Exploring the possibility of transforming food crops for salinity tolerance using the TMT gene encoding thiol methyltransferase enzyme

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

Soil salinity is a serious environmental stress threatening productivity of major crops worldwide. Among the various biotic and abiotic strategies that exist, transgenic technologies provide a promising avenue to reduce yield losses in crops under saline environments. Recently, transgenic technology involving the *TMT* gene encoding thiol methyltransferase enzyme has been suggested as an effective solution for engineering a chloride detoxification capability into a high value crops to improve tolerance against chloride ion toxicity under saline environments. This proposed mechanism, however, results in the emission of methyl chloride (CH_3Cl) from plants, which has deleterious effects on stratospheric ozone. This study was performed to examine the relationship between salt tolerance and chloride volatilizing capacity of transgenic plants containing *TMT* gene as well as to explore the possibility of generating transgenic rice crop containing *TMT* gene for salinity tolerance. To achieve these objectives, transgenic tobacco plants containing TMT gene were grown in comparison with wild type tobacco plants under three levels of sodium chloride (NaCl) salinity (0, 100 and 200 mM), three levels of soil water content (40%, 60% and 80% of the field capacity) and their tolerance to NaCl and water stress was studied. Plant growth parameters recorded included plant height, number of leaves, leaf area, stem dry weight, leaf dry weight, root dry weight, plant dry biomass and root/shoot ratio. Similarly, both types of plants were exposed to five levels of NaCl concentrations (0, 50, 100, 150 and 200 mM) and three levels of soil water content (40%, 60% and 80% of the field capacity), and the quantity of CH_3Cl

emitted was recorded. Significant decrease in plants growth parameters of both types of plants were recorded upon exposure to salinity and water stress. Under 100 mM NaCl, however, transgenic plants showed better tolerance to salinity by suffering less reduction in growth parameters compared to wild type plants. Under 200 mM NaCl, growth of both types of plants was completely inhibited. The interactive effects of salinity and water stress were more pronounced in wild type plants than in transgenic plants. Results also showed that all engineered plants acquired an ability to efficiently transform chloride ion to CH_3Cl, and the rate of such transformation was higher under greater NaCl and soil water content compared to lower NaCl concentrations and soil water content. In order to explore the possibility of generating a transgenic food crop using TMT gene, a hypothetical transgenic rice crop was grown over 27 million hectares of the saline coastal areas of south and southeast Asia and the possible emission of CH_3Cl from such ecosystem was inferred based on the CH_3Cl emission data obtained from transgenic tobacco plants. The estimates showed that the possible CH_3Cl emission from such ecosystem would be 219.21 Gg which is equivalent to 5.36 % of the global atmospheric emissions of CH_3Cl.

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Dedication

This thesis is dedicated to my parent for their endless love, support and encouragement.

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Chapter 1

General Introduction

1.1 Soil salinity

Loss of arable land via salinization is a major factor undermining the productivity of modern agricultural systems (Galvani, 2007). Salinization of agricultural soils occurs primarily due to agricultural practices, including poor water management, high evaporation, heavy irrigation and previous exposure to sea water (Pitman & Lauchli, 2002). Currently, approximately 6% of the world's land area, which is equivalent to 800 million hectares, is affected either by salinity or sodicity (FAO, 2008). In addition, salinity affects 20% of the world's irrigated land, which accounts for one-third of the world food production (Chinnusamy et al., 2005; FAO, 2008). It has been estimated that salinity is affecting 3 hectares of additional arable land each minute worldwide (FAO, 2008). This constant salinization of arable land is expected to have overwhelming global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang et al., 2003). This progressive loss of arable land has potentially serious consequences for the expanding global population, which is steadily increasing towards seven billion, and set to increase by a further 50% by 2050 (FAO, 2009a). Recent changes in global climate are likely to further exacerbate the problem of soil salinity. Variation in important climate variables including temperature and precipitation are expected to decrease water for irrigation and impose high evapotranspiration losses (Yeo, 1999). The resulting drier conditions will further raise irrigation demands which are often

met with poor quality of water containing dissolved salts. These conditions will be more critical for arid and semi arid regions which are already at limit with respect to water availability (Chartzoulakis & Psarras, 2005; Sivakumar et al., 2005). The decrease in good quality of water in these areas will accelerate the use of saline water for irrigation which will raise salt accumulation in soils, and increase the extent of secondary salinization (Yeo, 1999).

1.2 Raising crop productivity under salinity

Numerous physical and chemical approaches exist for improving agricultural productivity in saline environments (Rains & Goyal, 2003). These include drainage and leaching of excess salt from the root zone, chemical amelioration of soils, and crop-based management practices (Goyal et al., 1999). However, apart from being extremely costly and time-consuming, these techniques are non-applicable at many instances due to the unavailability of improved irrigation and drainage systems (Sharma & Manchanda, 1996). Alternatively, researchers have been working towards developing salt-tolerant crop varieties using selective breeding techniques over the past century; however, none of those efforts has proven successful (Ashraf, 2010; Yamaguchi & Blumwald, 2005). During the last decade, developing salt tolerant plants through modern biotechnology has been accorded very high research priority in plant biotechnology research and development. Recently, transgenic technology has been perceived as a viable option for generating plants with innate ability to tolerate different level of salts (Wang et al., 2003). Numerous developments have been reported in generating plants using transgenic

technology where overexpressing a single gene has conferred high salinity tolerance in plants (Wang et al., 2004; Zhang & Blumwald, 2001; Zhang et al., 2001).

Research has shown that certain organisms including marine alga *Endocladia muricata*, wood-rotting fungus *Phellinus pomaceus* and a halophytic plant *Mesembryanthemum crystallinum* possess a methyl chloride transferase (MCT) enzyme that can methylate Cl^- to CH_3Cl (Wuosmaa & Hager, 1990). Because two of these species survive under saline conditions, this enzyme has been suggested as a possible mechanism for Cl^- detoxification through its volatilization as CH_3Cl. To experimentally verify this theoretical probability, Saini et al. (1995) undertook a survey of 118 herbaceous species, using a simple method where leaf discs from these plants were floated on potassium iodide solution and the resultant methyl iodide (CH_3I) production was measured. From this study, they revealed that the majority of plants were able to methylate iodide ion (I^-) to CH_3I, which was considered to be an indication of methyltransferase activity. To further investigate the biochemical basis for these emissions, Attieh et al. (1995) isolated and characterized the enzyme S-adenosyl-L-methionine: halide/bisulphide methyltransferase from *Brassica oleracea*, which is capable of methylating halides ions into methyl halides. Attieh et al (2000) subsequently performed kinetic studies on this enzyme and found that the purified enzyme preferred thiol ions (thiocynate 4,4–thiobisbenzenethiol, thiophenol, and salicylic acid) as substrates. They named this enzyme as thiol methyltransferase (TMT).

Prompted by these findings, a cabbage gene encoding the TMT enzyme was isolated (Attieh et al., 2002) and engineered into tobacco plants that otherwise lack the enzyme (Kaur, 2006). Detailed physiological studies on these TMT containing transgenic plants revealed that the engineered plants acquired an ability to efficiently transform Cl^- to CH_3Cl. Further, these transgenic plants developed a high degree of tolerance to NaCl salinity as compared to their wild type counterparts (Kaur, 2006). Based on the result of this study, the author suggested the enzymatic conversion of Cl^- to CH_3Cl as a possible way of engineering Cl^- detoxification capability into high value crops to improve their tolerance against salinity. Though the TMT gene incorporation into tobacco plants showed substantial increase in salinity tolerance, initial studies have shown that these transgenic plants emitted considerable amounts of CH_3Cl in the presence of NaCl (Kaur, 2006). Emission of CH_3Cl is of great environmental concern since CH_3Cl originating from natural sources show high ozone depletion potential (Harper, 2000; Schafer et al., 2007).

1.3 Methyl halides and ozone depletion

Ozone layer, which is found between 15 to 35 km above the Earth's surface, is crucial to life on the Earth, as ozone absorbs potentially harmful ultraviolet-B (UV-B) radiation from the sun (Gebhardt, 2008), which otherwise, upon reaching the earth surface, would cause serious damage to human and animal health, terrestrial biosphere, aquatic ecosystems, biochemical cycles, and other materials (UNEP, 2006) . The chlorine and bromine containing compounds that are actively involved in ozone depletion include

chlorofluorocarbons (CFCs), carbon tetra chloride (CCl_4), methyl chloroform (CH_3CCl_3), hydrochlorofluorocarbons (HCFCs), hydrobromofluorocarbons (HBFCs), and halons (WMO, 2007). All these compounds primarily originate from anthropogenic sources (McConnell & Jin, 2008). In addition, ozone layer is also depleted by CH_3Cl and CH_3Br (methyl bromide) which originate from natural sources such oceans, fungi and plants (Harper, 2000). Due to the high global warming potential (GWP) of these monohalomethanes, their presence even in low concentrations may have considerable impact on atmospheric warming when compared to other greenhouse gases with low GWP (WMO, 2007). Because these halocarbons are extremely stable, they are not degraded in the troposphere. In the stratosphere, however, they are chemically separated, and release chlorine and bromine, which deplete the ozone layer (Velders et al., 2000).

1.4 Research questions

The goal of this research was to look into the prospects of deploying TMT gene for salinity tolerance on commercial scale in an environmentally friendly manner. Specific objectives of the study were as follows:

1. To determine the differences in growth between transgenic and wild tobacco plants exposed to different NaCl concentrations and soil water content.
2. To estimate the quantity of CH_3Cl produced by transgenic and wild type tobacco plants as influenced by the different NaCl concentrations and soil water content.

Chapter 2

Review of Literature

2.1 Environmental Constraints to global food production

With the global human population steadily increasing towards seven billion, and set to increase by a further 50% by 2050 (UN, 2008), and with 800 people lacking access to adequate food (Pretty et al., 2002), the global need for food production has never been greater. Further, the increase in demand for food is likely to occur in the developing countries where 90% of the population growth is expected to take place, and where food insecurity is already a major issue (Gidamis & Chove, 2009). In addition, several environmental problems such as climate change, ozone depletion, drought, desertification, flooding, soil salinity, and soil erosion further endanger our capacity to meet food demands (Godfray et al., 2010). Specifically, changes in global climate due to increasing greenhouse gases are likely to aggravate current problems of global agricultural system and may erode all global efforts to achieve food sufficiency (Aggarwal & Singh, 2010). Climate change is likely to bring changes in global temperature and amount and pattern of precipitation (IPCC, 2007) . Agriculture, being intimately tied to nature, is likely to face severe losses due to these predicted changes in important climate variables as well as their associated impacts on water availability and increase in weeds and pests proliferation (Stern, 2007). Particularly, changes in the duration and pattern of rainfall will result in shortage of water in rain-fed regions, and

will also indirectly reduce storage of water, thereby limiting water availability for irrigation (IWMI, 2007). Furthermore, changes in these key parameters of global climate are likely to deteriorate soil quality by influencing soil water content, runoff, erosion, soil temperature, salinization, and soil biodiversity leading to an adverse effect on crop production (Aggarwal & Singh, 2010). Also, climatic change is expected to accelerate the problem of ozone depletion allowing the penetration of UV-B radiation reaching the Earth surface leading to serious consequences for crop productivity (Caldwell et al., 2007).

The prevailing fewer natural resources particularly land and water and their deterioration due to intensive agriculture is also a serious constraint to meeting global food needs (Khan & Hanjra, 2009). In order to feed the increasing population from existing natural resources, significant advances are required in the field of agriculture production which is possible either by bringing more area under cultivation or increasing productivity from land already under cultivation. Recruiting new arable land under agricultural system is unlikely because of the limited amount of land suitable for agriculture (Kendall & Pimentel, 1994). This option is further offset by the urbanization, soil degradation, and depletion of water supplies (Khan & Hanjra, 2009). Fresh water supplies, essential for modern high-input agriculture, are dwindling because of the increased human and agricultural use, and are being polluted by agricultural run-off, and widespread use of agrochemicals (Khan & Hanjra, 2009). This clearly demonstrates that increasing arable land is a less effective measure than increasing crop productivity from

the existing land, and that any future increase in crop production will have to occur through increase in production per unit area. Increasing agricultural productivity from the existing arable land in an environmentally friendly manner is, however, a big challenge for the global agricultural system (Robertson & Swinton, 2005). A possible way forward is to increase efficiency and sustainability of current crop production practices along with incorporating modern agricultural biotechnology (McMichael, 2001), and to take abrupt actions to preserve the natural resources in the form of soil and water (Khan & Hanjra, 2009). More specifically, increased efforts are needed to raise crop productivity from salt affected land and water by combining crop production and management practices and genetic improvement that are environmentally sustainable and socially acceptable (Galvani, 2007; Pitman & Lauchli, 2002).

2.2 The issue of soil salinity

Soil salinity is a major environmental issue threatening agricultural productivity worldwide (Wang et al., 2003). It has been estimated that soil salinity, along with other abiotic stresses, is responsible for more than 50 percent crop production losses in major field crops (Mahajan & Tuteja, 2005). Saline soil is one with an electrical conductivity of the saturation extract (ECe) of 4 ds m^{-1} (equivalent to 40 mM) or more, and soils with ECe's exceeding 15 dS m^{-1} are considered strongly saline (FAO, 1997). The three common cations associated with salinity include Na^{+}, Ca^{2+}, Mg^{2+}; whereas the common anions include Cl^{-}, SO_4^{-2}, and HCO_3^{-}. However, the most damaging ions are Na^{+} and Cl^{-}

because excessive Na^+ causes deterioration of the soil structure, and both Na^+ and Cl^- can be toxic to plants (Dudley, 1994; Hasegawa et al., 2000).

