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Effect of genetically engineering chloride methylation into bacteria and plants on their salinity tolerance

par

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Effect of genetically engineering chloride methylation into bacteria and plants on their salinity tolerance

présenté par

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ABSTRACT

The discovery that man-made halogenated gases such as chlorofluorocarbons have a role in the depletion of ozone in the stratosphere has provoked a growing interest in identifying and quantifying the principal sources and sinks of atmospheric CH_3Cl along with numerous attempts to understand the mechanisms of its biosynthesis and degradation in nature. CH_3Cl is produced from natural sources at a rate of 2.5-5 x 10^9 kg $year^{-1}$ [60, 80] and presently accounts for 15-16% of the atmospheric chlorine content and involved in ozone depletion in the stratosphere stratosphere [19, 35, 85].

New intensive measurements of oceanic emissions suggest that the global emission from this source is 310 x 10^6 kg $year^{-1}$ [43]. Man-made sources of CH_3Cl contribute 30 x 10^6 kg $year^{-1}$ [34, 60, 67]. Several terrestrial biological sources of CH_3Cl have been identified. Biomass burning is known to be a significant source of atmospheric CH_3Cl. The CH_3Cl is released during the rotting of wood by many fungi. The biosynthesis is widespread in the Hymenochaetaceae, a large family of wood-rooting fungi comprised of nearly 63 species. Over half of these species released CH_3Cl during growth, particularly those in the genera *Phellinus* and *Inonotus* [33, 37]. *Phellinus pomaceus* can volatilize over 90% of inorganic chloride, even at extremely low concentration of the ion [33]. Annual global flux from such fungi has been estimated at 160,000 tones [77]. Emission of CH_3Cl by higher plants represents another potential source of atmospheric CH_3Cl. Varns [75] first reported release of gaseous CH_3Cl by freshly harvested tubers of the potato (*Solanum tuberosum*). Similarly, a survey of 60 potato cultivars by Harper *et al.* [36] has demonstrated the emission of CH_3Cl by freshly harvested tubers ranged from 4 to 650 ng g^{-1} fwt d^{-1}. A survey of 118 species of flowering plants showed that leaf discs of a majority of these species produce halomethanes when floated on solution of 100 mM iodide ion [62].

Several organisms possess enzymes that can convert chloride ions to chloromethane gas through one-step enzymatic methylation of the former. For example, Wuosmaa and Hager (1990) reported that a marine alga *Endocladia muricata*, wood-rotting fungus *Phellinus pomaceus* and a halophytic plant *Mesembryanthemum crystallinum* posses a MCT that can methylate Cl^- ions to CH_3Cl [80]. Presence of this enzyme activity in

certain organisms that live in saline habitats has been interpreted as a possible mechanism for chloride detoxification via its volatilization. This possibility has never been experimentally tested. Dr. Saini's research group has cloned a cabbage gene encoding a thiol methyltransferase (TMT) enzyme that can methylate thiocyanate (^-SCN), bisulphate ion (HS^-) and a variety of aromatic thiolates ($R\text{-}S^-$) in the presence of S-adenosyl-L-methionine (AdoMet) as follows:

$^-SCN + AdoMet \rightarrow CH_3SCN + AdoHcy$

$HS^- + AdoMet \rightarrow CH_3SH + AdoHcy$

$RS^- + AdoMet \rightarrow CH_3R + AdoHcy$

TMT can also methylate halide ions, including chloride, through an analogous reaction:

$X^- + AdoMet \rightarrow CH_3X + AdoHcy$

Where: X = chloride (Cl^-), bromide (Br^-) or iodide (I^-)

Dr. Saini's group has engineered the enzyme into *E. coli* and root culture of potato and tobacco and tobacco plants that otherwise lack the enzyme as well as its usual metabolic context (Koonjul, Babayeva and Saini, unpublished). This group has also isolated *TMT1* over expressing and T-DNA insertion mutants of *Arabidopsis thaliana* in which the *TMT* gene has been silenced. These transgenic and mutant organisms were used to test whether the presence of *TMT* gene and the enzyme it encodes confers the ability to methylate Cl^- to CH_3Cl gas and whether this Cl^- volatilizing reaction is associated with the overall salinity tolerance of the species. Detailed physiological studies were performed to examine the relationship between salt tolerance and chloride volatilizing capacity of transgenic organisms.

The data presented in this study on bacterial cells demonstrate that the presence of TMT promotes the growth of bacterial cells; however it does not improve their tolerance to NaCl salinity despite the fact that their production of CH_3Cl from Cl^- containing medium increases at a higher rate. Furthermore, the results obtained with potato and tobacco roots indicate that all the *+TMT1* roots had better growth than *-TMT1* roots and were able to volatilize Cl^- to CH_3Cl.

Further experiments were undertaken at different growth stages of tobacco plants. Germination rate of seeds of +*TMT1* plants was similar to -*TMT1* plants under absence of NaCl. Although there was a reduction in the germination of seeds as the concentration of NaCl increased; no major differences were observed between these two plants. During early growth stage, all types of seedlings experienced similar growth in the absence of NaCl. Although, the growth of +*TMT1* plants was negatively impacted with an increase in NaCl, they were still able to maintain their growth and volatilized Cl^- at much higher levels as compared to -*TMT1* plants. In contrast, the growth of all the -*TMT1* plants under salinity stress was severely undermined and no detectable quantities of CH_3Cl were recorded.

The introduction of *TMT1* gene dramatically enhanced the tolerance of tobacco plants to sodium chloride salinity at later growth stage. The +*TMT1* and -*TMT1* plants experienced comparable growth in all aspects of the analysis when grown without NaCl. Furthermore, when exposed to moderate levels of salt stress, both +*TMTI* and -*TMT1* plants were able to survive, flowered and set viable seeds. However, +*TMT1* plants performed better in all aspects of growth as compared to -*TMT1* plants. Next, when both +*TMT1*, and -*TMT1* plants were exposed to higher levels of salt stress (200 mM), +*TMTI* plants were able to flower and set viable seeds; whereas -*TMT1* plants could not tolerate this concentration and ultimately died. These results suggested that TMT can play a role in the adaptation to salinity when it is introduced into a species that does not contain its normal metabolic context, and this may be useful in engineering salt tolerant crop plants using this new pathway.

In contrast, there was no change in salt tolerance when *tmt1* gene was silenced or over-expressed in *Arabidopsis*. It was further noted that at 200 mM NaCl, wild type, *TMT1* over-expressing and TMT-mutated *Arabidopsis* plants were severely inhibited and ultimately died. This is presumably because *Arabidopsis* naturally contains the *tmt1* gene, and when its natural thiol substrates are present TMT does not efficiently methylate Cl^-.

The results presented here indicate a correlation between ability of TMT to volatilize Cl^- and confirm a role of TMT in enhancement of salt tolerance. Taken together, results showed that all the engineered species acquired an ability to efficiently transform chloride ions to chloromethane. Parallel with this, the transformed plants developed a high degree of tolerance to NaCl salinity, which was toxic to the untransformed counterparts. The results

convincingly demonstrate that volatilization of chloride is a detoxification event that can contribute to the plant's ability to withstand salinity stress.

