006) has elegantly demonstrated the utility of *TMT* gene insertion in conferring salt tolerance to tobacco plants. Though *TMT* insertion might appear as a beneficial and effective tool to confer and enhance salt tolerance by converting the toxic Cl^- into CH_3Cl , the emission of this gas into the atmosphere is of concern. Indeed, CH_3Cl is considered to be an ozone-depleting substance, responsible for about 16% of the ozone destruction. Both CH_3Cl and C_2H_4 are of

particular interest in the present study. In addition to being involved in plant stress resistance, both share a common feature of using SAM for their synthesis: as a methyl group donor for

The present study deals with two of the major routes of stress resistance in tobacco plants: the stress hormone, ethylene, and salt tolerance through Cl^- detoxification by *TMT* insertion. The present work is the first experimental report dealing with the impact of *TMT* gene insertion, and hence the ability to methylate halides into their corresponding methyl halide, on SAM availability for ethylene synthesis in engineered tobacco plants.

The results of *in vitro* experiments presented in this study led to two diverging situations: When leaf discs were overnight incubated in the presence of 0.1 M NaBr or incubated in the presence of increasing NaBr concentrations or 0.1 M NaCl – added from the beginning of the experiment– ethylene emission from these leaves was altered. In contrast, exposing leaf discs of transgenic tobacco to 0.1 M NaCl, NaBr or KI–added after two hours from the beginning of the experiment–did not alter ethylene emission. The decrease in ethylene accumulation observed in the first case could be explained by the detachment of the tissue from its natural environment, and hence increased wound-induced production of ethylene combined with the faster exhaustion of SAM and other substrates, by a greater toxic effect of salts on tissues when cut leaf disks are bathing in salt solutions, by an enzymatic denaturation or even by an artifact response. The second set of *in vitro* results support the hypothesis that *TMT* insertion is unlikely to alter ethylene emission in transgenic tobacco. Because of the discrepancy in the *in vitro* results, *in vivo* experiments were conducted in order to find out more reliable results. *In vivo* results obtained in the present study tend to reject the hypothesis of a possible SAM limitation for C_2H_4 synthesis in the additional presence of halide methylation as ethylene emission was not altered. Based on the *in vivo* and part of the *in vitro* results, tobacco is capable of producing good

amounts of SAM in response to the increased demand as CH_3X production pathway is added to the basal C_2H_4 biosynthetic activity. Thus, TMT insertion is unlikely to limit SAM availability for ethylene synthesis. Moffat (2001) stated that even if there is a limitation in SAM, plant cells can respond to this limitation by increasing the activity of SAM synthetase (SAMs), although it is not clear how plant cells can monitor SAM levels. Furthermore, according to Yang (1979), since only a small amount of the SAM pool is used for ACC synthesis, SAM was unlikely to limit ethylene production.

Because of its involvement in various metabolic reactions, SAM should be maintained at a steady level, which is possible through the Yang Cycle. MTA, produced when SAM is converted into ACC, is used for the synthesis of new Met. This recycling, named the Yang Cycle, enables maintenance of Met at a constant level, even when C_2H_4 is rapidly synthesized (Yang & Hoffman, 1984). The Yang Cycle consists of four steps, as shown in Figure 16. Initially, MTA is hydrolyzed into 5'-methylthioribose (MTR), and then into adenine (Ade) by MTA nucleosidase. The second step is a phosphorylation of MTR into MTR1-P by MTR kinase, before being further metabolized to KMB. Finally, KMB is transaminated into Met. The Yang Cycle preserves the CH_3S (methyl thiol group) at the cost of one ATP molecule. Without this recycling, the amount of reduced sulphur present would limit the available Met and thus C_2H_4 synthesis. Thanks to this cycle, C_2H_4 can be formed constantly without demanding an increase in Met pool.