Salinization of soils develop due to two sources; primary and secondary salinization. Primary salinization occurs due to natural processes including weathering of minerals and soils derived from saline parent rocks whereas secondary salinization results from improper agricultural management practices including poor water management, high evaporation, heavy irrigation and previous exposure to sea water (Galvani, 2007; Pitman & Lauchli, 2002). Of these two types of soil salinity, secondary salinization of arable land is a sources of major concern because it has adversely affected approximately one third of the world's agriculturally productive land (FAO, 2008). Such increased salinization of productive land works against the needs of expanding global population, which is projected to reach 9 billion by the year 2050 (FAO, 2009a), and is expected to require an increase in food production of 20% in developed countries and 60% in developing countries over the next 30 years (Galvani, 2007).

2.3 Effect of salinity on plant growth

Salinity adversely affects important physiological and biochemical processes in plants ultimately leading to reduction in plant growth and development (Munns, 2002; Tester & Davenport, 2003). These adverse effects are induced by either restricting the flow of water and nutrients into the plants or by direct injury to plant cells through the accumulation of toxic ions (Apse & Blumwald, 2002). Plant response to salinity stress

occurs in two phases: an initial and rapid response to the elevation in external osmotic pressure and a slower response due to the buildup of Na^+ inside the plant cells (Munns et al., 2006). Osmotic effect, which develops due to increasing salt concentration in the root medium, is a primary contributor in growth reduction in the initial stages of plant growth (Munns & Tester, 2008). This stage can be characterized by reduction in generation of new leaves, leaf expansion, development of lateral buds leading to fewer braches or lateral shoots formation in plants (Munns & Tester, 2008). When salt concentrations in the soil medium increase, the osmotic potential of the medium decreases, restricting the flow of water and nutrients through the root membrane leading to overall reduction in plants growth and development (Volkamar et al., 1998). Other effects of osmotic stress include inhibition of root growth, decrease in stomatal conductance leading to reduction in the rate of photosynthesis (Munns, 1993; Munns et al., 2002).

The ion toxicity occurs when certain ionic species from irrigation water make their way into the plant, altering K^+/Na^+ ratios, and increasing Na^+ and Cl^- ion concentrations to those that are detrimental to plants because of their negative effects on important processes in plants, including enzymatic activity, protein metabolism, and balance of plant growth regulators (Munns et al., 2002; Tester & Davenport, 2003). When salt concentration increases inside the plant, the salt starts to accumulate inside the older leaves and eventually they die (Munns, 2002). If these older leaves die at a rate greater than that at which new leaves generate, it reduces the capacity of plants to supply

the carbohydrate requirements of younger leaves leading to reduction in their growth rate (Munns et al., 2006). This phase may be recognized by the appearance of some specific symptoms of plant damage in the leaves such as color change, tip burn, marginal necrosis and succulence (Munns & Tester, 2008). The shortening of life time of individual leaves results in growth and yield reductions in plants which ultimately leads to reduction in overall crop productivity (Munns et al., 2002). Osmotic stress is more injurious to plants than ionic stress, except in plant species which lack the ability to store Na^+ in the cell vacuole or in situations when salt concentrations in very high in the growth medium (Munns & Tester, 2008; Tester & Davenport, 2003). In addition, salinity brings about several nutritional disorder in plants by affecting nutrient availability, their transport, and partitioning within plants (Grattan & Grieve, 1999).

Several authors have reported the negative effect of NaCl on plant growth and development. For instance, a significant growth reduction has been reported in Amaranth (Omami, 2005), tomato (Mohammad et al., 1998), and sugarbeet (Ghoulam et al., 2002). Similarly, Liu et al (2008) reported significant reduction in the dry biomass of halophyte *Suaeda salsa* when exposed to different concentration of NaCl under different water regimes. The different water regimes markedly influence the plant response to salinity, with the highest growth being recorded at 85% field capacity under higher salinity concentrations. Likewise, Sifola and Postiglion (2002) reported significant reduction in the plant height and leaf yield of tobacco plants under elevated salt concentrations

whereas these plants showed better response to higher concentrations of salinity under higher soil moisture levels.

2.4 The effect of moisture stress on plant growth

Water stress is one of the most serious environmental factors undermining plant growth and development (Farooq et al., 2009). Water stress, induced either by the low soil water content or by the increasing concentrations of soluble salts in the root zone, restricts the ability of plants to get enough water from the soil to compensate for the water loss through transpiration, and water stress conditions develop (Griffiths & Parry, 2002). Plants suffer from water stress both at cellular and plant level. Under moderate water stress, plant regulates the function of stomata which start opening partially to conserve water (Chaves et al., 2003). Because stomata play an important role in CO_2 absorption for photosynthesis, their closure adversely affects the exchange of water, CO_2 and oxygen leading to reduction in photosynthesis and other important metabolic processes in plants. Continued water deficit situations may lead to dehydration and disorganization of the protoplasm and death of most organisms (Kramer & Boyer, 1995)

At plant level, the initial effect of water deficit is the poor germination and seedling growth (Okcu et al., 2005). Plant growth is the combined result of cell division, cell enlargement, and differentiation, and all these processes are extremely sensitive to water stress (Farooq et al., 2009). Water stress also cause reduction in vegetative growth by reducing number of leaves, leaf area, plant height and total plant biomass (Boutraa,

2010; Wu et al., 2008). Likewise, several plant physiological processes that determine yield such as tiller development, grain formation and grains filling are sensitive to water stress (Fredrick et al., 2001; Samarah, 2005). Water stress has been reported to significantly reduce the yield of many crops including maize (Cattivelli et al., 2008), soybean (Fredrick et al., 2001), cotton (Pettigrew, 2004) and rice (Yang et al., 2007).

Plants use several strategies to escape or avoid water stress. Some plants, such as Mediterranean annuals, escape drought by shortening their life cycle (Boyer, 1982). Other plants slow their growth which enables these plants to switch assimilates and energy into protective molecules to fight stress (Zhu, 2002). Plants use drought avoidance mechanism by maintaining high tissue water potential either by improving water acquisition through better root growth or minimizing water loss through stomatal control and reduced shoot growth (Kavar et al., 2007; Osorio et al., 1998). Some plants have the ability to tolerate water stress conditions through the over-production and accumulation of organic and inorganic solutes which lower the cell osmotic potentials, thereby enabling the maintenance of water absorption and cell turgor at lower soil water potentials (Morgan, 1984).

2.5 Salinity tolerance mechanisms in plants

The ability of plants to grow and survive under restrictive growth conditions imposed by salinity is known as salinity tolerance (Moller & Tester, 2007). These characteristics vary among plant species, and they can be accordingly classified as

glycophytes or halophytes (Flowers & Flowers, 2005). Glycopytes are plants that are unable to complete their life cycle in elevated concentrations of salt whereas halophytes have the ability to tolerate the harsh condition imposed by salinity. Plants use two mechanisms to tolerate salinity, salt avoidance and tissue tolerance (Volkamar et al., 1998). Salt avoidance is the process whereby plants keep the ions away from their sensitive parts through the passive exclusion of ions by a permeable membrane, the active expelling of ions by ion pumps, and by dilution of ions in the tissue of plants (Munns, 2002). These plants adjust their osmotic pressure by producing compatible solute such as proline, glycinebetaine and sugars (Munns & Tester, 2008). These compatible solutes have low molecular weight, are highly soluble in water, are electrically neutral, and do not interfere with plant's metabolic processes (Ashraf & McNeilly, 2004). Tissue tolerance occurs when the ions have already accumulated in the tissue of the plant, and they are then compartmentalized into plant's vacuole, thus controlling the salt concentration in the cyctosol and maintaining a high cystolic K^+/Na^+ ratio in their cells (Chinnusamy et al., 2006). This protects the cytoplasm from ion toxicity, and prevents build up of salts in the cell wall (Apse & Blumwald, 2002). Such compartmentalization of Na^+ is facilitated by the action of the Na^+/H^+ antiporters on the tonoplast (Yamaguchi & Blumwald, 2005). The vacuolar Na^+/H^+ antiporters, which are found in several plants species, utilize the proton motive force generated by vacuolar ATPases and pyrophosphates to move Na^+ from cytoplasm into vacuoles (Blumwald et al., 2000; Yamaguchi & Blumwald, 2005). Such gathering of salt from the cytoplasm into the

vacuole generates a strong osmotic rise across the vacuolar membrane (Volkamar et al., 1998). This osmotic gradient is balanced by an accumulation of high concentrations of either organic ions or low molecular weight organic solutes which are not detrimental to cellular biochemistry (Ashraf & McNeilly, 2004; Blumwald et al., 2000).

2.6 Environmental effect and salinity tolerance

Plant's adaptability to salinity depends on the interaction between salinity and different environmental factors such as soil, water and climatic conditions (Mass, 1993). For instance, some plants become susceptible to salinity when raised under hot and dry conditions than under cool and humid conditions (Mass & Hoffman, 1977). Salinity can cause greater reduction in crop yield under dry and hot conditions as compared to under cool and humid conditions. This can be linked to less ion accumulation and improved water relations in the latter case (Salim, 1989). The problem of soil salinity is expected to become severe with the changes in climatic variables such as temperature and rainfall patterns, specifically in the arid and semi arid regions of the world relying on rain-fed and non-irrigated agriculture (Pauw et al., 2000).

2.7 Evaluation of salinity tolerance in plants

Salt tolerance of plants is assessed either by the percent biomass production or by yield in saline versus control conditions over prolonged period of time (Munns et al., 2002). Such assessment can be carried out by growing plants under controlled environments such as hydroponics or solution culture, pot culture in greenhouse or

growth chambers or field conditions. Field screening in soils are made difficult by the high spatial and temporal variability in the chemical and physical properties of the soil as well as seasonal fluctuation of rainfall (Munns et al., 2006). Screening of plant genotypes under controlled conditions has been a widely used technique, which allow uniform and precise stress conditions to assess salinity tolerance (Houshmand & Arzani, 2005). Specifically, screening plants in pots under greenhouse conditions have remained an efficient criterion.

Plants are screened for salinity tolerance with respect to their agronomic and physiological traits. Under controlled environments, important agronomic traits include germination, leaf area, dry matter yield, phenotypic expression, and stability of traits over environment. Important physiological traits include Na^+ and K^+ uptake ratio, Na^+ and Cl^- exclusion, K^+/Na^+ or Ca^{++}/Na^+ discrimination, leaf water retention, and photosynthesis (El-Hendawy et al., 2007; Meneguzzo et al., 2000).

2.8 Approaches for alleviating the impact of salinity

A variety of biotic and abiotic approaches exist to alleviate the problem of soil salinity, and improving agricultural productivity in saline environments (Rains & Goyal, 2003). The common abiotic approaches include leaching of salts, managing crops, chemical amelioration of soils and use of salt tolerant plants (Goyal et al., 1999). These techniques are, however, expensive, laborious and provide only short-term solution (El-Hendawy et al., 2009; Singh & Singh, 2000). During the last couple of decades,

application of biotic approaches for improving plants' adaptability to salinity has attracted significant attention (Ashraf & McNeilly, 2004). Important biotic approaches include conventional breeding, transcriptional profiling technologies, protoplast fusion, and molecular biotechnology (Ashraf & McNeilly, 2004; Yamaguchi & Blumwald, 2005). Although scientists have been striving to develop salt-tolerant crop varieties using selective breeding techniques through the past century, none of those efforts has proven successful (Flowers & Yeo, 1995; Yamaguchi & Blumwald, 2005). In addition, such breeding approaches are time consuming, laborious, and allow transfer of undesirable genes along with desirable ones (Ashraf, 2010). Alternatively, modification of plants to fit the restrictive growing conditions imposed by salinity has been given high research priority in plant biotechnology to solve the problem of food insecurity. Recently, plant biotechnology has been perceived as an efficient, and economic means of tailoring plants for salinity tolerance, and has been pursued vigorously to improve the quantitative and qualitative traits of crop plants including tolerance to biotic and abiotic stresses in different crops (Wang et al., 2003).

Plant biotechnology enables breeders to make highly specific, and rapid agronomic and qualitative changes in the characteristics of plants in ways not possible with traditional breeding methods (Khan & Liu, 2009). Application of genetically modified (GM) technology in crop plants started in the 1980s. Since then, these engineering techniques and applications have developed rapidly in agriculture leading to an increase in the area under GM crops (James, 2008). To date, a variety of commercially

released GM crops conferring diverse traits have been successfully released. The focus of these developments has been increasing shelf life of agricultural products, conferring resistance to insect pests or viruses, and pesticides, and improving the nutritional value of crops (Ashraf & Akram, 2009). These crops are under commercial cultivation in both developed and developing countries with US, Argentina, Brazil, Canada, India and China occupying 118.3 million ha or 95% of the total worldwide GM crop area (James, 2008). Similarly, considerable research is underway to develop plants with enhanced resistance to abiotic such as salinity, drought or freezing, and biotic stresses such as pathogen (Snow et al., 2005).

2.9 Plant biotechnology for developing salinity tolerant plants

Agricultural biotechnology can provide powerful solution to the problem of maintaining agricultural productivity in saline soils. Application of crop biotechnology can expedite the development of new crop varieties that would be more tolerant to physical stresses such as saline soils, and drought, in an environmentally friendly way (Arzani, 2008). In the recent past, considerable research has been undertaken to indentify, isolate and characterize several genes that could potentially improve salt tolerance in various ways (Flowers, 2004). Because of the complexity of salt tolerance, it is hard to assume that transferring a single gene encoding a single specific stress protein to a transgenic plants could dramatically improve salt tolerance in plants (Wang et al., 2003).