These results demonstrate a major role in developing salt tolerant plants by means of introducing *TMT* gene and suggest possibilities for engineering a chloride detoxification capability into a high value crop to improve tolerance against chloride ion toxicity under saline conditions.

RÉSUMÉ

La découverte des effets néfastes des gaz halogénés fabriqués par l'homme tels que les chlorofluorocarbones sur la couche d'ozone dans la stratosphère, a suscité un intérêt croissant pour l'identification et la quantification de sources et puits majeurs de chlorométhane CH_3Cl. De plus, des efforts de recherche ont été déployés afin de mieux comprendre les mécanismes de sa biosynthèse et de sa dégradation chez les êtres vivants. Le CH_3Cl d'origine naturelle est produit à un taux de 2.5 à 5 x 10^9 kg an-^{1} [60, 80], représente 15 à 16% du chlore atmosphérique et cause des dommages significatifs à la couche d'ozone stratosphérique [19, 35, 85].

Des mesures récentes d'émissions océaniques suggèrent que cette source produit 310 x 10^6 kg an^{-1} de CH_3Cl [43]. Les activités humaines contribuent à la production de CH_3Cl à hauteur de 30 x 10^6 kg an^- [34, 60, 67]. Plusieurs sources biologiques terrestres de CH_3Cl ont été identifiées. La combustion de biomasse est une source significative de CH_3Cl atmosphérique, ainsi que le pourrissement du bois par divers champignons. En effet, la biosynthèse de CH_3Cl est très répandue chez les Hymenochaetaceae, une famille de pourritures du bois comprenant près de 63 espèces. Plus de la moitié produisent du CH_3Cl lors de leur croissance, en particulier celles du genre *Phellinus* et *Inonotus* [33, 37]. *Phellinus pomaceus* peut volatiliser plus de 90% de chlorure (Cl^-) inorganique, même à très basse concentration de l'ion [33]. Le flux global annuel à partir de tels champignons a été estimé à 160 000 tonnes [77]. L'émission de CH_3Cl par les plantes supérieures représente une autre source potentielle de CH_3Cl atmosphérique. Varns [75] a été le premier à documenter la production de CH_3Cl gazeux par des tubercules de pomme de terre (*Solanum tuberosum*) fraîchement récoltés. De même, une étude sur 60 cultivars de pomme de terre par Harper *et al.* [36] a montré que leurs tubercules fraîchement récoltés émettent 4 à 650 ng g^{-1} pf j^{-1} de CH_3Cl. Dans une étude portant sur 118 espèces de plantes à fleurs, la plupart dont les disques foliaires ont été mis à flotter sur une solution d'ion iodure de 100 mM ont produit des halométhanes.

Plusieurs organismes sont capables de convertir l'ion Cl^- en CH_3Cl gazeux par une réaction enzymatique de méthylation en une étape. Par exemple, Wuosmaa et Hager (1990) ont montré que l'algue marine *Endocladia muricata*, la pourriture du bois *Phellinus*

pomaceus et la plante halophytique *Mesembryanthemum crystallinum* possèdent toutes une chlorure-méthyltransférase (MCT) qui peut méthyler Cl^- en CH_3Cl. La présence de cette enzyme chez certains organismes vivant dans des milieux salins a suggéré son implication possible dans un mécanisme de détoxification du chlorure par volatilisation. Cette possibilité n'a jamais été testée de façon expérimentale.

Le groupe de recherche du Dr. Saini a cloné un gène du chou qui code pour une thiol-méthyltransférase (TMT) capable de méthyler le thiocyanate (^-SCN), l'ion bisulphate (HS^-) et divers thiolates aromatiques ($R\text{-}S^-$) en présence de S-adénosyl-L-méthionine (AdoMet), comme suit :

$^-SCN + AdoMet \rightarrow CH_3SCN + AdoHcy$

$HS^- + AdoMet \rightarrow CH_3SH + AdoHcy$

$RS^- + AdoMet \rightarrow CH_3R + AdoHcy$

La TMT peut aussi méthyler les ions halogénures, notamment le chlorure, par une réaction analogue :

$X^- + AdoMet \rightarrow CH_3X + AdoHcy$

Où: X = chlorure (Cl^-), bromure (Br^-) ou iodure (I^-).

Le groupe du Dr. Saini a introduit la TMT par transgenèse dans *E. coli*, dans des cultures racinaires de la pomme de terre et du tabac, et dans des plants de tabac naturellement dépourvus de cette enzyme et de son contexte métabolique (Koonjul, Babayeva et Saini, non publié). Ce groupe a également transformé des plants d'*Arabidopsis thaliana* pour sur-exprimer *TMT1*, et isolé des mutants d'*Arabidopsis* n'exprimant plus le gène *TMT* suite à une insertion d'ADN-T. Ces transformants ont servi à tester si la présence du gène *tmt* et de l'enzyme qu'il code permet de méthyler Cl^- en CH_3Cl, et si la réaction de volatilisation de Cl^- est associée à une tolérance à la salinité. Des études physiologiques détaillées ont été menées sur les organismes transgéniques afin d'examiner la relation entre leur tolérance au stress salin et leur capacité à volatiliser l'ion chlorure.

Les données de cette étude sur *E. coli* démontrent que la présence de la TMT a stimulé la croissance des bactéries. Cependant, elle n'a pas amélioré leur tolérance au stress du NaCl malgré une forte production de CH_3Cl à partir du milieu contenant l'ion Cl^-. Par ailleurs, les résultats obtenus avec les racines de pomme de terre et de tabac indiquent que

toutes les racines +*TMT1* avaient un taux de croissance plus élevé que les racines -*TMT1* et pouvaient volatiliser Cl^- en CH_3Cl.

D'autres expériences ont été réalisées sur les plants de tabac à différents stades de croissance. Le taux de germination de graines +*TMT1* était comparable à celui de graines -*TMT1* en l'absence de NaCl. Bien que le taux de germination ait baissé à des concentrations plus élevées de NaCl, aucune différence majeure n'a été observée entre les deux types de plants. Aux premiers stades de développement, tous les types de graines ont montré un même taux de croissance en l'absence de NaCl. Même si des concentrations plus élevées de NaCl ont eu un impact négatif sur la croissance des plants +*TMT1*, ceux-ci ont pu maintenir leur croissance et volatiliser Cl^- à des taux plus élevés que les plants
-*TMT1*. Au contraire, le stress salin a sévèrement réduit la croissance des plants -*TMT1* et dans ce cas, les niveaux de CH_3Cl produits n'ont pu être détectés.

L'introduction du gène *TMT1* dans les plants de tabac a renforcé leur tolérance au stress salin aux stades ultérieurs de croissance. En l'absence de NaCl, les plants +*TMT1* et -*TMT1* ont eu une croissance semblable selon les divers critères d'analyse. Les plants +*TMT1* et -*tmt1* ont survécu à des niveaux modérés de stress salin, ont pu fleurir et donner lieu à des graines viables. Toutefois, la croissance des plants +*TMT1* était meilleure que celle des plants -*TMT1*. Par ailleurs, lorsque les plants +*TMT1* et -*TMT1* ont été exposés à un fort stress salin (200 mM de NaCl), les plants +*TMT1* ont pu fleurir et produire des graines viables alors que les plants -*TMT1* n'ont pu tolérer cette concentration et ont fini par mourir. Ces résultats suggèrent que la TMT peut jouer un rôle dans l'adaptation au stress salin si elle est introduite dans une espèce dépourvue d'un tel contexte métabolique, et que cette voie métabolique peut être utile à la création de plantes génétiquement modifiées pour résister au stress salin.