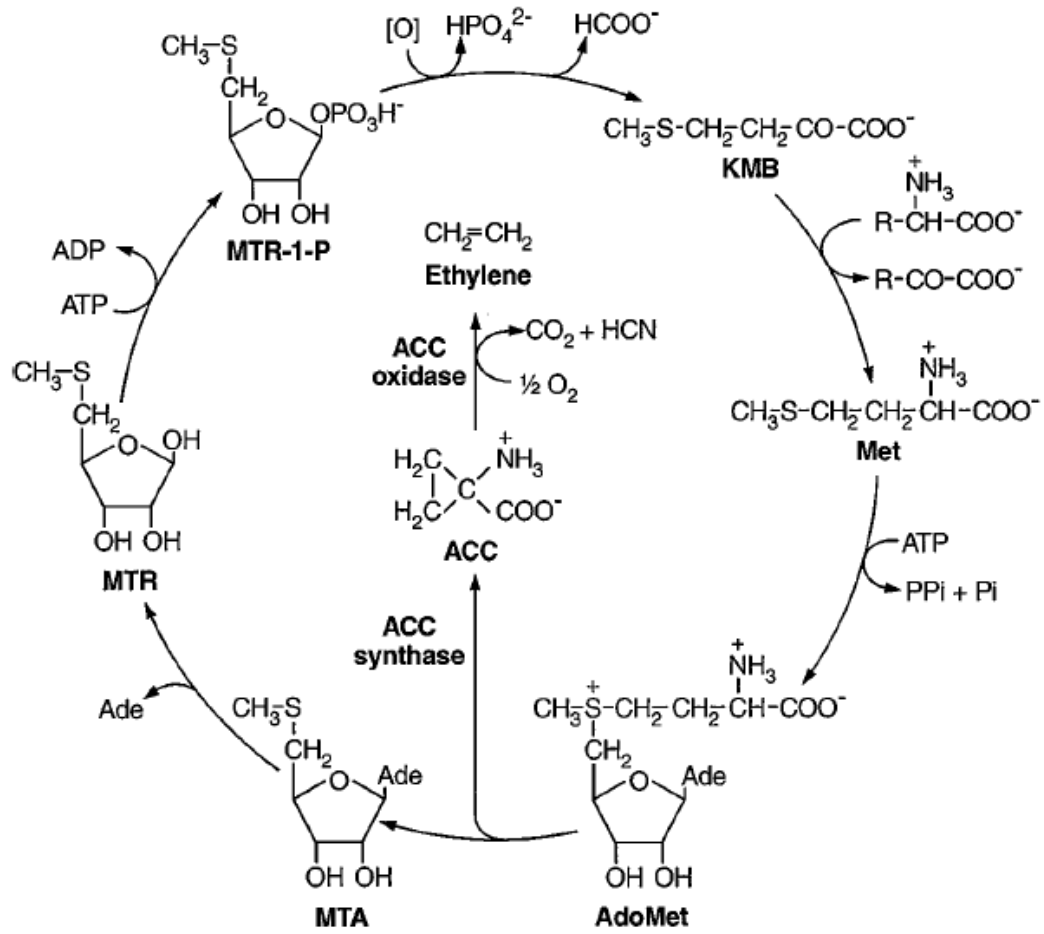


Figure 16. Yang cycle and ethylene biosynthetic pathway (Bleecker & Kende, 2000).

At present, there is a dearth of knowledge regarding concentrations of SAM in plants. Estimating the SAM demand for ethylene and methyl halide synthesis is complicated, as the requirements for SAM vary widely under different growth conditions. For this reason, any measurement is merely a snapshot of a very dynamic situation, that varies temporarily and spatially among cells (Moffat & Weretilnyk, 2001). For instance, it was found that SAM amounts in *Nicotiana suaveolens* flowers fluctuate rhythmically during day and night between 10 to 50 nmol/g fresh weight (Roeder et al., 2009). Furthermore, based on labelling experiments conducted on the aquatic plant *Lemna paucicosta*, Datko (1990) proposed a model for the fluxes of SAM into the different pathways as shown in Figure 17. Met and SAM together in a reaction catalyzed by Methionine S-methyltransferase (MMT) lead to the formation of S-methyl methionine (SMM), known to be a major transport molecule of reduced sulphur in some plants and a storage form of Met. Experiments conducted in *Lemna paucicosta* showed that SMM cycle prevents the depletion of the Met pool when an overshoot in SAM synthesis occurs and acts as a reserve for methyl groups (Mudd & Datko, 1990). This Cycle may function in plants other than *Lemna* and thus, SAM limitation is unlikely to occur or alter ethylene synthesis when another metabolic feature using SAM –halide methylation– is inserted into a plant.