Current efforts to tailor plants tolerant to salinity through genetic manipulation have started to bear fruits. In recent years, many transgenic crop varieties have been developed both through over-production of compatible solutes, and by over-expressing a vacuolar Na^+/H^+ antiporter gene in transgenic plants. For instance, Tarezynski et al (1993) first engineered transgenic tobacco overexpressing mannitol. Similarly, transgenic crops including Arabidopsis (Hayashi et al., 1997), rice (Mohanty et al., 2002), wheat (Abebe et al., 2003) and tobacco (Hong et al., 2000) have been generated by overexpressing of compatible osmlytes in these plants. The resultant transgenic plants have been reported to show better germination, seedling growth and seed production under high salt and osmotic stresses.

Likewise, transgenic plants have been generated by over expressing Na^+/H^+ antiporter gene in Arabidopsis (Apse et al., 1999), tomato (Zhang & Blumwald, 2001), *Brassica napus* (Zhang et al., 2001), maize (Yan et al., 2004), wheat (Xue et al., 2004) and tobacco (Wang et al., 2004). These transgenic plants exhibited improved salt tolerance compared to their wild type progenitors.

2.10 Employing TMT gene for salinity tolerance

To counter the effect of salinity, Kaur (2006) has suggested engineering crop plants for salinity tolerance using a gene coding for the enzyme thiol methyltransferase (TMT). This gene, and the enzyme it encodes, confers an ability in plants to methylate Cl^- to CH_3Cl. Wuosmaa and Hager (1990) initially reported that a marine alga *Endocladia*

muricata, wood-rotting fungus *Phellinus pomaceus* and a halophytic plant *Mesembryanthemum crystallinum* possess a methyl chloride transferase (MCT) enzyme that can methylate Cl^- to CH_3Cl. Because two of these organisms survive under saline habitats, the presence of this enzyme has been suggested as a possible mechanism of salinity tolerance in these organisms via volatilization of Cl^- as CH_3Cl. To experimentally test this possibility, Saini et al. (1995) conducted a survey of 118 herbaceous species, using a simple method where leaf discs from these plants were floated on potassium iodide solution and the resultant methyl iodide (CH_3I) production was measured. They found that majority of plants were able to methylate halide ions to methyl halides, and this ability was considered to be an indication of methyltransferase activity. In addition, low enzymatic activities were recorded in the halophytic or salt tolerant plants, including *Mesembryanthemum crystallinum*, whereas the highest methyltransferase activities were found in the Brassicaceae and Resedaceae families of the order Capparales. Further investigations revealed that *Brassica oleracea* and 19 other species possessing halide methyltransferase activity were also able to methylate bisulphide (HS^-) ion to methanthiol (CH_3SH). Subsequently, Attieh et al. (1995) purified and characterized a methyltransferase enzyme from cabbage, which can methylate halide (X^-) and HS^- ion to monohalomethanes and methanethiol (CH_3SH) in the presence of S-adenosyl-L-methionine (AdoMet) which act as a catalyst. Kinetic studies showed that the purified enzyme preferred thiol ions (thiocynate 4,4–thiobisbenzenethiol, thiophenol, and salicylic acid) as substrates and had Michaelis-Menten (*Km*) values of 11, 51, 250 and 746 nM for

thiocyanate 4,4' –thiobisbenzenethiol, thiophenol, and thiosalicylic acid, respectively (Attieh et al., 2000). Based on the results of the kinetic study, these authors named this enzyme as thiol methyltransferase (TMT).

In order to dissect this pathway, and to understand its molecular regulation, physiological role, and ecological significance, a TMT gene was isolated from cabbage (Attieh et al., 2002), and was engineered into *E. coli*, and several plant species, that otherwise lack the enzyme as well as its usual metabolic context (Kaur, 2006). The author treated these TMT containing organisms with different salt concentrations under controlled environments. The results revealed that transgenic organisms containing methyltransferase enzyme produced methyl chloride when exposed to different salt concentrations in the growth medium. In addition, these transgenic organisms showed greater tolerance to different salt concentration by volatilizing Cl^- as CH_3Cl. The author suggested the enzymatic volatilization of Cl^- as CH_3Cl to be a possible mechanism of improving tolerance of high valuable crops against Cl^- toxicity under saline conditions. However, before such a possibility can be turned into reality, it is highly important to carefully estimate the possible emissions of CH_3Cl from such crops prior to introduction on commercial scale.

2.11 Methyl Chloride (CH_3Cl)

The contribution of CH_3Cl to the total stratospheric chlorine is approximately 15%, and as such it plays an important role in the atmospheric chemistry and depletion of

ozone layer (Montzka & Fraser, 2003). The global average atmospheric concentration of CH_3Cl is approximately 550±30 parts per trillion by volume (pptv) (Simmonds et al., 2004) with a total global emission of 4,089 Gg yr^{-1} (Xiao et al., 2009). CH_3Cl originates both from natural and anthropogenic sources (Harper & Hamilton, 2003), with natural emissions far exceeding those from the anthropogenic sources (Xiao et al., 2009). The known sources of CH_3Cl include biomass burning (Lobert et al., 1999), oceans (Moore et al., 1996) incineration/industrial emissions (McClulloch et al., 1999), wood-rotting fungi (Watling & Harper, 1998), salt marshes (Rhew et al., 2003), and higher plants (Yokouchi et al., 2002). Initially, scientists believed the oceans to be the dominant source of CH_3Cl to the atmosphere (Singh et al., 1983). However, recent investigations have revealed that the contribution of oceans to the total atmospheric CH_3Cl budget is only 12% (Moore et al., 1996), and that most of CH_3Cl released to the atmosphere is of terrestrial origin dominated by biogenic sources (Harper & Hamilton, 2003; Xiao et al., 2009). This has initiated research to identify potential sources of biogenic emissions of CH_3Cl and the biochemical mechanisms controlling these emissions. Higher plants emit CH_3Cl (Harper et al., 1999; Saini et al., 1995; Varns, 1982), and exploring the mechanism of CH_3Cl production by plants has attracted a great deal of attention in the recent past.

2.12 Methyl chloride production by higher plants

Over the last couple of decades, a number of attempts have been made to identify the potential biological sources, their extent of contribution to the global atmospheric CH_3Cl budget along with the mechanism of production. Since the initial discovery by

Varns (1982) that fresh potato tubers could emit methyl chloride, several studies have been conducted to identify potential plant sources and their contribution to the global CH_3Cl budget. Wuosmaa and Hager (1990) revealed that the halophytic plant *Mesembryanthemum crystallinum* released small of amount of CH_3Cl when incubated in 100 mM KCl. Based on these findings, the authors further suggested a survey of methyl chloride by other halophytes. Subsequently, Saini et al (1995) carried out a survey on 118 herbaceous plants, including 21 halophytes, and observed that the majority of these tested showed the capability to emit CH_3Cl and other halides, with the family of Brassicaceae being particularly efficient. In continuation to these findings, research studies were directed to measure CH_3Cl release in the field from individual plants or plant communities by enclosure techniques. Varner et al (1999) performed in situ measurement of CH_3Cl flux from individual plants or plant communities under normal growing conditions in the field. They estimated an annual global CH_3Cl emissions at two wetland sites in the northeastern United States dominated by *Sphagnum spp.*, *Carex spp.*, and ericaceous shrubs to be 48 Gg $year^{-1}$. Dimmer et al. (2001) reported CH_3Cl emission from the west coast of Ireland, which after extrapolating to global peatlands, were equal to 5 Gg $year^{-1}$. Lee-Taylor and Redeker (2005) recorded a significant release of methyl halide emission in a rice field. They observed emission of methyl halides from flooded rice fields, which depended on rice growth stage, soil organic content, halide concentration, and field water management. They estimated that 1% of atmospheric methyl chloride and 5% of methyl iodide arise from rice fields worldwide. The next most

intriguing and interesting findings regarding methyl chloride and methyl bromide emission are from salt marshes. Large fluxes of methyl chloride and methyl bromide are released by salt marshes (Rhew et al., 2000). A study carried out in two salt marshes in California at different seasons showed greater emission of methyl halides from the middle and upper middle vegetation zones where halophytes such as *salicornia spp*., *Batis maritina* and *Frankenia grandifolia* were dominant vascular plants. The estimated global emission of CH_3Cl from this source was reported to be 170 Gg yr^{-1}. These researchers also observed great variation in the fluxes in terms of growing season and growth stages.

Similarly, tropical forest plants were also found to contribute to the global CH_3Cl budget. Yokouchi et al. (2002) studied CH_3Cl emission from the tropical plants in the lowland-forest selection of tropical rain-forest in Tsokuba Botanical Garden, where more than 200 representative species from the lowland tropical forest of south east Asia were grown. They observed large emission of CH_3Cl from common tropical plants (including some ferns and members of *Dipterocarpaceae* family) ranging from 01 to 3.7 µg of dry leaf per hour. Based on their preliminary findings, they estimated the CH_3Cl flux from *Dipterocarpaceae* family in Southeast Asia alone to be 910 Gg yr^{-1}. In another study, Manley et al. (2006) used a bottom-up approach derived from up-scaling individual estimates of methyl halide emission from costal salt marshes plants to the global level derived from considerations of global surface area coverage of coastal salt marsh forests. The authors collected preliminary methyl halide emissions data from two plants species

at leaf level and then was extrapolated to the global costal salt marshes surface coverage, which yielded an annual global emissions of 160 Gg CH_3Cl. These authors applied similar approaches to estimate the contribution of mangrove forest to the global methyl chloride budge (Manley et al., 2007). Recently, Yokouchi et al. (2007) reported considerable emission of CH_3Cl by subtropical forests. Tropical rain forests have also been reported to contribute significantly towards the global atmospheric budget (Blei et al., 2010). These authors collected preliminary data on CH_3Cl emission from individual tropical rainforest plants using enclosure techniques and the resultant plant level fluxes were extrapolated to the existing global area under tropical rainforest, which yielded to an annual global emission of 1500 Gg CH_3Cl.

2.13 CH_3Cl and ozone depletion

Ozone depletion is the reduction in ozone layer which shields the Earth from harmful UV-B radiation. Ozone is the most important chemically active trace gases in the Earth's atmosphere because of its ability to selectively absorb deleterious UV-B radiation (wavelength 280-320 nm) (Gebhardt, 2008). Ozone depletion takes place when certain chemicals containing chlorine and bromine, produced through both natural and industrial process, reach the stratosphere where they take part in photooxidation reactions that destroy ozone (Baldwin et al., 2007). The reduction in stratospheric ozone concentration allows more of the UV-B light (280-320 nm) to reach the Earth's surface. UV-B radiation poses serious threats to human beings, terrestrial biosphere and aquatic ecosystems (UNEP, 2006). Increased UV-B radiation can lead to the development of skin cancer and

cataract formation in eyes in humans (Gruijl et al., 2002). Exposure to UV-B can also damage immune systems which make human and other animals more susceptible to infectious diseases (UNEP, 2006). Increased intensity of UV-radiation causes degradation of photosynthetic pigments in plants, thereby reducing plant's photosynthetic ability leading to reduced leaf area, reduced shoot elongation, and lower biomass production, causing lower growth and productivity with significant implications for food security (Caldwell et al., 2007; Xiong & Day, 2001). UV-B radiation affects some fungi, bacterial communities, and soil fauna that could play a role in decomposition and nutrient cycling (Caldwell et al., 2007) . Similarly, changes in the intensity of solar UV-B radiation brings significant changes in aquatic ecosystems (DAy & Neale, 2002). UV-B radiation increases the photodegradation of the colored dissolved organic matter (CDOM), which controls the penetration of UV-B radiation into water bodies (Hader et al., 2007). Such penetration of UV-B has detrimental effects on the growth and development of aquatic primary producers including phytoplankton, microalgae, which reduce the CO_2 uptake capacity of oceans which may result in increase atmospheric CO_2 concentrations. (Clements et al., 2008). Other deleterious atmospheric consequences of increased UV-B radiation include its acceleration of carbon monoxide production from dead plant matter in terrestrial ecosystem, and nitrogen oxide production from Arctic and Antarctic snow-packs (Zepp et al., 2003). These changes in plants and soil and aquatic microorganisms may alter the atmospheric cycling of carbon, nitrogen and other

elements, and may have serious implications for atmospheric biogeochemical cycles (Zepp et al., 2006).

2.14 Ozone depletion, global warming and climate change

Initially, scientists conceived ozone depletion and climate change as two serious environmental issues with little in common. Recent scientific evidence, however, suggests that ozone depletion, and climate change are interlinked, and that continued global warming will accelerate stratospheric ozone depletion (Newman et al., 2009). Global warming means an average increase in Earth's temperature resulting mainly from the increased concentration of greenhouse gases in the atmosphere including, carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), which in turn cause changes in the climate (Houghton, 2005). Global warming influences atmospheric heat distribution by trapping heat in the troposphere causing stratospheric cooling leading to increase in ozone depletion (Shine et al., 2003). In addition, projected increase in the global average temperature may increase the rate of halocarbon emissions from biogenic sources, which will further accelerate ozone depletion (Redeker & Ciceron, 2004).