Par contre, aucun changement dans la tolérance au sel n'a été observé pour les plants d'*Arabidopsis* dont le gène *TMT1* a été sur-exprimé ou rendu silencieux. De plus, il a été noté qu'à 200 mM de NaCl, les plants d'*Arabidopsis* sauvages, sur-exprimant *TMT1* ou mutés dans le gène *TMT* n'ont pu maintenir leur croissance et ont fini par mourir. Une explication possible est qu'*Arabidopsis* exprime le gène *TMT1* et qu'en présence de ses substrats thiols naturels, la TMT ne méthyle pas Cl^- efficacement.

Les résultats présentés ici indiquent une corrélation entre la capacité de volatiliser Cl^- et la tolérance au stress salin, et confirment ainsi le rôle de la TMT dans la tolérance accrue au stress salin. Dans leur ensemble, les données montrent que toutes les espèces manipulées génétiquement ont acquis la capacité de transformer les ions chlorure en gaz chlorométhane. De plus, les plants transformés ont développé un haut degré de tolérance à des niveaux de stress au NaCl qui se sont révélés toxiques pour les plants non transformés. Les données prouvent que la volatilisation du chlorure est un moyen de détoxification qui peut permettre à la plante de survivre dans des conditions de stress salin.

Ces résultats démontrent qu'il est possible de développer des plantes tolérantes au sel par introduction du gène *TMT* dans leur génome, et suggèrent de nouveaux moyens d'améliorer la tolérance de plantes d'intérêt économique à la toxicité de l'ion chlorure en conditions de forte salinité.

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ABBREVIATIONS

CH_3Cl	:	Chloromethane
CH_3I	:	Idomethane
CH_3SH	:	Methanthiol
CH_3X	:	Halomethane
ESP	:	Exchangeable Na^+ percentage
H/BMT	:	Halide/bisulfide methyltransferase
HS^-	:	Bisulphate ion
GC	:	Gas chromatograph
IPTG	:	Isopropyl β-D-thiogalactopyranoside
MCT	:	Methyl chloride transferase
MS	:	Murashige and Skoog basal salt mixture
PEG	:	Polyethylene Glycol 8000
R-NCS	:	Isothiocyanates
$R\text{-}S^-$	:	Organic thiolates
AdoMet	:	S-adenosyl-L-methionine
AdoHcy	:	S-adenosyl-L-homocystene
SCN	:	Thiocyanate
TMT	:	Thiol methyltransferase
+*TMT1*	:	Organism transformed with *TMT1* gene
-*TMT1*	:	Organism transformed without *TMT1* gene
f. wt	:	Fresh weight

ACKNOWLEDGEMENTS

As I stand at end my journey through the Masters Program, a number of friendly faces come to mind when I think back at this incredible voyage. There have been some bumps along the road and some smooth rides.

First, I would like to extend my gratitude to my supervisor, Deep Saini for making this expedition a great learning experience for me. Deep taught me how to approach all sorts of problems and provided guidance on tackling different issues that I faced in my research. His continuous support throughout this program has been a tremendous help and the reason for successful completion of my Masters Program and Research initiatives. Above is only an attempt to put my appreciation for Deep's support in words, however there is a lot more that I would like to express but will hold it until I find the right ones.

There are many other helping faces that come to mind, and without their support and encouragement my masters program would not have been as enjoyable and resourceful. Priyum Koonjul and Sima Babayeva are two of these friendly and supportive faces from my lab that assisted me and answered any questions posed to them during my research. They are not just my colleagues but good friends that made my experience both in Canada and in my research a pleasant one. I would also like to thank Eric Claeyssen for his help. In addition, I would like to thank everyone else in the lab for their support and help with my research.

I am also thankful to my co-supervisor Anja Geitmann for her help. Furthermore, I would like to thank other faculty members that assisted me throughout my program. Also the green house staff has been a great help with my education and research and for that I extend my special appreciation to them.

Last but not least I would like to thank my family for all their support and encouragement throughout my education. Again words come short to show even a shred of appreciation for what they have done for me; all I can say is "Thank You".

DEDICATION

To my Mom and Dad and to my Uncle.

-with all my love.

1. INTRODUCTION

1.1. Historical view

The discovery that man-made halogenated gases such as chlorofluorocarbons have a role in the depletion of ozone in the stratosphere has provoked a growing interest in identifying and quantifying the natural emissions of chlorine-containing gases. Chloromethane (CH_3Cl) is the most important naturally occurring gas among a handful of chlorine-containing trace gases in the atmosphere; it presently accounts for 15-16% of the atmospheric chlorine content, and involved in ozone depletion in the stratosphere [19, 35, 85]

It is estimated that CH_3Cl, the most abundant halocarbon in the atmosphere, is produced from natural sources at a rate of 2.5-5 x 10^9 kg $year^{-1}$ [60, 80]; the contribution of 30 x 10^6 kg $year^{-1}$ from man-made sources is negligible in comparison [34, 60, 67]. Although historically the oceans have been regarded as the major source of atmospheric CH_3Cl [66, 67], new intensive measurements of oceanic emissions suggest that the global emission from this source is 310 x 10^6 kg $year^{-1}$ [43]. This source is, therefore considerably smaller than previous estimates of about 2.1 x 10^9 kg $year^{-1}$ [42, 48, 67, 69], raising the question of where the remainder comes from. The terrestrial sources of the atmospheric CH_3Cl, which are predominately tropical, dominate the atmospheric budget [48, 61, 69, 85], suggesting the greater importance of tropical islands as a source. Several terrestrial biological sources of CH_3Cl have been identified. Biomass burning is known to be a significant source of atmospheric CH_3Cl [14]. The CH_3Cl is released during the rotting of wood by many fungi. The biosynthesis is widespread in the *Hymenochaetaceae*, a large family of wood-rooting fungi comprised of nearly 63 species. Over half of these species released CH_3Cl during growth, particularly those in the genera *Phellinus* and *Inonotus* [33, 37]. *Phellinus pomaceus* can volatilize over 90% of inorganic chloride, even at extremely low concentration of the ion [33]. Annual global flux from such fungi has been estimated at 160,000 tones [77]. Emission of CH_3Cl by higher plants represents another potential source of atmospheric CH_3Cl. Varns [75] first reported release of gaseous CH_3Cl by freshly harvested tubers of the potato (*Solanum tuberosum*). Similarly, a survey of 60 potato cultivars by Harper *et al.* [36] demonstrated the emission

of CH_3Cl by freshly harvested tubers ranged from less than 4 to 650 ng g^{-1} fwt d^{-1}. A survey of 118 species of flowering plants showed that leaf discs of a majority of these species produce halomethanes when floated on solution of 100 mM iodide ion [62].

With a view to explaining the biochemical basis for CH_3Cl emissions, Wuosmaa and Hager [80] reported a methyl chloride transferase (MCT) that catalyzes the methylation of halide ions (X^-) to halomethanes (CH_3X). The enzyme activity was found in a wood-rotting fungus (*Phellinus pomaceus*), a marine alga (*Endocladia muricata*) and a halophytic plant (*Mesembryanthemum crystallinum*). The fact that two of these species grow under saline conditions, suggested that the enzyme could be part of a salt-tolerance mechanism involving volatilization of chloride (Cl^-) to CH_3Cl.