In the present study, it was difficult to estimate the exact amounts of SAM, as well as its turnover rate for both C₂H₄ and CH₃Cl biosynthetic pathways. Furthermore, it was arduous to speculate on the consequences of SAM drainage for both pathways on other metabolic reactions. But generally, in the present study, the plant growth was not affected by the competition for the use of SAM between CH₃X and C₂H₄ synthetic pathways.

The importance of this work resides in the possibility of creating transgenic salt-tolerant plants without compromising their ethylene emission. Salinity, defined as the presence of

excessive amounts of soluble salts inhibiting plant growth, is a major land degradation problem worldwide (Chinnusamy et al., 2005; Sairam & Tyagi, 2004). Salinity costs include a reduction in agricultural productivity and quality, a decline in the quality of water supplies for drinking, irrigation and industrial use, and loss of biodiversity in both terrestrial and aquatic ecosystems. The US Department of Agriculture estimates that, worldwide, 10 million hectares of arable land are lost because of irrigation salinity every year. This type of salinity is mainly caused by an over-irrigation of agricultural land without adequate drainage, and inefficient water use. In this context, transgenic technology that confers plant salt tolerance, combined with traditional breeding, could have potential benefits in improving plant productivity in stressful environments. Although a wide array of traits has been inserted into many species of plants, the issue of transgenic plants is still problematic due to the environmental impacts of this technology (Lovei, 2001). Indeed, there are serious concerns surrounding it. Among the perceived risks of transgenic crops are the likely effects on biodiversity, including soil microorganisms, pollinating insects, and non-target organisms. Furthermore, the risks of putative invasiveness and of the horizontal and vertical transgene flow, are considered potentially significant hazards of transgenic technology (Lovei, 2001). Although transgenic plants may harm the ecosystem components as well as human health, some studies have proven their efficacy in the detoxification of pollutants (Bizily et al., 2000) or in conferring salt resistance (Kaur, 2006).

In relation to the present study, if TMT engineered crops are to be commercialized or brought to saline fields, they might add extra amounts of CH_3Cl to the global atmospheric budget. In this context, through an assumptive study on transgenic rice transformed by *TMT* insertion, Ali (2010) reported that the introduction of transgenic rice crops in saline coastal areas of Asia and Southeast Asia could add an extra 5.36% to the global CH_3Cl input. Currently CH_3Cl is mainly

derived from natural sources, with a small part of this gas produced by anthropogenic sources (less than 10% of the total known sources) (WMO, 2003). The natural key emitters of CH₃Cl include higher plants, tropical and rainforest ecosystems (Gebhardt, 2008; Yokouchi et al., 2007), and rice (Redeker et al., 2004), as well as salt marshes (Rhew et al., 2000). As emissions from natural sources outweigh CH₃Cl sinks (Gebhardt, 2008; WMO, 2003), any additional CH₃Cl emission from transgenic plants would account for higher fluxes of this gas in the atmosphere, contributing to further ozone depletion.