On the other hand, stratospheric ozone depletion accelerates global warming (IPCC, 2007). Ozone itself and some other important ozone-depleting substances such as CFCs and HCFCs are also powerful greenhouse gases (Kaniaru et al., 2008) because of their ability to absorb terrestrial infrared radiation (McFarlane, 2008). Ozone depletion, and the resulting UV-B radiation are detrimental to the survival of phytoplankton (UNEP,

2006). Plankton plays an important role in the ability of oceans to absorb carbon dioxide from the atmosphere, thereby making oceans important carbon sinks (Hader et al., 2007). Loss of plankton may reduce the capacity of oceans to absorb CO_2 and may boost the rate of buildup of CO_2 in the atmosphere which may further accelerate global warming (Revere, 2000). Also, other findings reveal that changes in stratospheric circulation may alter weather patterns in the troposphere (Shine, 2000).

2.15 **Specific objectives of the study**

1) To determine the differences in growth between transgenic and wild tobacco plants exposed to different NaCl concentrations and soil water content.
2) To estimate the quantity of CH_3Cl produced by transgenic and wild type tobacco plants as influenced by the different NaCl concentrations and soil water content.

2.16 **Hypothesis and null hypothesis**

1. Transgenic plants will produce improved growth compared to wild type plants when exposed to salinity under different soil water content.

   $H_{0:}$ There will be no differences in growth between transgenic and wild type tobacco plants as influenced by the different NaCl concentrations and soil water content.
2. Transgenic plants will emit CH_3Cl and the rate of emission will by higher under higher NaCl concentrations and soil water content

$H_{0:}$ There will be no effect of NaCl and soil water content on the quantity of CH_3Cl released by transgenic plants

Chapter 3

Materials and methods

This study was conducted to investigate the salinity tolerance of transgenic tobacco plants, and estimate the corresponding emissions of CH_3Cl.

3.1 Planting material and growth conditions:

Transgenic tobacco (*Nicotiana tabacum)* plants (containing TMT gene) (Kaur, 2006) and wild type tobacco plants were used in this study. The genetic identity of these primary transgenics was verified by polymerase Chain Reaction (PCR). These plants were grown in the greenhouse (Department of Biology, University of Waterloo, Canada) under natural temperature, light and relative humidity. Seeds were sown directly in germination trays containing artificial soil medium (Sunshine Mixture # 1). Before the start of the experimental treatments, all pots were provided with 100 ml of 20-20-20 fertilizer solution (1g L^{-1}) (Plant Products, Brampton, Ontario, Canada). Before transplanting of seedlings into pots, amount of soil moisture was calculated to bring the amount of water required to bring the pots to 40%, 60% and 80% field capacities. For this purpose, a soil sample was taken and weighed, and then oven dried at 90°C for 24 h, and weighed again. The difference in weight before and after drying was determined as the amount of water present in the soil mixture. This dried soil sample was saturated with a known quantity of water and was left to drain for 24 h. This procedure was replicated twice. The amount of water required to bring the soil to field capacity was calculated by

subtracting the amount of water drained from the soil sample after 24 h from the total amount of water applied to the pot. The amount of water required to bring the pots to 40%, 60% and 80% field capacity was calculated from the amount of water retained by the soil mixture at field capacity. Double deionized water was used for irrigation purpose throughout the experiment. Two-weeks old seedlings were transferred to previously weighed small plastic pots (15 cm diameter) filled with soil mixture # 1, irrigated normally for two weeks to avoid any transplantation stress. Before the start of the experimental treatments, these pots were weighed again and the difference in weight was calculated to apply the required amount of soil moisture to bring these pots to desired water content (% of field capacity). A saucer was placed under each pot to prevent soil loss from the pot. The pots were weighed daily and the appropriate amount of water was added to maintain the plants at the required water content. Because the daily increment of plant mass was small in comparison to water lost, the former was not taken into account. These plants were watered with normal or saline water for five weeks.

3.2 Experimental design and treatments

The experimental was a factorial combination of two plant types (transgenic and wild type tobacco plants), NaCl concentrations (0, 100, 200 mM) and soil water content (40, 60, 80% of the field capacity). The experimental design was randomized complete blocks of eighteen treatments with three replications, each replicate consisting of four plants in individual plastic pots. For estimating the quantity of CH_3Cl produced, NaCl levels used were 0, 50, 100, 150 and 200 mM.

3.3 Data collection and analysis:

At the end of the experiment (35 days), the plants were harvested and the data were collected on the following growth variables:

- **Plant height:** measured from the soil surface to the top of the main stem
- **Number of leaves per plant:** Number of leaves (including tips of newly emerging leaves) was counted.
- **Leaf surface area:** leaf surface area was measured with the LI-COR (LI-3000) portable area meter.
- **Plant dry weight:** At the end of the experiment, three plants collected from each treatment were washed with deionized water. The plants were divided into roots, leaves and stems. The roots were removed from the soil and washed initially with tap water and then with deionized water. The dry weight (DWt) was measured after the fresh material was oven dried at 48^{o}C for 48 hours.
- **Root/Shoot ratio:** Dry root and shoot portions of each plant were weighed separately. The root/shoot ratio was calculated as root dry weight/shoot dry weight.

3.4 Measuring CH_3Cl emission from TMT containing transgenic plants

After two-week exposure to salinity, data collection on CH_3Cl emission was started from both salt-treated and control plants. From each treatment, a total of six plants were selected, and from each plant three gas samples were taken at 5 minutes intervals. A bag enclosure method was employed to collect foliar emissions of CH_3Cl. A healthy and young leaf of a plant in each treatment was enclosed in "light run" Tedlar bags (5" by 4") with a volume of 400 ml (Qubit Systems Inc. Kingston, Onatario, Canada), and the top of each bag was closed carefully with a clamp (Fig. 1) (Alejar et al., 1988). Care was taken to avoid any damage to the leaf. After 30 minutes of sample incubation, 1 ml gas sample from the bag was collected with a 1 ml syringe and was immediately injected into gas chromatograph (GC). These gas samples were analyzed using a Hewlett Packard Series 5890-II gas chromatograph (GC) equipped with a 80-100 mesh Porapak-Q (Supelco Canada) packed column (0.3 cm x 210 cm) and a flame ionization detector. Before the sample was injected, GC was calibrated using standard CH_3Cl gas (Linde Canada Limited, Mississauga, Canada). The flow rate of the carrier gas (He) was maintained at 40 ml min^{-1} and the column temperature was kept at 130^{o}C. The injector and detector temperatures were 120^{o}C and 200^{o}C, respectively.

The portion of the leaf enclosed in the bag was dried at 80^{o}C for 48 hours. The emission rate of CH_3Cl (µg g^{-1} h^{-1}) was calculated using the following equation (Yokouchi et al., 2007)

$E = C \times 400 / (W \times T)$,

Where C is the measured concentration of CH_3Cl in the bag (ng ml^{-1}), 400 ml is the volume of the bag, W is the weight of the oven dried leaf (g), and T is the elapsed time between sealing the bag and analyzing the head-space gas sample (h).

3.5 Statistical analysis

Data on all growth parameters of wild type and transgenic crops was statistically analyzed at type 1 error rate of 5% using factorial design, and least significant means were compared using Turkey's multiple comparison test (Bowley, 2004). If the data was not normally distributed, it was transformed to natural log in order to meet the RCBD additive linear model requirements of random, independent, normally distributed error with zero mean and common variance. The residuals were plotted against predicted residuals and normalcy of residuals were tested. Statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC).

Figure 1: A young tobacco leaf is enclosed in a Tedlar bag and the open end of the bag is closed with a clamp.

Chapter 4
Results

4.1 Evaluation of salinity tolerance of transgenic tobacco plants grown under different NaCl concentrations and soil moisture content

In order to assess the tolerance of transgenic tobacco plants to salinity, they were exposed to different concentrations of NaCl and soil water content. Data recorded on height, number of leaves, leaf area, stem dry weight, leaf dry weight, root dry weight, total plant dry biomass and root/shoot ratio is presented in Table 3.

Statistical analysis of the data showed that both water stress and salt stress significantly affected the plant height, number of leaves, leaf area, dry stem weight, dry leaf weight, dry root weight, dry biomass and root/shoot ratio of both types of plants (see ANOVA Tables). At 100 mM NaCl concentrations, growth of both wild type and transgenic tobacco plants was significantly reduced, whereas at 200 mM NaCl, severe growth inhibition occurred in both types of plants. Differences between wild type and transgenic plants under stress treatments for all characters were also highly significant. Interaction between plant type, salinity, water stress was significant for all growth parameters except was root/shoot ratio. Reduction in growth parameters under salinity was greater under lower soil water content than under higher soil water content and the extent of such reduction were greater in wild type plants than in transgenic plants.

All the stress treatments significantly reduced the plant height of both types of plants compared to control plants (p<0.0001) (Table 1) and such reduction in plant height was significantly higher in transgenic plants than in wild type plants (p<0.0001) (Table 1). For instance, the reduction in plant height of transgenic plants at 100 mM NaCl and 80% field capacity was 47% compared to 39% in wild type plants. Similarly, water stress caused significantly greater reduction (p<0.0001) (Table 1) in wild type plants (25%) than in transgenic plants (13%) with respect to their control plants (Table 9). The interactive effect of salt and water stress was also significant (p<0.0001) for plant height, and under high salt concentration (100 mM) and low soil water (40%), such effect was greater in transgenic plants (55%) than in wild type plants (52%) (Table 9) (Fig. 2).

In plants submitted to different stress treatments, number of leaves was significantly reduced with respect to their control (p<0.0001) (Table 2) and significantly greater (p<0.0001) reduction in number of leaves in wild type plants was recorded than in transgenic plants. For instance, in wild type plants, salinity stress of 100 mM NaCl at 80% field capacity reduced number of leaves by 17% compared to 15% in transgenic plants (Table 9). Likewise, the reduction in number of leaves due to water stress was significantly greater in wild type plants (19%) compared to transgenic plants (14%) (Table 9). The interactive effect of different NaCl concentrations and soil water content was also significant (p<0.0001), and under high salt concentration (100 mM) and low water content (40%) such effect was significantly higher in wild type plants (37%) than in transgenic plants (29%) (Table 9) (Fig. 3).

Table 1: Analysis of variance of plant height (cm) of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0.03813	0.063040	.60	0.2727
Residual	0.4434	0.1075	4.12	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	**1**	**34**	**8495.86**	**<.0001**
NaCl	**2**	**34**	**8671.80**	**<.0001**
Water	**2**	**34**	**413.56**	**<.0001**
Plant*NaCl	**2**	**34**	**393.00**	**<.0001**
Plant*Water	**2**	**34**	**153.17**	**<.0001**
NaCl*Water	**4**	**34**	**33.50**	**<.0001**
Plant*NaCl*Water	**4**	**34**	**11.19**	**<.0001**

Table 2: Analysis of variance of number of leaves of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0.028320	.04441	0.64	0.2618
Residual	0.2865	0.06948	4.12	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	**1**	**34**	**2823.49**	**<.0001**
NaCl	**2**	**34**	**641.93**	**<.0001**
Water	**2**	**34**	**225.20**	**<.0001**
Plant*NaCl	**2**	**34**	**195.14**	**<.0001**
Plant*Water	**2**	**34**	**23.53**	**<.0001**
NaCl*Water	**4**	**34**	**2.49**	**<.0001**
Plant*NaCl*Water	**4**	**34**	**1.71**	**<.0001**

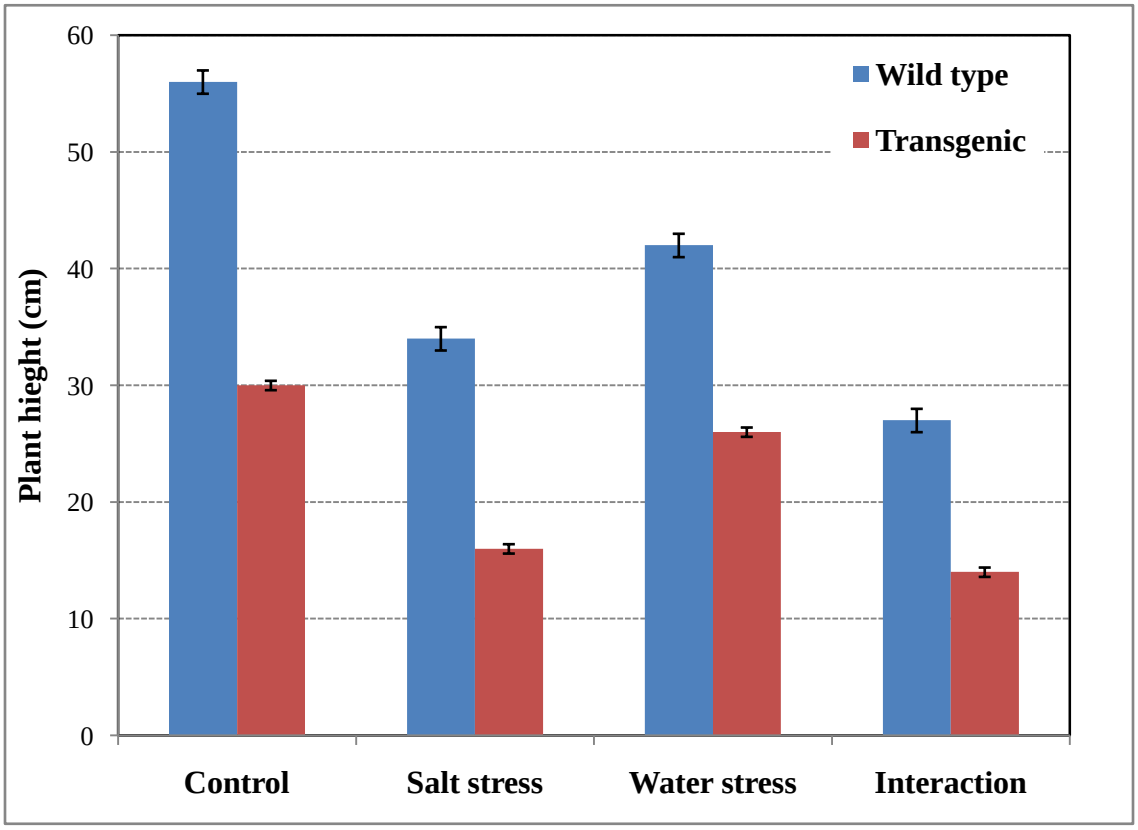

Figure 2: Plant height (cm) of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