To test whether there was an association between MCT activity and relative salt tolerance conferred by volatilization of Cl^- to CH_3Cl, a broader survey of 118 different species of higher plants was done in Dr. Saini's laboratory [62]. They found that the majority of higher plants tested possessed an enzyme capable of methylating I^- to CH_3I. KI was used as the substrate because the enzyme methylated I^- with the greater efficiency among other halides [62]. However, 21 species that are generally considered to be halophytic or salt tolerant, including *Mesembryanthemum crystallinum*, in which MCT activity was first reported, had relatively low emission rates. Further, halide methyltransferase activity did not increase under greenhouse conditions upon controlled salinization of three relatively salt tolerant species, *Portulaca oleracea*, *Beta vulgaris* and *M. crystallinum*. In fact, the activity was suppressed in *M. crystallinum*.

On the other hand, the highest activities were recorded in the order Capparales, represented by 15 species of the family Brassicaceae and one of Resedaceae. This suggested that a function other than or in addition to halide methylation may exist for this enzyme. Further results showed that *Brassica oleracea*, which had one of the highest emission rates in this survey, and other 19 species containing halide methyltransferase activity could also methylate bisulphate (HS^-) ions to methanthiol (CH_3SH).

To elucidate the biochemical basis for these emissions, Attieh *et al.* [8] purified and characterized an enzyme from cabbage, which catalyzed the *S*-adenosyl-L-methionine (AdoMet) dependent methylation of X^- and HS^- ion to monohalomethanes and CH_3SH, respectively. They tentatively named this enzyme halide/bisulfide

methyltrasnferase (H/BMT). Further biochemical evidence showed that the enzyme had a relatively high affinity for HS^- and a substantially lower affinity for Cl^-, which is its only physiologically relevant halide substrate [8]. These results suggested that the role of H/BMT was more likely to be in sulphur metabolism than in halide detoxification.

One possible role of H/BMT could be detoxification of excess HS^-, which is the end product of sulphate reduction. This idea was ruled out by the findings that chloroplasts, the primary site of sulphate reduction lacked this activity, the enzyme was predominantly cytosolic and a number of modulators of sulphate reduction, did not affect its activity [9]. An alternative is a role in the methylation of sulfur containing products arising from the catabolism of glucosinolates, which are secondary metabolites accumulated by all members of Brassicaceae. Further results show that glucosinolate accumulating plants had high levels of this activity, whereas members of Papaveraceae (family that lacks glucosinolates but from which many methyltransferases have been purified) and Alliaceae (which accumulate organic disulphides but not glucosinolates) lack this activity. Kinetic studies revealed that the purified enzyme preferred thiocycnate 4,4'-thiobisbenzenethiol, thiophenol, and salicylic acid as substrates. The enzyme had K_m values of 11, 51, 250 and 746 mM for thiocyanate 4,4'-thiobisbenzenethiol, thiophenol, and thiosalicylic acid, respectively [9]. Hence, it was renamed thiol methyl transferase (TMT).

The main role of TMT appears to be in the metabolism of glucosinolates. Glucosinolate containing plants have a glucohydrolase enzyme, myrosinase, which initiates the degradation of glucosinolates [7]. This enzyme comes in contact with the glucosinolates when tissue is disrupted, for example during attack by insects and other herbivores, and degrades them into a variety of compounds such as thiocyanates (R-SCN), isothiocyanates (R-NCS), thiocyanate ion (^-SCN), organic thiolates ($R\text{-}S^-$) and elemental sulphur. TMT catalyzes methylation of the hydrolysis products of glucosinolates.

Five isoforms of TMT were purified and characterized [10] and 2 gene encoding the enzyme TMT have been cloned in Dr. Saini's laboratory [7]. These isoforms could methylate ^-SCN, HS^- and a variety of aromatic $R\text{-}S^-$ in the presence of AdoMet as follows:

$^{-}SCN + AdoMet \rightarrow CH_3SCN + AdoHcy$

$HS^{-} + AdoMet \rightarrow CH_3SH + AdoHcy$

$RS^{-} + AdoMet \rightarrow CH_3R + AdoHcy$

TMT can also methylate halide ions, including chloride, through an analogous reaction:

$X^{-} + AdoMet \rightarrow CH_3X + AdoHcy$

Where: X = chloride (Cl^{-}), bromide (Br^{-}) or iodide (I^{-})

Similarly, putative methyl chloride transferases (MCTs) enzymes that have been reported in several organisms, such as marine algae, fungi, and halophytic plants, can convert chloride ions to chloromethane gas through one-step enzymatic methylation of the former. For example, Wuosmaa and Hager [80] reported that a marine alga (*Endocladia muricata*) and a halophytic plant (*Mesembryanthemum crystallinum*) posses an MCT that can methylate Cl^{-} ions to CH_3Cl. To date, the only known MCT has been characterized from *Batis maritima*, which is a halophytic species [57]. Presence of this enzyme activity in organisms that live in saline habitats has been interpreted as a possible mechanism for chloride detoxification via its volatilization. This possibility has never been experimentally tested. On the other hand, TMT enzyme can also methylate chloride, in addition to its natural thiol substrates, and it has been shown that the K_m value of TMT for Cl^{-} (85 mM) [8] is lower than that of the *B. maritima* MCT (155 mM) [57]. Dr. Saini's group has engineered the enzyme into *E. coli* and several plant species that otherwise lack the enzyme as well as its usual metabolic context (Koonjul, Babayeva and Saini, unpublished). This group has also isolated T-DNA insertion mutants of *Arabidopsis thaliana* in which the *TMT* gene has been knock-out. I have used these transgenic and mutant organisms to test whether the presence of *TMT* gene and the enzyme it encodes confers the ability to methylate Cl^{-} to CH_3Cl gas and whether this Cl^{-} volatilizing reaction is associated with the overall salinity tolerance of the species.

Several crop species, including rice, barley, soybean, grapevine, cotton, citrus and avocado, are known to be sensitive to Cl^{-} ion toxicity [31, 45, 62]. Therefore, if a positive association between the presence of TMT, Cl^{-} volatilization and salt tolerance could be confirmed, genetic engineering of *TMT* gene into TMT-lacking species could be used to

enhance their salt tolerance against chloride ion salinity and improve their yield under salt conditions.

1.2. Salt tolerance of plants

Approximately 7% of the world's land area, which amounts to 930 million hectares, is affected either by salinity or the associated conditions of sodicity [52]. A global study of land use found that 6% of world's land area become saline over 45 years [28]. It is estimated that about 25-50% of the world's irrigated land has been damaged by salinity [2, 64]. Irrigated land represents only 15% of total cultivated land of the world, but as irrigated land has at least twice the productivity of rain fed land, it produces one-third of the world's food [53].