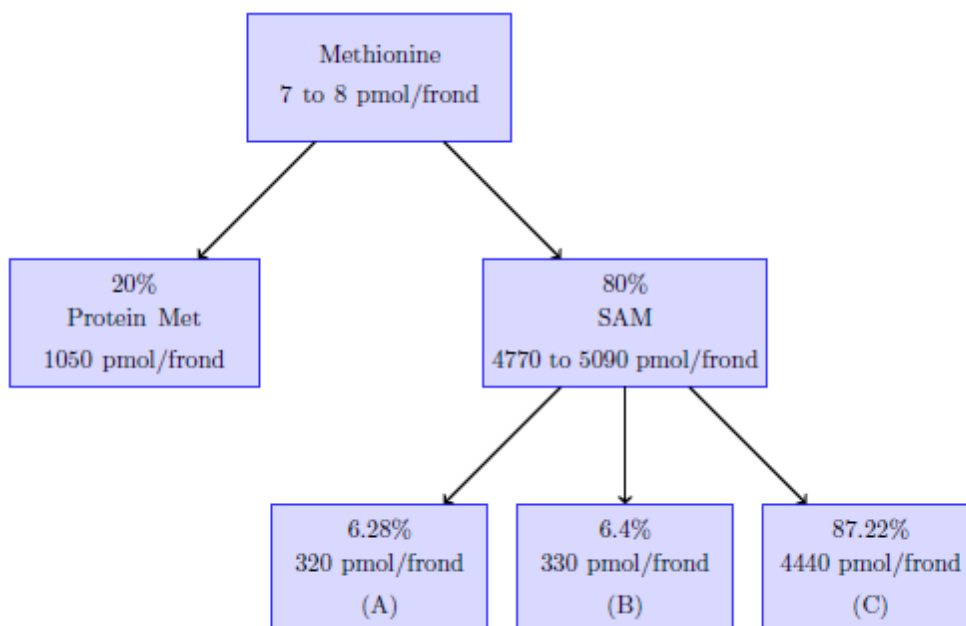


Figure 17. Estimation of the fluxes of SAM serving different metabolic pathways in *Lemna paucicosta* (Mudd & Datko, 1990). A: SMM; B: Spermidine synthesis and other reactions leading to methylthiol recycling; C: Non turning-over methylated products (including CH₃X and C₂H₄).

Conclusion and future prospects:

The present study provides experimental data on the effect of a novel thiol methyltransferase (*TMT*) gene insertion in tobacco plants on SAM availability for their ethylene emission. The potential effect of TMT insertion on ethylene emission was tested through *in vitro* and *in vivo* experiments.

The first section of the study consisted of a series of *in vitro* experiments in which tobacco leaf samples were exposed to various salt solutions. A part of the *in vitro* results showed a decrease in ethylene emission whereas the other part did not reveal any alteration in ethylene emission because of TMT insertion.

The second section of this study dealt with *in vivo* experiments which revealed that ethylene emission was not affected by TMT insertion. Tobacco plants, thus, are likely capable of producing sufficient quantities of SAM to support both ethylene and CH₃X synthesis pathways.

The final part of this study was to predict the amounts of SAM used for methyl halides and ethylene synthesis. Tobacco plants seem to produce good amounts of SAM to serve both pathways though it was not clear whether these amounts reflect the optimal SAM quantities required to drive both reactions at maximal rates.

In general, the fact that *TMT* gene insertion did not alter the *in vivo* C₂H₄ synthesis motivates further cloning of *TMT* gene into other high-value halo-intolerant plants and crops, to improve tolerance against Cl⁻ toxicity. To make good use of this innovative genetic technology, further experimental work and field investigations need to be conducted to assess the impacts of *TMT* gene insertion on other metabolic pathways and physiological traits in plants, and to create a way to mitigate the amounts of methyl halides emitted to the atmosphere because of this insertion.

More remains to be done, such as the development of quantitative metabolic models that faithfully reflect intracellular concentrations and compartmentalization of SAM, which will undoubtedly yield valuable insight into this area of metabolism.

References

- Adams, O. D., & Yang, F. S. (1977). Methionine metabolism in apple tissue: implication of S-Adenosylmethionine as an intermediate in the conversion of methionine to ethylene. *Plant Physiology*, *60*, 892-896.
- Adams, O. D., & Yang, F. S. (1979). Ethylene biosynthesis: identification of 1-aminocyclopropane-1- carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(1), 170-174.
- Ali, A. (2010). *Exploring the Possibility of Transforming Food Crops for Salinity Tolerance Using the TMT Gene Encoding Thiol Methyltransferase Enzyme.*, Master's thesis. University of Waterloo., Waterloo.
- Argueso, C. T., Hansen, M., & Kieber, J. J. (2007). Regulation of ethylene biosynthesis. *Journal of Plant Growth Regulation*, *26*, 92-105.
- Attieh, J., Djiana, R., Koonjul, P., Etienne, C., Sparace, S. A., & Saini, H. S. (2002). Cloning and functional expression of two plant thiol methyltransferases: a new class of enzymes involved in the biosynthesis of sulfur volatiles. *Plant Molecular Biology*, *50*, 511-521.
- Attieh, J., Kleppinger-Sparace, K. F., Nunes, C., Sparace, S. A., & Saini, H. S. (2000). Evidence implicating a novel thiol methyltransferase in the detoxification of glucosinolate hydrolysis products in *Brassica oleracea* L. *Plant, Cell and Environment*, *23*, 165-174.
- Bizily, P. S., Rugh, L. C., & Meagher, B. R. (2000). Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nature Biotechnology*, *18*, 213-217.
- Bleecker, A. B., & Kende, H. (2000). Ethylene: a gaseous signal molecule in plants. *Annual Reviews of Cell and Developmental Biology*, *16*, 1-18.