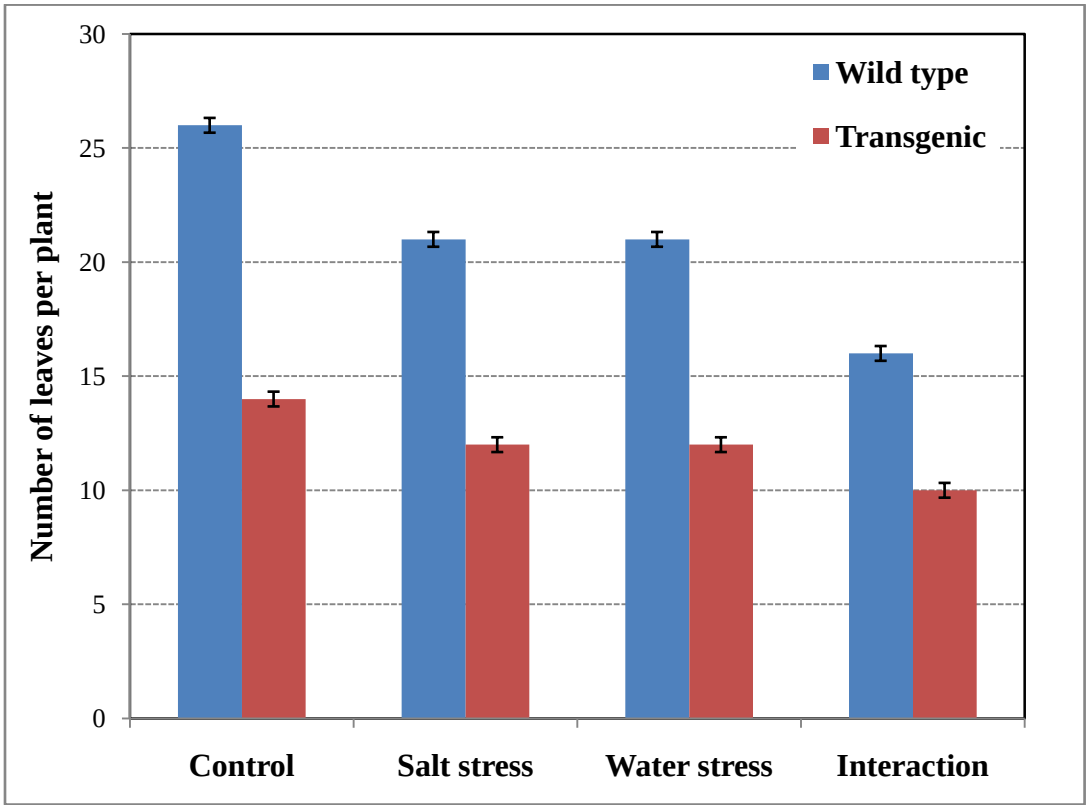

Figure 3: Number of leaves of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

Leaf area was also significantly reduced by all stress treatments with respect to control plants (p<0.0001) (Table 3) and at any particular treatment the reduction was significantly greater (p<0.0001) in wild type plants compared to that in transgenic plants (Table 3). For example, at 100 mM NaCl stress and 80% field capacity, wild type plants exhibited significantly greater reduction in leaf area (27%) compared to transgenic plants (18%) (Table 9). Similarly, reduction in leaf area under water stress was significantly greater (p<0.0001) (Table 3) in wild type plants (40%) than in transgenic plants (26%) (Table 3). The interaction between plant type, salinity and soil water content was also significant for leaf area (p<0.0001) and under high NaCl concentration of 100 mM and low soil water content of 40% field capacity such reduction was significantly greater (p<0.0001) in wild type plants (57%) than in transgenic plants (42%).

In plants submitted to different stress treatments, stem dry weight was significantly reduced with respect to control plants (p<0.0001) (Table 4). The effect of salinity stress (100 mM) on stem dry weight at 80% field capacity was significantly greater (p<0.0001) in transgenic plants (66%) compared to wild type plants (61%) (Table 10). Water stress also significantly lowered (p<0.0001) (Table 4) the stem dry weight of both types of plants. Such reduction was significantly lower (p<0.0001) in transgenic plants (22%) compared to wild type plants (32%) (Table 10). The interactive effect of salinity and water stress was also significant (p<0.0001) for stem dry weight and a greater interactive effect of salinity and water stress was noted in transgenic plants (74%) than in wild type plants (71%) (Table 10; Fig. 5).

Table 3: Analysis of variance of leaf area (cm^2) of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0	-	-	
Residual	34.74070	8.1885	4.24	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	2601.11	<.0001
NaCl	2	34	865.86	<.0001
Water	2	34	290.15	<.0001
Plant*NaCl	2	34	218.09	<.0001
Plant*Water	2	34	14.00	<.0001
NaCl*Water	4	34	16.48	<.0001
Plant*NaCl*Water	4	34	0.60	<.6656

Table 4: Analysis of variance of stem dry weight (g) of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0	-	-	-
Residual	0.002283	0.000538	4.24	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	2461.12	<.0001
NaCl	2	34	13420.8	<.0001
Water	2	34	386.72	<.0001
Plant*NaCl	2	34	409.21	<.0001
Plant*Water	2	34	28.92	<.0001
NaCl*Water	4	34	170.11	<.0001
Plant*NaCl*Water	4	34	38.13	<.0001

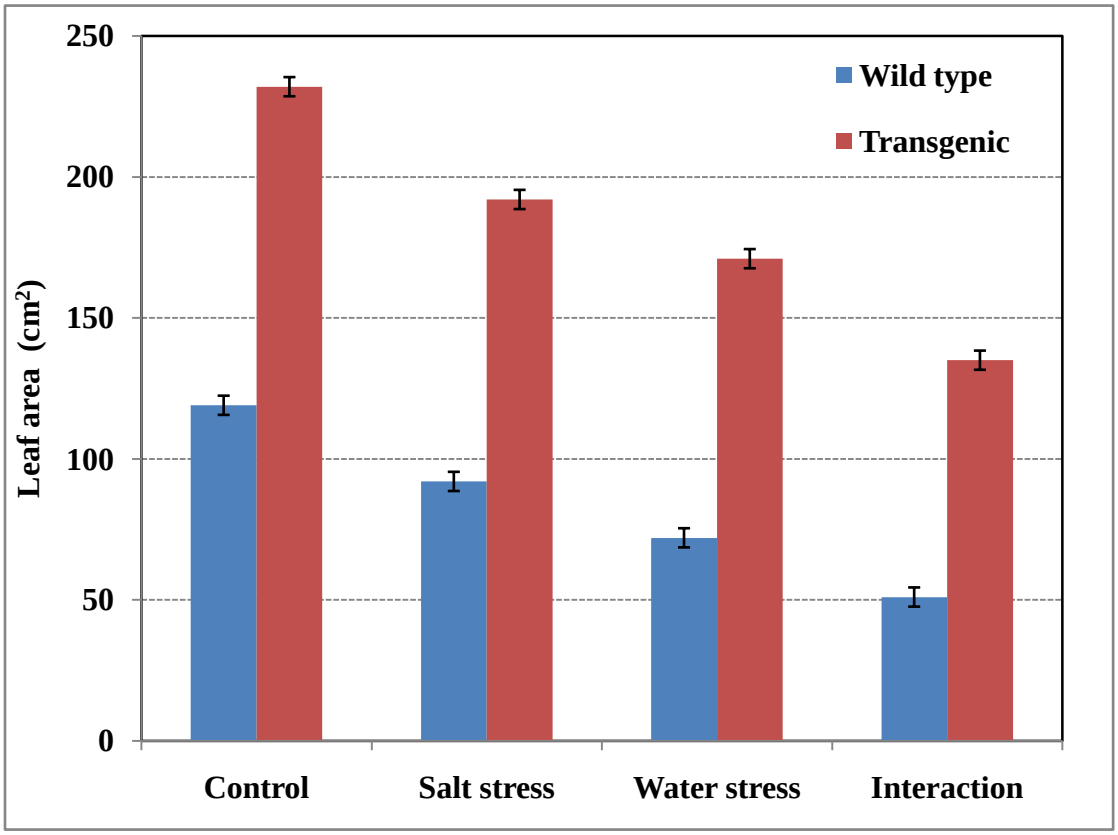

Figure 4: Leaf area (cm^2) of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

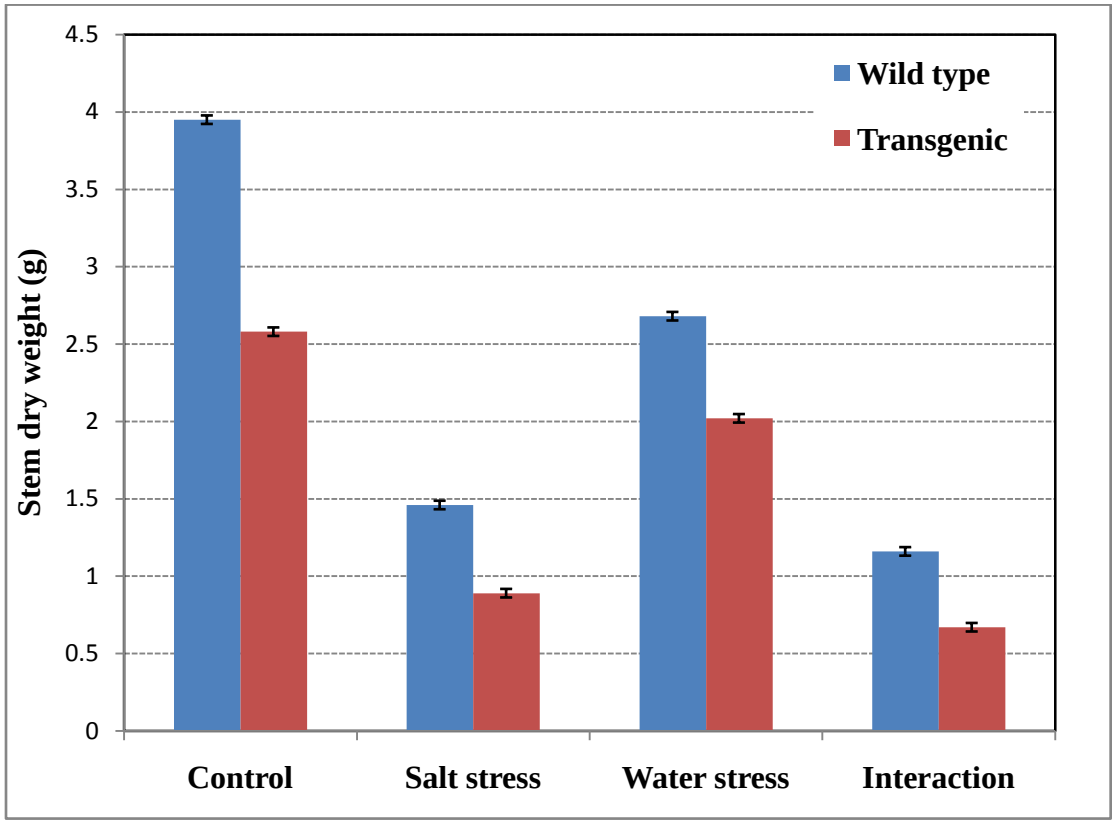

Figure 5: Stem dry weight (g) of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

Significant reduction occurred in leaf dry weight in all plants exposed to salinity stress, water stress and the interaction of water and salinity stress (p<0.0001) (Table 5). At 100 mM NaCl and 80% field capacity, the reduction in leaf dry weight was significantly greater (p<0.0001) in wild type plants (42%) compared to transgenic plants (20%) (Table 10). In plants submitted to water stress, leaf dry weight was significantly reduced (p<0.0001) (Table 5) and such reduction was significantly greater in wild type plants (33%) than in transgenic plants (27%) (Table 10). The interaction effect of high salt concentration (100 mM) and low soil water content (40% field capacity) for leaf dry greater was significantly greater (p<0.0001) (Table 5) in wild type plants (63%) than in transgenic plants (44%) (Table 10) (Fig. 6).

Both salinity and water stresses significantly reduced the root dry weight in all plants with respect to their control (p<0.0001) (Table 6). Wild type plants, however, experienced significantly greater reduction ((p<0.0001) in root dry weight (56%) compared to transgenic plants (52%) under 100 mM NaCl concentration and 80% field capacity (Table 10). The reduction in root dry weight under water stress was significantly greater (p<0.0001) (Table 6) in wild type plants (19%) compared to transgenic plants (9%) (Table 10). The interactive effect of salinity and water stress was also significant for root dry weight of both types of plants (p<0.0001). Transgenic plants, however, experienced less reduction (58%) than wild type plants (62%) under the interactive effect of salinity and water stress (Table 10) (Fig. 7).