Salinity is generally defined as presence of the excessive amounts of soluble salts that inhibit plant growth. It is measured in terms of electric conductivity (EC_e), or with the sodium adsorption ratio (SAR- proportion of sodium to multivalent ions in the soil solution, especially Ca^+ and Mg^{2+}) [11] or as the exchangeable Na^+ percentage (ESP- the ratio of sodium to the total cation exchange capacity). A soil is classified as saline or salt-affected when the EC_e of its saturated paste extract exceeds 4 dSm^{-1} equivalent to 40 mM NaCl [53, 74]. When saline soils have a low concentration of soluble salts but a high ESP, they are considered sodic. When ESP is $\geq$ 15 with a high pH of 8.5-10, they are classified as alkaline soils [74].

Under sodic conditions plant growth is mainly affected by poor soil conditions. In dry sodic soils, clay particles are linked with Na^+ ions and remain as aggregates [11]. On wetting by rain or fresh water of low salinity, Na^+ ions become highly hydrated leading to dispersion of clay particles as single individuals [11]. Because there is only a narrow range of available water over which plant roots can grow and sustain plant function in a sodic soil, root growth gets restricted either by too wet (immediately after rain or irrigation) or by too dry soil (after a few days of fine weather) [11]. Under saline conditions, plant growth is affected by either an osmotic effect, which reduces the plant's ability to take up water, or by direct toxicity of salt ions.

1.2.1. Physiological effects of salinity on plants

Salinity affects many aspect of plant metabolism, and as a result, it reduces plant growth. The excess amount of salt in soil water makes it harder for plants to extract water from the soil [13]. This leads to an overall reduction in growth rate. This is referred to as the osmotic or water-deficit effect of salinity. In addition, salt may enter the transpiration stream and eventually injure cells in the transpiring leaves. This is salt-specific effect of salinity [31, 52]. These two responses occur simultaneously, resulting in a two-phase growth response to salinity [51, 52]. The first phase of the growth response is due to the osmotic effect of salt in the soil solution, and is quickly apparent. The salt in the soil solution reduces leaf growth. It also inhibits the root growth but to a lesser extent and decreases stomatal conductance and thereby photosynthesis [51]. This leads to an overall reduction in growth rate and is presumably regulated by hormonal signals coming from the roots. These symptoms are identical to those in drought-affected plants. The second phase of effects on growth results from the toxic effects of salt inside the plant and takes time to develop. If an excessive amount of salts enters in the transpiration stream of plants, there is an injury to cells in the transpiring leaves [52]. As the concentration in the wall starts to rise, the cell will shrink, the concentration of ions inside will rise, and some will efflux. The ion concentration in the wall will rapidly escalate, and the cell will rapidly dehydrate [54]. Being the actively transpiring parts of the plant, the leaves accumulate salt over a long period of time, eventually resulting in very high Na^+ and Cl^- concentration, which leads to premature senescence of the leaves [52]. This reduces the amount of assimilate that the plant can produce, and a reduction in assimilate transport to the growing tissues may cause further reduction in growth [52].

1.2.2. Time-dependent changes

When plants are exposed to salinity under laboratory conditions, there is an immediate reduction in leaf and root elongation rate within the first few seconds or minutes, followed by a rapid partial recovery of growth [52]. This recovery remains steady over hours but with reduced rate of leaf and root elongation. Continued transport of salt into transpiring leaves eventually results in salt build up to excessive concentration causing injury in older leaves.

After weeks, injury becomes visible as yellowing or death of older leaves [52]. The rate of leaf death is crucial for the survival of plant. If the rate of production of younger leaves is greater than that at which older leaves die, there might be enough photosynthesizing leaves for the plant to produce some flowers and seeds [53]. However, if the rate of leaf death exceeds the rate at which new leaves are produced, the plant may not survive to produce seed [53]. After months, there is a clear difference between plants with high and low salt uptakes [52].

Under saline conditions, for an annual plant, there is a race against time to produce flowers and form seeds while there is still an adequate leaf area to supply the necessary photosynthate. In perennials, there is an opportunity to enter a state of dormancy, and thus survive the stress.

1.2.3. Mechanisms of salt tolerance

On the basis of two-phase effects of salinity on plant growth, mechanisms of salt tolerance fall into two main categories:

1. Mechanisms controlling the osmotic effect of salt
2. Mechanisms controlling the salt-specific effect of salt

Based on their capacity to grow on high salt medium, plants are classified as halophytes or glycophytes [23]. Most plants, including a majority of crop species, are glycophytes and cannot tolerate high salinity. Glycophytes have poor ability to restrict the uptake of salt and are also unable to compartmentalize salts once they buildup to toxic levels in the transpiring leaves [52]. Halophytes are tolerant to high salinity and show a wide range of adaptations from morphological to biochemical levels that include both type of mechanisms [22, 30, 71]. They have the ability to exclude salt at high rate, and effectively compartmentalize the salts that inevitably get into cell-vacuoles. This mechanism allows them to grow for a long period of time in saline soil [22, 52]. For example, salt concentration in the xylem sap of costal mangrove *Avicennia marina* stays as low as 9 mM for plants growing in 500 mM NaCl, that is, 98% exclusion is achieved [12]. Some halophytes also utilize salt-secreting glands to remove excess ions from their leaves [22]. For example, the salt glands of *Aegiceras*

corniculatum can excrete up to 90% of salt reaching their leaves under high salt conditions [12]

1.2.3.1. Mechanism controlling the osmotic effect of salt

Salts negatively affect the osmotic potential in the soil which reduces availability of water to roots. Plants must process a lower osmotic potential than their medium if they are to take up water and nutrients from a saline soil. An increased production of compatible solutes is an adaptive strategy to lower the osmotic potential of cell cytosol, found in plants in response to salt stress. They are termed compatible because they have low molecular weight, are highly soluble in water, are electrically neutral and do not interfere with the plant's metabolic processes [32]. They are also called osmolytes as they generate an osmotic potential high enough to have an important osmotic function, especially if concentrated in a small cell compartment. They protect plants from stress by increasing the osmotic potential and thus the retention of water in the cytoplasm, by detoxification of radical oxygen species (ROS) or by stabilization of quaternary structure of protein [16, 17, 82].

There are four main classes of organic solutes that accumulate most commonly and could have an osmotic or protective role: N-containing solutes such as proline and glycine betaine; sugars such as sucrose and raffinose; straight-chain polyhydric alcohols (polyols) such as manitol and sorbitol; and cyclic polyhydric alcohols (cyclic polyols) [53]. Different solutes accumulate in different species. For example, glycine betaine was found to be the major organic substrate that accumulates in a diverse range of plant species as well as in bacterial and animals [11]. Accumulation of glycine betaine has also been reported under salt stress, for example in barley [41]. Proline is regarded as a general stress metabolite and accumulates in all species up to enormous concentrations in a great variety of stress situations, particularly in relation to salt stress [68, 73]. There are many articles where genetically engineered over-production of these osmotic solutes has been shown to enhance salt tolerance. For example, coordinated expression of a bacterial choline dehydrogenase gene (betA) and betaine aldehyde dehydrogenase gene (betB) in tobacco conferred accumulation of glycine betaine and increased tolerance to salt [38]. In another example, Abebe *et al* [1] expressed bacterial mannnitol-1-phosphate dehydrogenase (mt1D) in wheat.

The transgenic plants were more tolerant to salt as compare with control plants. Similarly, expression of IMT1 (myo-inositol O-methyl transferase) of the common ice plant in tobacco conferred tolerance to drought and salinity stress [65].