- Buchanan, B. B., Gruissem, W., & Jones, R. L. (2000). Biosynthesis of hormones and elicitor molecules. In *Biochemistry and Molecular Biology of Plants* (pp. 895-901). Rockville, Maryland: American Society of Plant Physiologists.
- Butler, J. H. (2000). Atmospheric chemistry: better budgets for methyl halides? *Nature*, *403*, 260-261.
- Chinnusamy, V., Jagendorf, A., & Zhu, J. K. (2005). Understanding and improving salt tolerance in plants. *Crop Science Society of America*, *45*, 437-448.
- Collier, P., Conway, G., & Venables, T. (2008). Climate change and Africa. *Oxford Review of Economic Policy*, *24*(2), 337-353.
- Dauncey, G., & Mazza, P. (2001). *Stormy Weather: 101 Solutions to Global Climate Change*. Canada: New Society Publishers.
- Downing, E. T., Ringius, L., Hulme, M., & Waughray, D. (1997). Adapting to climate change in Africa. *Mitigation and Adaptation Strategies for Global Change*, *2*(1), 19-44.
- Even-Chen, Z., Mattoo, A. K., & Goren, R. (1982). Inhibition of ethylene biosynthesis by aminoethoxyvinylglycine and by polyamines shunts label from 3, 4-[¹⁴C] methionine into spermidine in aged orange peel discs. *Plant Physiology*, *69*, 385-388.
- Gebhardt, S. (2008). *Current State of Knowledge on Atmospheric Halocarbons. I. Biogenic Emissions of Halocarbons.*, Phd thesis. University of Gutenberg, Mainz, Germany.
- Gibson, R. B., Hassan, S., Holtz, S., Tansey, J., & Whitelaw, G. (2005). Criteria: Sustainability requirements as the basis for decision making. In *Sustainability Assessment: Criteria, Processes and Applications*. (pp. 98-107): Earthscan.

- Godfray, H. C., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., & Toulmin, C. (2010). Food security: the challenge of feeding 9 billion people. *Science*, 327(5967), 812-818.
- Gribble, W. G. (2004). Amazing organohalogens. *American Scientist*, 92, 342-349.
- Hamilton, J. T. G., McRoberts, W. C., Keppler, F., Kalin, R. M., & Harper, D. B. (2003). Chloride methylation by plant pectin: an efficient environmentally significant process. *Science*, 301, 206-208.
- Harper, D. B. (1993). Biogenesis and metabolic role of halomethanes in fungi and plants. In H. Sigel & A. Sigel (Eds.), *Metal Ions in Biological Systems* (Vol. 29, pp. 345-385): Marcel Dekker.
- Harper, D. B. (1994). Biosynthesis of halogenated methanes. *Biochemical Society Transactions*, 22(4), 1007-1011.
- Howden, S. M., Soussana, J. F., Tubiello, F. N., Chhetri, N., Dunlop, M., & Meinke, H. (2007). Adapting agriculture to climate change. *Proceedings of the National Academy of Sciences of the United States of America*, 104(50), 19691-19696.
- ILO. (2008). International Chemical Safety Card (ICSC). Available online at:
http://www.ilo.org/legacy/english/protection/safework/cis/products/icsc/dtasht/_icsc04/icsc0419.htm
http://www.ilo.org/legacy/english/protection/safework/cis/products/icsc/dtasht/_icsc01/icsc0109.htm
http://www.ilo.org/legacy/english/protection/safework/cis/products/icsc/dtasht/_icsc05/icsc0509.htm