Table 5: Analysis of variance of leaf dry weight (g) of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0.002205	0.003534	0.62	0.2664
Residual	0.02367	0.005741	4.12	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	1436.44	<.0001
NaCl	2	34	2302.40	<.0001
Water	2	34	367.73	<.0001
Plant*NaCl	2	34	256.49	<.0001
Plant*Water	2	34	3.49	<.0420
NaCl*Water	4	34	23.72	<.0001
Plant*NaCl*Water	4	34	0.65	<.6300

Table 6: Analysis of variance of root dry weight (g) of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0	- -	-	
Residual	0.00087	0.00021	4.24	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	16.78	<.0001
NaCl	2	34	7294.76	<.0001
Water	2	34	99.35	<.0001
Plant*NaCl	2	34	21.52	<.0001
Plant*Water	2	34	2.16	<.1305
NaCl*Water	4	34	15.47	<.0001
Plant*NaCl*Water	4	34	5.36	<.0019

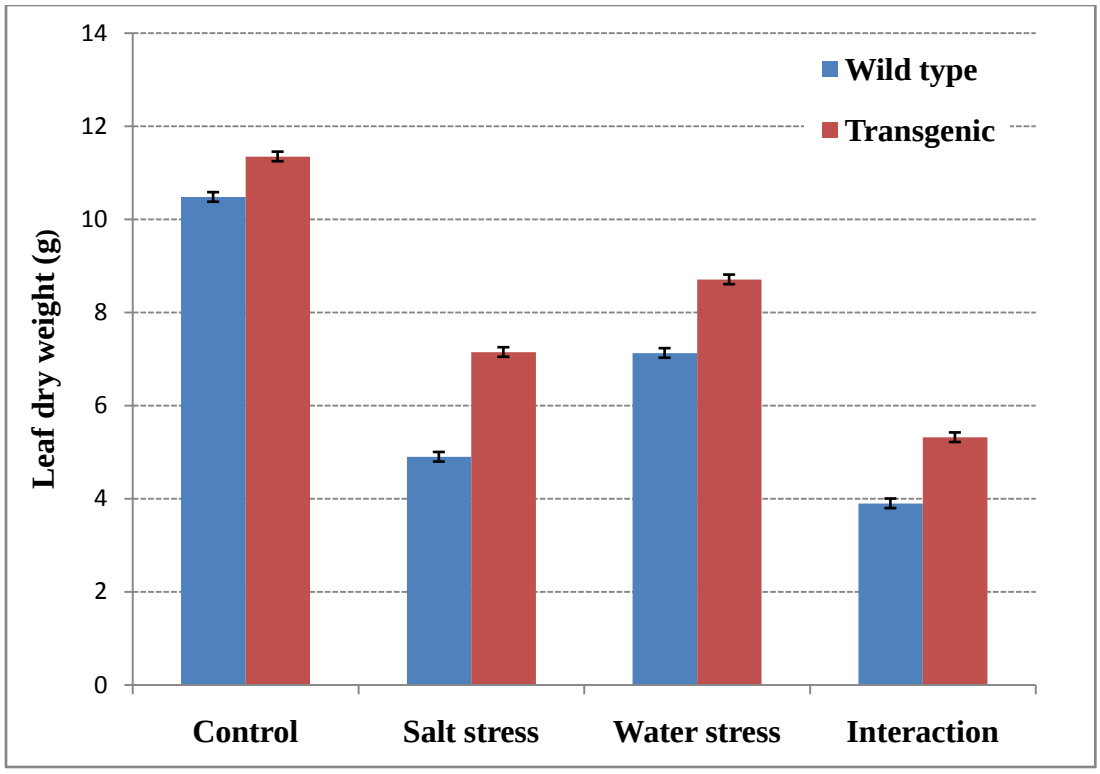

Figure 6: Leaf dry weight (g) of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

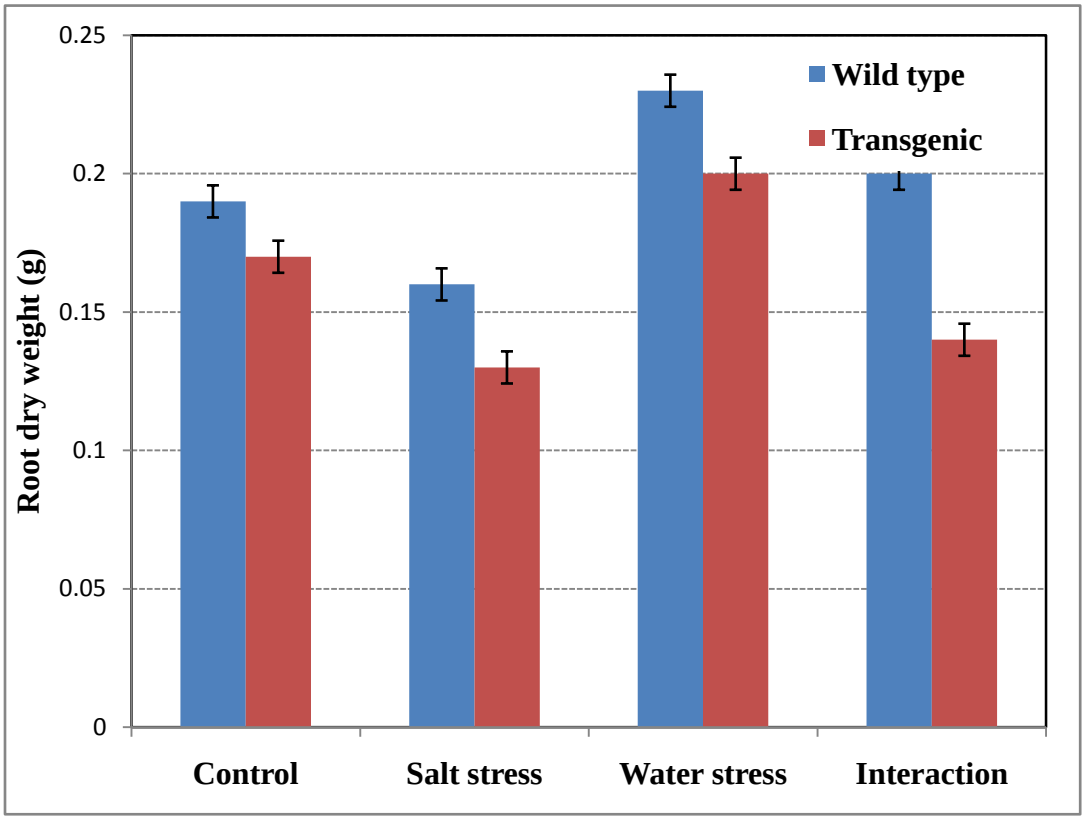

Figure 7: Root dry weight (g) of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

Both wild type and transgenic plants exhibited significant reduction in total plant dry biomass when exposed to water and salinity stress ($p<0.0001$) (Table 7). The reduction in plant dry biomass under 100 mM NaCl salinity and 80% field capacity was significantly greater ($p<0.0001$) (Table 7) in wild type plants (51%) compared to transgenic plants (37%). In plants exposed to water stress, plant dry biomass was significantly reduced ($p<0.0001$) in wild type plants (30%) than in transgenic plants (23%) (Table 10). The interactive effect of salinity and water stress also caused significant reduction in plant dry biomass ($p<0.0001$), the extent of such reduction, however, was significantly greater in wild type plants (63%) than transgenic plants (53%) with respect to their control (Table 10) (Fig. 8).

Salinity and water stress caused significant effect on root/shoot ratio ($p<0.0001$) (Table 8). Under the moderate salinity of 100 mM NaCl and 80% field capacity, the reduction in root/shoot ratio was significantly greater ($p<0.0001$) in transgenic plants (24%) compared to wild type plants (16%) (Table 10). Water stress increased the root/shoot ratio in transgenic plans by 24% compared to 21% in wild type plants (Table 10). Interactive effect of plant type, salinity and water stress was not significant for root dry weight.

Table 7: Analysis of variance of dry biomass (g) of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0.001235	0.002945	0.42	0.3375
Residual	0.03027	0.007342	4.12	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	412.60	<.0001
NaCl	2	34	7718.29	<.0001
Water	2	34	565.39	<.0001
Plant*NaCl	2	34	125.23	<.0001
Plant*Water	2	34	0.07	<.0001
NaCl*Water	4	34	68.52	<.0001
Plant*NaCl*Water	4	34	5.90	<.0001

Table 8: Analysis of variance of root/shoot ratio of wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters (Cov Parm) estimates. (*N*=3)

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0.00001	0.000015	0.66	0.2545 -
Residual	0.00009	0.000024.12	<.0001	
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	107.47	<.0001
NaCl	2	34	67.58	<.0001
Water	2	34	62.13	<.0001
Plant*NaCl	4	34	8.61	<.0009
Plant*Water	2	34	2.80	<.0747
NaCl*Water	4	34	1.21	<.3246
Plant*NaCl*Water	4	34	1.59	<.1986

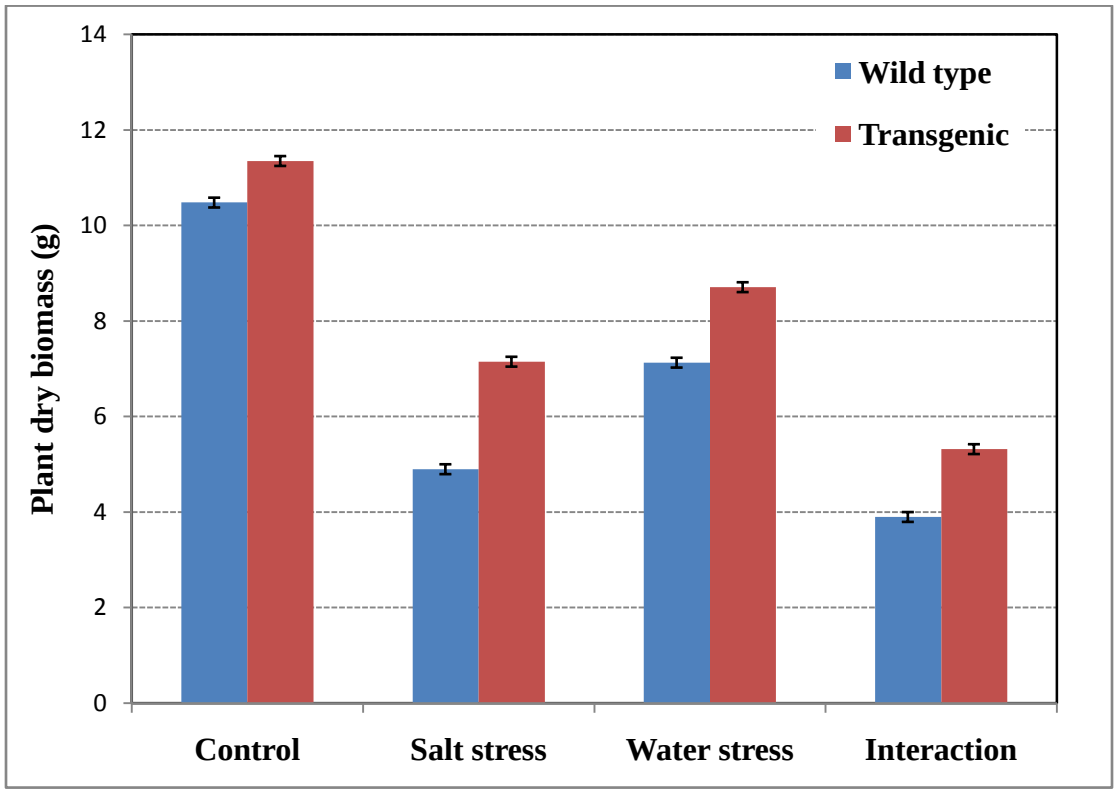

Figure 8: Plant dry biomass (g) of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

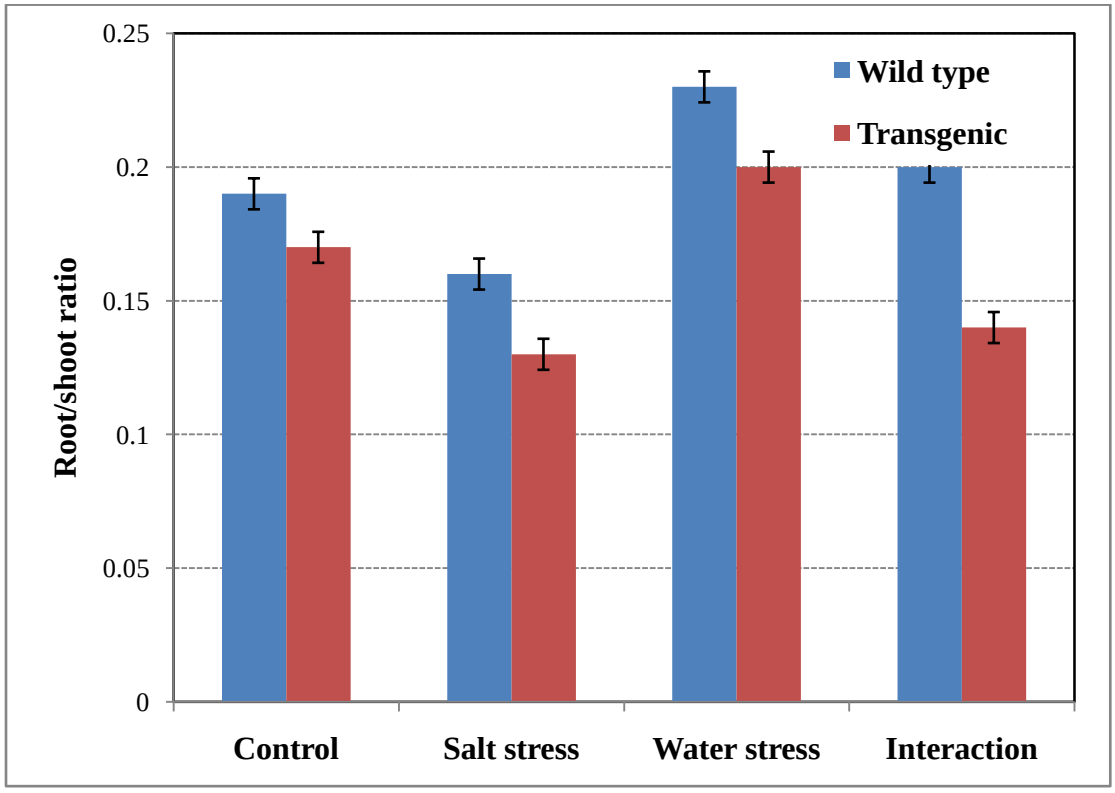

Figure 9: Root/shoot ratio of wild type and transgenic tobacco plants under normal growth conditions (0 mM NaCl and 80% FC) and under conditions of salt stress (100 mM NaCl at 80% FC), water stress (40% FC) and their interaction.