1.2.3.2. Mechanism controlling the salt-specific effect of salinity

The mechanisms that operate to impart tolerance fall into two categories: those minimizing the entry of salt into the plant and those minimizing the concentration of salt in the cytoplasm.

Mechanisms minimizing the entry of salt into the plant:

Under conditions of low salinity, tolerance in a plant can be due to minimized salt uptake. This is known as salt exclusion. According to Munns (2002) "salt exclusion functions to reduce the rate at which salt accumulates in transpiring organs". Depending on the weather, plants transpire 30 -70 times more water than they retain in their leaves [52]. Consequently, any soil solute not excluded by roots will end up in leaves and will be 30-70% more concentrated than in the soil solution [52]. A plant must exclude most of the salts in the soil solution. For example, if a plant is transpiring 40 times more water than it retains and lets in only 2.5 % of the salt in the soil solution and excludes the other 97.5%, the salt concentration in leaves would stay comparable to soil salt concentration and the plant would survive indefinitely in saline soil. All plants exclude salt in the soil solution to some extent, but certain species exclude salt reasonably well, allowing very little to be transported in the xylem of the shoots. For example, barley, which is a salt-tolerant species and considered as a strong excluder can exclude 94% of the Na^+ and 91% of the Cl^- at 50 mM NaCl in the soil solution, and thus concentration of these ions in the leaves can reach up to 3.2 and 4.7 mM, respectively [50]. Bread wheat excludes 98% of the Na^+ at 50 mM NaCl in the soil solution so the concentration does not buildup in leaves more than 2 mM [78]. In contrast, lupin is a salt sensitive species and considered as a weak excluder had a poor ability to regulate xylem salt concentration once the soil solution exceeded about 125 mM [11].

At whole plant level, salt exclusion takes place at four different sites in the plant: 1) selectivity of uptake by root cortex from the soil solution, 2) at the loading of the xylem, 3) at the retrieval from the xylem in the upper part of the plant and 4) loading of phloem [52]. The first three processes function to reduce the transport to the leaves, and tolerance of glycophytes towards salt mostly depends on these three mechanisms to various degrees. Export from leaves in the phloem is another important control point that can help to maintain low salt concentration and ensure that salt is not redirected to growing tissues of the shoot. Only halophytes have an additional mechanism: specialized cells (salt glands or bladders) to excrete salt from leaves. However, all halophytes also rely on the first four mechanisms to control the uptake and transport of salt to the leaves. In addition, excretion is an essential strategy for plants growing in very saline environment and for perennial species.

The contributory factors that are involved in maintaining low rates of salt accumulation in leaves are high shoot: root ratio and high relative growth rates [55, 59]. According to Munns 2006 [55] "the shoot ion concentration is the result of the rate at which ions arrive in the shoot, the rate at which they are reexported back to the root, and the rate at which the shoot expands (the relative growth rate)". There are certain ions such as Na^+ and Cl^- for which a very small proportion of import in the xylem translates into export from the shoot. Thus the concentration in the shoot originates fundamentally from the uptake rate divided by the corresponding growth rate, as shown in equation 1 [55]:

$$\text{Ion concentration in shoot (mol } g^{-1}) = \text{Ion uptake rate in shoot (mol } g^{-1}\ d^{-1}) / \text{Relative growth rate (g } g^{-1}\ d^{-1}) \qquad \text{Equation 1}$$

This equation shows that the relative growth rate (g g^{-1} d^{-1}) influences the ion concentration in the shoot, not the absolute growth rate (g d^{-1}) itself.

Mechanisms minimizing the concentration of salt in the cytoplasm:

Compartmentalization of ions within the leaf vacuoles is another strategy used by the species that cannot exclude at least 98% of the salt from the transpiration stream. This protects the cytoplasm from ion toxicity and prevents buildup of salts in the cell wall, which would cause water deficit in the leaf [83]. In the absence of this strategy, salt concentration in the older leaves would eventually become high enough to kill the cells.

In general, cytoplasmic NaCl concentrations above 100 mM start to inhibit most enzymes activity [11]. Ideally, Na^+ and Cl^- should be sequestered in the vacuole of the cell and removed from major metabolic processes. However, if Na^+ and Cl^- are sequestered in the vacuole, an osmotic counter balance is necessary in the cytoplasmic compartment. N-Containing solutes, such as proline and glycine betaine, are accumulated most commonly to achieve this balance, although other molecules can accumulate to lesser degree.

The ability to compartmentalize Na^+ in the vacuole is an important strategy used by most plant species to tolerate salt stress. Vacuolar compartmentalization of Na^+ is achieved by the action of Na^+/H^+ antiporters on the tonoplast [81]. The proton gradient that drives the antiporter is generated by two electrogenic H^+ pumps: vacuolar type H^+-ATPases and the vacuolar pyrophosphatases (H^+-PPases) [15, 81]. Salt stress has been shown to increase Na^+/H^+ antiports activity in both glycophytes and halophytes for example, in *Arabidopsis* [6], and in the halophyte *Suaeda salsa* [76], respectively.

1.2.4. Approaches to enhancing salt tolerance in agriculture

According to Flowers and Yeo (1995) [21] at least 5 possible ways exist for improving salt tolerance of a given crop or cultivated species:

1. Develop halophytes as alternative crops by breeding and selection for agronomic characterstics.
2. Use interspecific hybridization to raise the tolerance of current crops.
3. Use conventional breeding and the variation already present in existing crops.
4. Generate variation within existing crops by using recurrent selection, mutagenesis or tissue culture. The main advantages of tissue culture are the large number of individual cells that can be handled and the ease of screening.
5. Breed for yield rather than tolerance.

There is another approach suggested by Bohnert and Jenson (1996) [17]. According to them "tolerance breeding must be accompanied by transformation" and that "successful releases of tolerant crops will require large-scale metabolic engineering which must include the transfer of many genes." This approach has now been widely accepted by scientists.

Direct introduction of a small number of genes by genetic engineering seems to be a more attractive and rapid approach to improving salt tolerance as compared to traditional breeding and marker-assisted selection programs. Salt tolerant plants developed through genetic engineering are already a reality. In recent years, there are numerous articles on genetic modification of plants directed to make them more tolerant to salt.

Considerable work has been carried out to genetically engineer overproduction of compatible osmolytes in transgenic plants such as *Arabidopsis*, rice, wheat, and tobacco. For example expression of a bacterial glycine betaine biosynthesis genes in tobacco, a species that does not accumulate glycine betaine, conferred increased tolerance to salt stress [38]. Similarly over-expression of enzymes leading to increased production of proline in tobacco [39], trehalose synthesis in rice [26], mannitol in tobacco [70], wheat [1] and *Arabidopsis* [72], D-ononitol production in tobacco [65], and fructan synthesis in tobacco [58], conferred increased tolerance to salt stress.

There are also numerous examples where compartmentalization of Na^+ in the vacuole increased the salt tolerance of most plant species, which depend on Na^+/H^+ antiporters as well as V-type H^+-ATPases and H^+-PPases. Over-expression of vacuolar H^+-PPases- AVP1 in *Arabidopsis* enhanced sequestration of Na^+ into the vacuole and showed increased tolerance to salt stress than that of wild type [27]. Similarly, over-expression of one of the vacuolar Na^+/H^+ antiporter AtNHX1 increased the salt of the transgenic *Arabidopsis* plants [6].