- IPCC. (2007). *Climate change 2007: Synthesis Report*. Contributions of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel of Climate change. Cambridge University Press, Cambridge, United Kingdom.
- Itoh, N., Toda, H., Matsuda, M., Negishi, T., Taniguchi, T., & Ohsawa, N. (2009). Involvement of S-adenosylmethionine-dependent halide/thiol methyltransferase (HTMT) in methyl halide emissions from agricultural plants: isolation and characterisation of an HTMT-coding gene from *Raphanus sativus* (daikon radish). *BMC Plant Biology*, 9(116).
- Kaur, S. (2006). *Effect of Genetically Engineering Chloride Methylation into Bacteria and Plants on Their Salinity Tolerance*. Master's thesis. University of Montreal, Montreal.
- Kende, H. (1989). Enzymes of ethylene biosynthesis. *Plant Physiology*, 91, 1-4.
- Lacis, A. A., Schmidt, A. G., Rind, D., & Ruedy, A. R. (2010). Atmospheric CO₂: principal control knob governing earth's temperature. *Science*, 330, 356-359.
- Lau, O. L., & Yang, S. F. (1976). Inhibition of ethylene production by cobaltous ion. *Plant Physiology*, 58, 114-117.
- Lovei, L. G. (2001). Ecological risks and benefits of transgenic plants. *New Zealand Plant Protection*, 54, 93-100.
- Manley, S. L. (2002). Phyto-genesis of halomethanes: a product of selection or a metabolic accident? *Biogeochemistry*, 60, 163-180.
- Moffat, B. A., & Weretilnyk, E. A. (2001). Sustaining S-adenosyl-L-methionine-dependent methyltransferase activity in plant cells. *Physiologia Plantarum*, 113, 435-442.
- Morgan, P. W., & Drew, M. C. (1997). Ethylene and plant responses to stress. *Physiologia Plantarum*, 100, 620-630.

- Mudd, H. S., & Datko, H. A. (1990). The S-Methylmethionine cycle in *Lemna paucicosta*. *Plant Physiology*, 93, 623-630.
- Nagahama, K., Ogawa, T., Fujii, T., & Fuduka, H. (1992). Classification of ethylene-producing bacteria in terms of biosynthetic pathways to ethylene. *Journal of Fermentation and Bioengineering*, 73(1), 1-5.
- Nagatoshi, Y., & Nakamura, T. (2007). Characterization of three halide methyltransferases in *Arabidopsis thaliana*. *Plant Biotechnology*, 24, 503-506.
- Nagatoshi, Y., & Nakamura, T. (2009). Arabidopsis Harmless to Ozone Layer protein methylates a glucosinolate-breakdown product and functions in resistance to *Pseudomonas syringae* pv. *maculicola*. *Journal of Biological Chemistry*, 284(29), 19301-19309.
- NASA. (2010). Ozone hole watch. Available online at: <http://ozonewatch.gsfc.nasa.gov/>
- Patz, J. A., & Kovats, R. S. (2002). Hotspots in climate change and human health. *British Medical Journal*, 325, 1094-1098.
- Ramussen, R. A., Ramussen, L. E., Khalil, M. A. K., & Dalluge, R. W. (1980). Concentration distribution of methyl chloride in the atmosphere. *Journal of Geophysical Research*, 85, 7350-7356.
- Redeker, K. R., Manley, S. L., Walser, M., & Cicerone, R. J. (2004). Physiological and biochemical controls over methyl halides emissions from rice plants. *Global Biogeochemical Cycles*, 18(1007), 1-14.
- Rhew, R. C., Miller, B. R., & Weiss, R. F. (2000). Natural methyl bromide and methyl chloride emissions from coastal salt marshes. *Nature*, 403, 292-295.
- Roeder, S., Dreschler, K., Wirtz, M., Cristescu, S. M., VanHarren, F. J. M., Hell, R., & Piechulla, B. (2009). SAM levels, gene expression of SAM synthetase, methionine