Table 9: Mean data of Plant height (cm), number of leaves and leaf area (cm^2) of wild type (WT) and transgenic (TR) tobacco plants under different NaCl concentrations and soil water content. Each value is mean±SE (N=3).

Growth parameters	Water content (% FC)	NaCl concentrations					
		0		100		200	
		WT	TR	WT	TR	WT	TR
Plant Height (cm)	80	56±0.4006	30±0.4006	34±0.4006	16±0.4006	14±0.4006	6±0.4006
	60	55±0.4006	29±0.4006	33±0.4006	15±0.4006	14±0.4006	6±0.4006
	40	42±0.4006	26±0.4006	26±0.4006	13±0.4006	12±0.4006	5±0.4006
No. of leaves	80	26±0.3239	14±0.3239	21±0.3239	12±0.3239	15±0.3239	6±0.3239
	60	26±0.3239	14±0.3239	21±0.3239	12±0.3239	15±0.3239	6±0.3239
	40	21±0.3239	12±0.3239	16±0.3239	10±0.3239	12±0.3239	5±0.3239
Leaf area (cm^2)	80	119±3.403	232±3.403	92±3.403	192±3.403	69±3.403	106±3.403
	60	106±3.403	218±3.403	75±3.403	181±3.403	57±3.403	97±3.403
	40	72±3.403	171±3.403	51±3.403	135±3.403	44±3.403	67±3.403

Table 10: Data of stem dry weight (g), leaf dry weight (g), root dry weight (g), dry biomass (g) and root/shoot ratio of wild type (WT) and transgenic (TR) tobacco plants under different NaCl concentrations and soil water content. Each value is mean±SE (N=3).

Growth parameters	Water content (% FC)	NaCl concentrations (mM)					
		0		**100**		**200**	
		WT	TR	WT	TR	WT	TR
Stem dry wt (g)	80	3.95±0.0276	2.58±0.0276	1.46±0.0276	0.89±0.0276	0.59±0.0276	0.36±0.0276
	60	3.87±0.0276	2.57±0.0276	1.44±0.0276	0.87±0.0276	0.55±0.0276	0.33±0.0276
	40	2.68±0.0276	2.02±0.0276	1.16±0.0276	0.67±0.0276	0.42±0.0276	0.27±0.0276
Leaf dry wt (g)	80	4.87±0.0923	7.13±0.0923	2.92±0.0923	5.45±0.0923	1.99±0.0923	2.30±0.0923
	60	4.81±0.0923	6.90±0.0923	2.83±0.0923	5.36±0.0923	1.94±0.0923	2.19±0.0923
	40	3.27±0.0923	5.20±0.0923	1.80±0.0923	3.96±0.0923	1.34±0.0923	1.55±0.0923
Root dry wt (g)	80	1.66±0.0171	1.64±0.0171	0.72±0.0171	0.81±0.0171	0.47±0.0171	0.40±0.0171
	60	1.59±0.0171	1.61±0.0171	0.69±0.0171	0.78±0.0171	0.42±0.0171	0.41±0.0171
	40	1.35±0.0171	1.49±0.0171	0.63±0.0171	0.69±0.0171	0.38±0.0171	0.34±0.0171
Dry biomass (g)	80	10.48±0.103	11.35±0.103	4.90±0.103	7.15±0.103	3.05±0.103	3.06±0.103
	60	10.27±0.103	11.08±0.103	4.96±0.103	7.03±0.103	2.9±0.103	2.92±0.103
	40	7.31±0.103	8.71±0.103	3.9±0.103	5.32±0.103	2.15±0.103	2.16±0.103
Root/shoot R	80	0.19±0.0058	0.17±0.0058	0.16±0.0058	0.13±0.0058	0.18±0.0058	0.15±0.0058
	60	0.18±0.0058	0.17±0.0058	0.16±0.0058	0.13±0.0058	0.17±0.0058	0.15±0.0058
	40	0.23±0.0058	0.20±0.0058	0.20±0.0058	0.14±0.0058	0.21±0.0058	0.19±0.0058

4.2 Differences in absolute and relative growth of tobacco plants

Mean data presented in Table 9 and Table 10 shows that both wild type and transgenic tobacco plants exhibited considerable differences in term of absolute and relative growth both under normal and stress growing conditions. Under normal growing conditions, wild type plants produced greater plant height, more number of leaves per plant and greater dry stem weight compared to transgenic plants. Transgenic plants, on the other hand, produced greater leaf area, leaf dry weight, root dry weight, and dry biomass. However, when these plants were exposed to NaCl and water stress, transgenic plants, relative to wild type plants, exhibited significantly less reduction in all growth parameters at 100 mM NaCl under all soil water contents with respect to their control, with the exception of plant height and stem dry weight where transgenic plants showed greater reduction relative to wild type plants. The interaction of salinity and water stress also caused significant reduction in all growth parameters in both types of plants. However, transgenic plants experienced less reduction under the combined effect of salinity and water stress than wild type plants with respect to their control. Under 200 mM NaCl, growth of both types of plants were severely inhibited.

4.3 CH_3Cl emission measurement

Data recorded on the CH_3Cl emission from transgenic tobacco plants under different NaCl concentrations and soil water content is presented in Table 13. Both NaCl salinity and soil water content significantly affected ($p<0.0001$) CH_3Cl emission from transgenic tobacco plants whereas no CH_3Cl emission was detected from wild type tobacco plants (Table 12). Similarly, the interaction between NaCl concentrations and soil water content was also significant ($p<0.0001$).

At 40% field capacity, when NaCl concentration was increased from 50 to 100 mM, CH_3Cl emission increased by 79%, followed by 86% and 82% increase in CH_3Cl emission when NaCl was increased from 50 to 150 and 200 mM, respectively. Likewise, at 60% field capacity, when NaCl concentration was increased from 50 to 100 mM, 53% increase in CH_3Cl emission was recorded, followed by 63% increase in CH_3Cl emission when NaCl was raised to 150 and 200 mM. Under 80% field capacity, when NaCl concentration was increased from 50 to 100 mM, a corresponding increase of 36% in CH_3Cl emission was noted. Under 80% field capacity, when NaCl concentration was raised from 50 to 150 mM and from 150 to 200 mM, an increase in CH_3Cl emission of 40% and 37% was recorded, respectively.

Under 50 mM NaCl concentration, when soil water content was lowered from 80% to 60%, and from 80% to 40%, CH_3Cl emission decreased by 15% and 38% respectively. Lowering soil water content from 80% to 60% and from 80% to 40% field capacity at 100 mM NaCl brought about 5% and 19% reduction in CH_3Cl emission

Table 11: Mean data analysis of variance of CH_3Cl emission (ng g^{-1} dry wt hr^{-1}) by wild type and transgenic tobacco plants under different NaCl concentrations and soil water content and covariance parameters estimates. *N*=6

Cov Parm	Estimate	Standard error	Z value	Pr > Z
Replications	0.000010	0.000015	0.66	0.2545
Residual	0.000092	0.000022	4.12	<.0001
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Plant	1	34	107.47	<.0001
NaCl	2	34	67.58	<.0001
Water	2	34	62.13	<.0001
Plant*NaCl	2	34	8.61	<.0001
Plant*Water	2	34	2.80	<.0001
NaCl*Water	4	34	1.21	<.0001
Plant*NaCl*Water	4	34	1.59	<.0001

respectively. Keeping NaCl at 150 mM, bringing soil field capacity 80% to 60% and from 80% to 40% yielded 1% and 17% reduction in CH_3Cl emission respectively. Likewise, keeping NaCl at 200 mM and lowering soil water content from 80% to 60% field capacity and from 80% to 40% field capacity resulted in 1% and 17% reduction in CH_3Cl emission respectively.

Taking average across all water contents, CH_3Cl emission increased by 53% as NaCl concentration was increased from 50 to 100 mM. However, when NaCl concentration was raised from 100 to 150 mM, the increase in CH_3Cl was only 4%. No significant increased in CH_3Cl emission was recorded when NaCl concentration was raised from 150 to 200 mM. Similarly taking average across all NaCl concentrations, decreasing soil water content from 80% to 40% caused 22% reduction in the CH_3Cl emission from the transgenic plants (Fig. 10).

4.4 Prospects of developing salt tolerance rice crop using TMT gene

The use of TMT gene technology appears to have some potential for developing transgenic crops, such as rice, thus bringing millions of hectares of salt affected soil into productive use. Rice is the most important food crop of the world, is most widely consumed cereal and is a source of staple food for 70% of the world's human population. Rice farms cover 11% of the world's arable land (IRRI, 2007). Asia dominates world rice production, and contains 90% of global rice area (FAO, 2002). Currently, south and southeast Asia occupies 54 million ha of saline soils, nearly 50% (27 million ha) of which are coastal saline soils located in the humid regions (Akbar & Ponnamperuma, 1982;

Massoud, 1974). These soils have great potential for rice cultivation because of favorable climatic conditions suitable for rice production (Thiruchelvam & Pathmarajah, 1999). The use of the TMT gene technology is an option to develop transgenic rice and thus bringing these millions of hectares of salt affected soil into productive use can be a major step forward in the global effort to boast food production and solve the problem of food insecurity.

However, a careful estimate of the possible CH_3Cl emission from such ecosystem is a pre-requisite before such a technology can be introduced. In order to carry out such estimation, the following three assumptions should be made: 1) that it is possible to transform rice plant with the TMT gene, 2) that transgenic rice plants emit similar quantity of CH_3Cl per gram dry biomass as do tobacco plant, and 3) that the prevailing salinity concentration under the proposed areas is 100 mM, and soil moisture content is 80% of the field capacity.

Prediction of CH_3Cl emission by transgenic rice crop grown under saline environments

- Total dry matter produced by rice per at maturity (at 100 mM NaCl) = 4.3 gm (Razzaque et al., 2009)
- Optimum Number of plant per hectare for rice crop = 250,000 (Hasanuzzaman et al., 2009)
- Total dry matter produced per hectare = 1,082,500 gm or 1,082.5 Kg

- CH_3Cl produced (ng/gm dry wt/hr) by transgenic tobacco plants = 1,728 (Table 13)
- CH_3Cl produced by transgenic rice plants gm^{-1} dry weight day^{-1} (at 100 mM NaCl and 80% FC) = 1,728* 24 = 41,472 ng = 0.0000415 gm
- CH_3Cl produced by rice plant gm^{-1} dry weight in 180 days = 0.0000415*180 = 0.0075 gm
- Total CH_3Cl produced by proposed rice crop per hectare in 180 days = 0.0075*1,082,500 gm = 8,118.75 gm
- CH_3Cl produced by the proposed rice crop under salinity on 27 million ha = 219,206,250,000 gm or 219,206,250 kg or 219.21 Gg

Now the total global emission of CH_3Cl to the atmosphere is 4,089 g per year (Xiao et al., 2009) ,which means that the possible emission of 219.21 Gg from transgenic rice crop grown under salinity over 27 million hectares will be only 5.36 % of the global emission of 4089 Gg. In order to investigate impact of such incremental change in the global atmospheric CH_3Cl budget over the stratospheric ozone, further laboratory and field experiments are needed.