In the past few years significant approaches have been employed to identify, isolate and characterize several genes that could potentially improve the salt tolerance in various ways. Because of the complexity of salt tolerance, it is hard to assume that altering a single gene in transgenic plants could dramatically boost salt tolerance, and it has been suggested that enhancement in tolerance of plants to saline conditions will be achieved only after expression of a number of genes, whereas each one alone could not confer a significant increase in salt tolerance [20, 84]. However, various authors have claimed that single-gene transfers have led to the enhancement of salt tolerance of plants [63, 86].

The present study was thus carried out to investigate the effect of engineering a single gene *TMT1* with respect to salinity tolerance. The gene was transferred in many organisms by Dr. Saini's research group. I have used these transgenic organisms to test whether

expression of *TMT1* enables the transgenic species to methylate Cl^- to CH_3Cl gas and whether this Cl^- volatilizing reaction is associated with the overall salinity tolerance of the species.

2. MATERIALS AND METHODS

2.1. Transgenic Materials

Transgenic *E. coli*, wild potato (*Solanum chacoense*) and tobacco (*Nicotiana tabacum*) roots, and tobacco plants used in this study were produced by Dr. Sima Babayeva and Dr. Priyum Koonjul in Dr. Saini's laboratory. This group has also isolated T-DNA insertion mutants of *Arabidopsis thaliana* in which the *TMT* gene has been knock-out. *TMT1* over-expressing *Arabidopsis* plants were produced by over-expression of cabbage *TMT1*.

The *TMT1* gene, encoding the TMT enzyme and isolated from red cabbage [7] was expressed in above mentioned organisms i.e. *E. coli*, wild potato (*Solanum chacoense*) and tobacco (*Nicotiana tabacum*) roots, and tobacco plants, that naturally do not contain TMT or other enzymes capable of methylating chloride. The detailed methodology for the production of this material will be published elsewhere, but briefly:

- M15 *E. coli* cells were transformed with either the expression vector PQE30 alone (-*TMT1*) or the vector containing the full-length *TMT1* gene (+*TMT1*).
- Wild potato (*Solanum chacoense*) and tobacco roots (*Nicotiana tabacum*) were transformed with *Agrobacterium rhizogenes* with the expression vector pBIN119 that either contained or lacked the *TMT1* gene (Koonjul, Babayeva and Saini, unpublished). Transformation was done by infecting petiole explants with the bacterium containing the appropriate vector. Roots emerged at the site of infection, and each root was considered an independent clone.
- Tobacco plants were transformed with *Agrobacterium tumefaciens* containing pBIN119 vector with or without the *TMT1* insert through agro-infiltration. Plants regenerated from the transformed calli, were grown to maturity and their seeds were collected.

2.2. Chemicals

Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Qiagen (Mississauga, ON, CA). Kanamycin, ampicillin, AdoMet, Murashige and Skoog basal salt mixture (MS), tryptone, phytagel, agar, yeast extract, sucrose, urea, NaCl and KI were all purchased from Sigma (St. Louis, Mo, USA). Polyethylene glycol 8000 (PEG) was purchased from Fisher Biotech (Fair Lawn, New Jersey, USA). Sodium phosphate and SDS were from the Laboratory MAT (Montreal, QC, CA). CH_3Cl and CH_3I were from Aldrich (Milwaukee, Wi., USA). Helium and nitrogen gases were obtained from Praxair (Montreal, QC, CA).

2.3. Buffers

The following buffers were used: Lysis buffer: 8M urea in 0.1M sodium phosphate and 0.01M Tris (pH 8); Buffer A: 100 mM Tris-acetate, pH 7.5 containing 10% (v/v) glycerol and 0.01% β mercaptoethanol. All buffers were filtered through a 0.22 μm membrane before use.

2.4. Culture media

MS Medium: One sachet of MS salt (4.3 g L^{-1}), 1 ml of MS vitamin, 30 g of sucrose and 4 g of phytagel in final volume of water made to 1 L. LB Medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar in final volume of water made to 1 L. All media were autoclaved for 25 minutes before use.

2.5. Determination of salt tolerance and CH_3Cl production of *E. coli* cells

2.5.1. Cell growth in response to NaCl

The *-TMT1* and *+TMT1* cells were grown on LB medium containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin at 37 °C until an OD_{600} of 0.6 was reached. The cells

were then induced with 1 mM IPTG, and an appropriate amount of the culture was transferred to tubes containing NaCl solution ranging in concentration from 25 mM to 1 M. The cells were allowed to grow for an additional 4 hr at 37°C, after which the OD_{600} was measured. One ml aliquot of the culture was centrifuged at 4,000g for 20 min, and the pellet was collected and lysed in 200 µl lysis buffer. Protein concentrations were estimated by the method of Bradford using the Gene quant pro RNA/DNA calculator.

2.5.2. Measurement of *in vivo* CH_3Cl emission

Separate cultures of *-TMT1*or *+TMT1* M15 *E. coli* cells were induced with IPTG as mentioned above. Five milliliter of each bacterial culture was transferred to 50 ml flasks containing 15 ml of LB and 100 or 250 mM NaCl. The flasks were closed with a rubber stopper and the cells were allowed to grow at 37°C for 4 hr. One ml headspace sample was analyzed by Hewlett Packard 5890 Series 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. The flow rate of the carrier gas (He) was 40 ml min^{-1} and the column temperature was 130°C. The injector and detector temperatures were 120°C and 200°C, respectively.

2.5.3. Measurement of *in vitro* enzyme activity

Fifty µl aliquot of the bacterial lysate (enzyme extract) added to total volume of 400 µl of buffer A was mixed with 25 µl of 10 mM AdoMet and 25 µl of 1 M NaCl in a 5 ml vial. The vial was closed with a screw cap lined with a Teflon septum and incubated for 30 min at the room temperature. One ml headspace sample was analyzed by GC.

2.6. Determination of salt tolerance and CH_3Cl emission of root cultures

Twenty transformed lines of wild potato and 7of tobacco were screened for CH_3I emissions in the presence of KI in order to select the 3 lines with the highest TMT activity. KI was used as the substrate because the enzyme methylated I^- with the greater

efficiency among halides [62]. In parallel, 3 lines of wild potato and 2 lines of tobacco that transformed with -*TMT*1 were also screened. For this purpose, 30-50 mg roots were incubated with 500 µl of 500 mM KI in 5 ml vial for three hours. The vial was closed with a screw cap fitted with a Teflon septum. CH_3I was quantified by analyzing one ml headspace gas sample by GC.

2.6.1. Root growth in response to NaCl

Four clones of potato, +*TMT1*-P1, +*TMT1*-P2, +*TMT1*-P3, and -*TMT1*-P1 and four clones of tobacco, +*TMT1*-T1, +*TMT1*-T2, +*TMT1*-T3 and -*TMT1*-T1 were selected. Each of these clones was sub-cultured on Petri plates containing MS and 0.4% (v/v) phytagel. One week later, 1 to 2 cm of root tip segments were cut and sub-cultured onto Petri plates containing MS and 0.4% (v/v) phytagel supplemented with NaCl ranging from 50 to 200 mM. Each treatment was given in 3 replicates. The root length was measured on the 7^{th} day.