- synthase and ACC oxidase, and ethylene emission from *N.suaveolens* flowers. *Plant Molecular Biology*, 70, 535-546.
- Roje, S. (2006). S-Adenosyl-L-methionine: Beyond the universal methyl group donor. *Phytochemistry*, 67, 1686-1698.
- Saini, H., Attieh, J., & Hanson, A. D. (1995). Biosynthesis of halomethanes and methanethiol by higher plants via a novel methyltransferase reaction. *Plant, Cell and Environment*, 18, 1027-1033.
- Sairam, R. K., & Tyagi, A. (2004). Physiology and molecular biology of salinity stress tolerance in plants. *Current Science*, 86(3), 407-420.
- Salisbury, F. B., & Ross, C. W. (1992). Hormones and growth regulators: Cytokinins, ethylene, abscissic acid and other compounds. In *Plant Physiology* (fourth ed., pp. 393-400). Belmont, California: Wadsworth Publishing Company.
- Saxena, D., Aouad, S., Attieh, J., & Saini, H. (1998). Biochemical characterization of chloromethane emission from the Wood-Rooting fungus *Phellinus pomaceus*. *Applied and Environmental Microbiology*, 64(8), 2831-2835.
- Schmidhuber, J., & Tubiello, F. N. (2007). Global food security under climate change. *Proceedings of the National Academy of Sciences of the United States of America*, 104(50), 19703-19708.
- Steck, T. L. (2010). Human population explosion. *The Encyclopedia of Earth*.
- Taiz, L., & Zeiger, E. (2006). Ethylene: The gaseous hormone. In *Plant Physiology* (Fourth ed., pp. 571-591). Sunderland, Massachusetts: Sinauer Associates, Inc.
- Walting, R., & Harper, D. B. (1998). Chloromethane production by wood-rotting fungi and an estimate of the global flux to the atmosphere. *Mycological Research*, 102, 769-787.

- Wang, K. L. C., Li, H., & Ecker, J. R. (2002). Ethylene biosynthesis and signaling networks. *The Plant Cell*, 14, 131-151.
- WHO. (2003). Stratospheric ozone depletion, ultraviolet radiation and health. In *Climate Change and Human Health- Risks and Responses. Summary*. (pp. 21-22). Geneva, Switzerland: Marketing and dissemination, World Health Organization.
- WMO. (2003). *Scientific Assessment of Ozone Depletion: 2002, Chapter 1: Controlled Substances and Other Source Gases*, Global Ozone Research and Monitoring Project- Report No 47, World Meteorological organization, Geneva, Switzerland, 1.1-1.43.
- WMO. (2009a). GAW research on stratospheric ozone. Available online at: <http://www.wmo.int/pages/prog/arep/gaw/ozone/>
- WMO. (2009b). WMO greenhouse gas bulletin.
- WMO, & UNEP. (2010). Scientific Assessment of Ozone Depletion: 2010. 1-33.
- Wreford, A., Moran, D., & Adger, N. (2010). *Climate Change and Agriculture: Impacts, Adaptation and Mitigation*.
- Wuosmaa, A. M., & Hager, L. P. (1990). Methyl chloride transferase: a carbocation route for biosynthesis of halometabolites. *Science*, 249, 160-162.
- Yang, F. S. (1974). The biochemistry of ethylene: biogenesis and metabolism. *Recent Advances in Phytochemistry*, 7, 131-164.
- Yang, F. S., & Adams, O. D. (1979). Ethylene biosynthesis: identification of -1-aminocyclopropane-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Sciences of the United States of America*, 76(1), 170-174.
- Yang, F. S., & Hoffman, E. N. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology*, 35, 155-189.

Yokouchi, Y., Noijiri, Y., Barrie, L. A., Toom-Sauntry, D., Machida, T., Inuzuka, Y., Akimoto, H., Li, H. J., Fujinuma, Y., & Aoki, S. (2000). A strong source of methyl chloride to the atmosphere from tropical coastal land. *Nature*, *403*, 295-298.

Yokouchi, Y., Saito, T., Ishigaki, C., & Aramoto, M. (2007). Identification of methyl chloride-emitting plants and atmospheric measurements on a subtropical island. *Chemosphere*, *69*, 549-553.