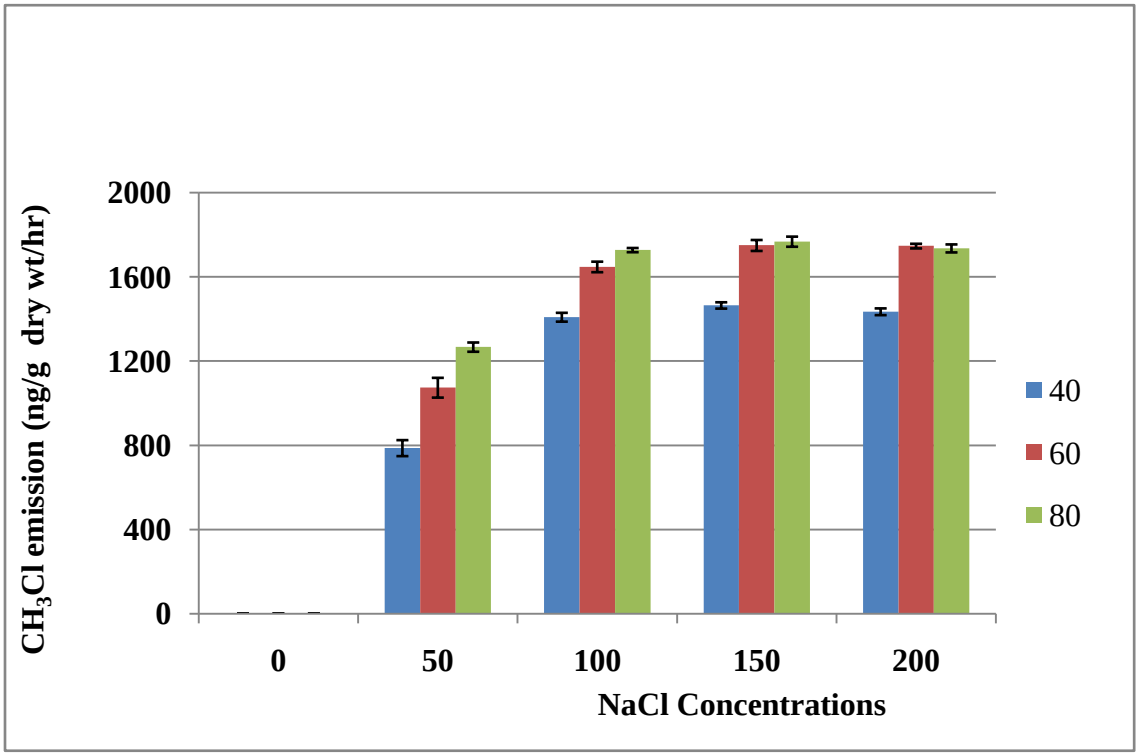

Figure 10: CH_3Cl emission (ng g^{-1} dry wt hr^{-1}) by transgenic transgenic tobacco plants under different NaCl concentrations and soil water content.

Table 12: Mean data of CH_3Cl emission (ng g^{-1} dry wt hr^{-1}) of wild type (WT) and transgenic tobacco (TR) plants under different NaCl concentrations and soil water content. Each value is mean±SE (N=6).

NaCl (mM)	CH_3Cl Emission (ng/gm dry wt/hr)					
	40% FC		60% FC		80% FC	
	WT	TR	WT	TR	WT	TR
0	-249E-15	7.74E-13	-219E-15	7.61E-13	8.16E-13	-321E-15
50	7.26E-14	787±6.11	-337E-16	1074±6.11	3.19E-13	1267±6.11
100	-798E-16	1409±6.11	-839E-16	1648±6.11	6.06E-13	1728±6.11
150	-573E-16	1465±6.11	-179E-16	1750±6.11	4.94E-13	1768±6.11
200	-257E-15	1435±6.11	-175E-15	1747±6.11	-573E-15	1736±6.11

Chapter 5

Discussion

Development of salt-tolerant crop varieties using selective breeding techniques has not proven successful (Ashraf, 2010; Yamaguchi & Blumwald, 2005), and developing salt tolerant plants through modern biotechnology has been considered a high research priority. The use of transgenic technology has been considered a viable option for generating plants with the ability to tolerate salts up to some extent (Wang et al., 2003). Several successful studies have shown that transgenic plants over-expressing a single gene can acquire high salinity tolerance in plants (Wang et al., 2004; Zhang & Blumwald, 2001; Zhang et al., 2001). The current research further explores the possibility of generating transgenic food crops having ability to tolerate the adverse conditions of salinity. A cabbage gene encoding thiol methyltransferase (TMT) enzyme was isolated (Attieh et al., 2002) and engineered into tobacco plants that otherwise lack the enzyme (Kaur, 2006). Studies with these TMT transgenic plants have revealed that they acquire the ability to transform Cl^- to CH_3Cl as well as a high degree of tolerance to NaCl salinity as compared to the untransformed plants (Kaur, 2006). These results clearly demonstrate that volatilization of chloride is a detoxification event that can contribute to the plant's ability to withstand salinity stress. The present study was designed to estimate the impact of CH_3Cl released by these transgenic plants on the atmospheric budget of CH_3Cl, with a view to determining if this technology could be successful deployed in the field to enhance salt tolerance in plants without major adverse effects on the environment.

Our results with tobacco transformed with TMT gene showed that both salinity and water stress caused significant reductions in plant height, number of leaves per plant, leaf area, root dry weight, stem dry weight, total plant dry weight of both transgenic and wild type plants. Under moderate salinity stress (100 mM NaCl), transgenic plants showed better growth as compared to the wild type plants. However, at high salinity stress (200 mM NaCl), growth of both transgenic, and wild type plants was completely inhibited. This effect was greatly dependent upon the soil moisture content. Plants growing under lower soil moisture level demonstrated stunted growth; however, an increase in soil moisture (from 40% to 80 % field capacity) resulted in an increase in growth of both wild type and transgenic plants. Both transgenic and wild tobacco plants experienced significant reduction in root/shoot ratio under salinity. Generally, the ratio declined with increasing salinity levels and with the increasing soil moisture content whereas it increased under water stress as well as under combined effect of salinity and water stress. The negative effect of salinity on the growth and development of plants has been well documented. Aragon and Alvarez (1988) reported significant reduction in tobacco yield under saline environments. In another study, exposure of tobacco plants to NaCl concentration of 200 mM resulted in significant reduction in the shoot dry weight but recorded hardly any difference in root dry weight or plant fresh weight (Flower et al., 1986). Similarly, Niknam et al (2004) observed significant reduction in the fresh, and dry weight of tobacco plants under salinity. Salinity caused significant reduction in tobacco plant height, plant dry matter, and markedly altered the dry matter partitioning between

stem and leaf (Sifola & Postiglione, 2002). These authors observed increased dry matter partitioning towards the leaf compared to the stem under elevated salinity concentrations. Several studies have shown that the ability of plants to growth is directly proportional to the availability of soil moisture (Biglouei et al., 2010; Chartzoulakis et al., 2002)

The interactive effect of salinity and water stress on tobacco plant growth may be linked to the restricted growth environment limiting the plant physiological access to water. Both these stresses lower the soil water potential, thereby making it hard for water to move into the plants (Munns & Tester, 2008) leading to water stress in plants causing inhibition of photosynthesis (Fisarkis et al., 2001). Reduction in the rate of photosynthesis leads to reduction in the rate of leaf surface expansion leading to cessation of expansion (Wang & Nil, 2000). Further, reduction in the vegetative growth, specifically leaf area, in plants implies that the area available for assimilation, and assimilate production would be reduced which would in turn reduce the overall growth and development of plants. Reduction in plant growth under lower soil moisture content can also be linked to reduction in nutrient uptake by the roots and transport from the roots to the shoots (Alam, 1999).

Production of many salinity-tolerant transgenic plants by single gene manipulation has been reported in the recent past. The two most common approaches employed for generating such transgenic plants have been engineering genes for ion transporters and osmoprotectants (Ashraf & Akram, 2009). Several studies have demonstrated that compartmentalization of Na^+ in the vacuole increases the salt tolerance

of most plant species, which depend on Na^+/H^+ antiporters as well as V-type H^+ -ATPases and H^+-PPases. For instance, over-expression of HbNHX1, a vacuolar Na^+/H^+ antiporter gene in tobacco resulted in increased tolerance in transgenic plants against salinity (Lu et al., 2005). Over-expressing of vacuolar Na^+/H^+ antiporter AtNHX1 resulted in about 18% improvement in shoot dry weight and 43% in root dry weight of tall fescue plants over control (Zhao et al., 2007). Transgenic *Arabidopsis* plants over-expressing vacuolar H^+ -PPases- AVP1 showed increased tolerance to salinity by sequestering Na^+ into the vacuole (Gaxiola et al., 2001). Zhang and Blumwald (2001) demonstrated the role of Na^+ compartmentation in plant salt tolerance by over-expressing AtNHX1 in tomato plants. These plants were able to grow, flower and set fruit in the presence of 200 mM NaCl. Similarly, the over-expression of Na+/H+ antiporter AlNHX1 in transgenic tobacco plants resulted in about 150% increase in relative dry weight per plant (Zhang et al., 2008). Likewise, the introduction of a vacuolar Na^+/H^+ antiporter from halophyte *Atriplex gmelini* conferred salt tolerance in rice (Ohta et al., 2002). It has also been demonstrated that the over expression of AtNHX1 resulted in enhanced salt tolerance in transgenic maize (Yan et al., 2004) and wheat (Xue et al., 2004). Similarly, the over-expression of BnNHX1 (*Brassica napus*), HbNHX1 (barley) and GhNHX1 (cotton) resulted in enhanced salt tolerance in transgenic tobacco plants (Lu et al., 2005; Wang et al., 2004; Wu et al., 2004). Also, salinity tolerance of tobacco (Karakas et al., 1997), wheat (Ma et al., 2008), tomato (Cortina & Culianez-Marcia, 2005), cabbage (Bhattacharya et al.,

2004) and rice (Su & Wu, 2004) crops have been improved through engineering genes for osmoprotectants.

Results also showed that CH_3Cl emission increased with the increase in NaCl concentrations; however, with 200 mM NaCl it reached plateau. Water deficit also remarkably reduced the rate of CH_3Cl emission. Decreasing soil moisture level (from 80% to 40% field capacity) caused substantial reduction. In contrast, there was no detectable amount of CH_3Cl emission from wild type plants. Our results are in conformity with the previous findings that transgenic plants containing TMT gene volatilize Cl^- to CH_3Cl in the presence of NaCl, and that the emission of CH_3Cl by transgenic plants is directly proportional to the concentration of NaCl in the growth medium (Kaur, 2006).

Although, tobacco plants containing TMT gene showed enhanced level of salinity tolerance, the fact that these plants volatilize Cl^- and contribute to the atmospheric budget of CH_3Cl is of great concern. The production of CH_3Cl as a result of chloride ion methylation may have serious consequences for the stratospheric ozone chemistry. Hence, to enable the successful utilization of this new mechanism for salinity tolerance, additional laboratory, and field experiments are required to reveal both the effects of environmental factors on the CH_3Cl emissions, and the influence of these emissions on the atmospheric budget of CH_3Cl.

The contribution of CH_3Cl to the total stratospheric chlorine is 15%, and it is a involved in depleting ozone layer (Montzka & Fraser, 2003). Any addition of CH_3Cl from transgenic plants would be in addition to the considerable amounts of this gas that originate from both natural and anthropogenic sources (Harper & Hamilton, 2003), with natural emissions far exceeding those from the anthropogenic sources (Xiao et al., 2009). Of the known sources, salt marshes (Rhew et al., 2003) and higher plants, tropical vegetations (Yakouchi et al., 2002; Yokouchi et al., 2007), rice (Redeker et al., 2004), and mangrove Forests (Manley et al., 2007) (Yokouchi et al., 2002) are the major contributors of CH_3Cl emissions.

In conclusion, results obtained in this study indicate a correlation between the ability of plants containing TMT gene to volatilize Cl^- to CH_3Cl, and their salinity tolerance. Transgenic plants developed a high degree of tolerance to salt stress as compared to wild type plants. All the transgenic plants were able to efficiently transform Cl^- to CH_3Cl. And finally, methylation of Cl^- to CH_3Cl in plants is a detoxification process that may contribute to plant's ability to become tolerant to saline environments.

Conclusion and future prospects

This study provides experimental data on the effect of engineering a novel thiol methyltransferase (TMT) gene into tobacco plants on their tolerance to sodium chloride stress. This enzyme has the ability to methylate Cl^- to CH_3Cl. Detailed experimental studies were conducted to evaluate the relationship between salt tolerance and chloride

volatilization capacity of transgenic plants. The first part of this study was to evaluate the salinity- and water-stress-tolerance of both transgenic and wild type plants. For that purpose, both types of plants were exposed to different salinity and water stress conditions. Results showed that under normal growing conditions, transgenic plants surpassed wild type plants in all growth parameters except plants height, number of leaves and dry stem weight. Under salinity and water stress conditions, transgenic plants showed better tolerance to these stresses by suffering less reduction in all growth parameters compared to wild type plants. Similarly, the interactive effect of salinity and water stress was more pronounced in wild type plants than in transgenic plants.

The second part of this study was to examine the chloride volatilizing capacity of transgenic plants. Both transgenic and wild type plants were grown under different NaCl concentrations and soil water content. Results showed that transgenic plants acquired an ability to efficiently transform Cl^- to CH_3Cl. Further, CH_3Cl emissions were higher under higher concentrations of NaCl and higher soil water content. No CH_3Cl emission was detected from wild type plants.

The final part of this study was to predict the possible global emissions of CH_3Cl from TMT engineered food crops if introduced on commercial scale on saline environments. To achieve that objective, an assumptive study was performed using a transgenic rice crop containing TMT gene grown over the saline environments in the saline coastal areas of south and Southeast Asia. Estimated data resulted from such study

revealed that the introduction of a transgenic rice crop in these areas covering 27 million hectares could add an extra 5.36% to the global CH_3Cl budget.

In general, results from this experiment clearly shows that the introduction of TMT gene confers salinity tolerance to plants, and suggests the possibilities for engineering a chloride detoxification capability into food crops to improve tolerance against chloride ion toxicity under saline environments. However, the production of CH_3Cl as a result of chloride ion methylation may have serious consequences for the stratospheric ozone chemistry. In order to enable the successful utilization of this new mechanism for salinity tolerance, additional laboratory, and field experiments are required to assess the influence of the possible CH_3Cl emissions from such ecosystems on the global CH_3Cl atmospheric budget.

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