2.6.2. CH_3Cl emission from root cultures

Separate cultures of potato and tobacco root clones were sub-cultured as described in the section. After one week, 1 to 2 cm of root tips were cut and sub-cultured onto 50 ml flasks containing MS and 0.4% phytagel supplemented with 0, 50, 100 and 200 mM NaCl. Each treatment was done in triplicate. The flasks were closed with a rubber stopper and 1 ml headspace sample was analyzed by GC at different time intervals as described earlier. Fresh weight of each sample was measured at the end of the experiment.

2.7. Determination of salt tolerance and CH_3Cl production ability of whole plants

Tobacco:

2.7.1. Effect of NaCl on seed germination of tobacco plants

Seeds of -*TMT1*-T1 plants and 3 independent lines of transgenic plants +*TMT1*-T1, +*TMT1*-T2, and +*TMT1*-T3 were surface sterilized with 50% (v/v) ethanol for 1 min and then 50% NaClO (v/v) and 0.2% (v/v) SDS for 15 min and washed with distilled water. The seeds were sown on agar plates containing MS medium maintained at 24°C under constant fluorescent lighting, and their germination was recorded for two weeks.

2.7.2. Effect of NaCl on seedling growth

Seeds were surface sterilized and germinated on MS medium as described above. Two week old seedlings were transferred to MS Petri plates supplemented with 0, 50, 100, and 200 mM NaCl, and were grown at 24°C under constant fluorescent lighting. Growth was monitored for 21 days and photographs were taken on the 21st day.

2.7.3. CH_3Cl emission from plants

Seeds were surface sterilized and grown on MS medium as described above. Two week old seedlings were transferred to 50 ml flasks containing MS and 0.8% (v/v) agar supplemented with 0, 50, 100 and 200 mM NaCl. Each treatment had 3 replicates. The flasks were closed with a rubber stopper and 1 ml headspace samples, taken on alternate days for 15 days, were analyzed by GC. Growth was monitored for 15 days, and fresh weight and height of each sample was recorded.

2.7.4. Greenhouse experiment

Whole-plant responses to salinity were studied in a greenhouse experiment. Seeds of +*TMT1* and -*TMT1* tobacco were surface sterilized and sown on agar plates as mentioned above. After two weeks, seedlings were transferred to 9 cm (height) x 11.4 cm (diameter) pots containing perlite soaked with half strength MS solution. Saline solution, in MS solution, was applied to 3-week old plants over a period of four weeks. NaCl

concentrations were 50, 100 and 200 mM. For osmotic stress, polyethylene glycol (PEG-8000) at concentrations of 141.6 g L^{-1} and 200 g L^{-1} was applied, which was approximately equal to the osmotic potential of the 100 mM (-0.52 MPa) and 200 mM (-1 MPa) NaCl solution, respectively. Each treatment had 3 replications. Plants were watered every day with approximately 25 ml solution per pot. The experiment was conducted twice as follows:

In one experiment, plants were harvested after 4 weeks of NaCl treatments at the age of 7 weeks. Plant height and number of leaves per plant were recorded. Root and shoot part of each plant was separated, and shoot fresh weight was measured. Total leaf surface area of each plant was measured using an area meter.

In the second experiment, 7 week old plants were transferred to 14 cm (height) x 24.5 cm (diameter) pots containing soil. Salinity treatment was applied twice weekly and plants were fertilized (200 ppm of N: P: K = 20: 20: 20) once a week, up to 13 weeks, which was the end of the experiment. Plant height, number of leaves per plant, and diameter of stem were recorded and the seeds were collected when the plants had reached the age of 20 weeks.

Arabidopsis:

2.7.5. CH_3Cl emission from *Arabidopsis* plants

Seeds of wild type plants, *tmt1* knock-out mutants and plants over-expressing *+TMT1* were surface sterilized and grown on MS medium as described above. Sixteen day old seedlings were transferred to 50 ml flasks containing MS medium and 0.4% (w/v) phytagel supplemented with 0, 50, 100 and 200 mM NaCl. Each treatment had 3 replications. The flasks were closed with a rubber stopper and 1 ml headspace samples, taken every other day for 11 days, were analyzed by GC. Plant growth was monitored for 11 days and fresh weight was recorded.

2.7.6. Effect of NaCl on growth of *Arabidopsis* plants

Seeds of wild type plants, knock-out mutants and *TMT*1 over-expressing *Arabidopsis* plants were grown in soil in 9 cm (height) x 11.4 cm (diameter) pots. NaCl solution at concentrations of 50, 100 and 200 mM was applied to 2 weeks old plants and the treatment lasted until the end of the experiment. Each treatment had 3 replications. Plants were watered with NaCl (approximately 25 ml solution per pot) twice a week and fertilized (200 ppm of N: P: K = 20: 20: 20) once a week. Plant height, number of leaves per plant, number of stems, number of flowers and fresh weight were recorded at the end of the experiment.

3. Results

A gene encoding TMT enzyme (*TMT1*) isolated from red cabbage was introduced into *E. coli* cells, root cultures of potato and tobacco, and tobacco and *Arabidopsis* plants (Koonjul, Babayeva and Saini, unpublished). Effects of these genetic transformations on the salt tolerance of the recipient organisms and their ability to convert Cl^- ions to chloromethane are described below.

3.1 Effect of *TMT1* insertion on bacterial cells

Figure 1 shows the effect of NaCl on the growth of *E. coli* cells that either contain or lack the *TMT1* insert. For this purpose, different concentrations of NaCl ranging from 0 to 1000 mM were used. The +*TMT1* bacterial cells grew significantly faster than -*TMT1* cells in the absence of NaCl. NaCl inhibited the growth of both cell types to a similar extent relative to the growth of respective no-salt controls, although the absolute growth of +*TMT1* cells was faster at all NaCl concentrations owing to the higher growth rate in no-salt medium. These results show that the presence of TMT promotes the growth of bacterial cells, but does not improve their tolerance to NaCl salinity.

All the +*TMT1* bacterial cells produced CH_3Cl in response to different concentration of NaCl (Figure 2). The rate of CH_3Cl production increased with the increase in NaCl concentration in the medium from 0 to 250 mM. Virtually all the CH_3Cl accumulation in the culture flasks occurred during the initial five hours, reaching a plateau thereafter. There was no detectable amount of CH_3Cl produced by the -*TMT1* cells. In conclusion, despite acquiring the ability to produce CH_3Cl from Cl^- containing medium (Figure 2), +*TMT1* cells did not acquire greater tolerance to NaCl (Figure 1).

In order to determine the effect of exposure of cells to NaCl on the extracted activity of TMT, the activity was determined in the *E. coli* lysate with 250 mM NaCl in the reaction mixture (Figure 3). The highest activity, 10 pmol $\mu g\ protein^{-1}\ hr^{-1}$ was recorded in the lysate of +*TMT1* cells that had not been treated with NaCl. The enzyme activity decreased linearly with the increase in NaCl concentration in the cell growth medium and was virtually eliminated by 500 mM NaCl. There was no detectable amount of CH_3Cl produced by the lysate of -*TMT1* cells grown at any salt concentration.

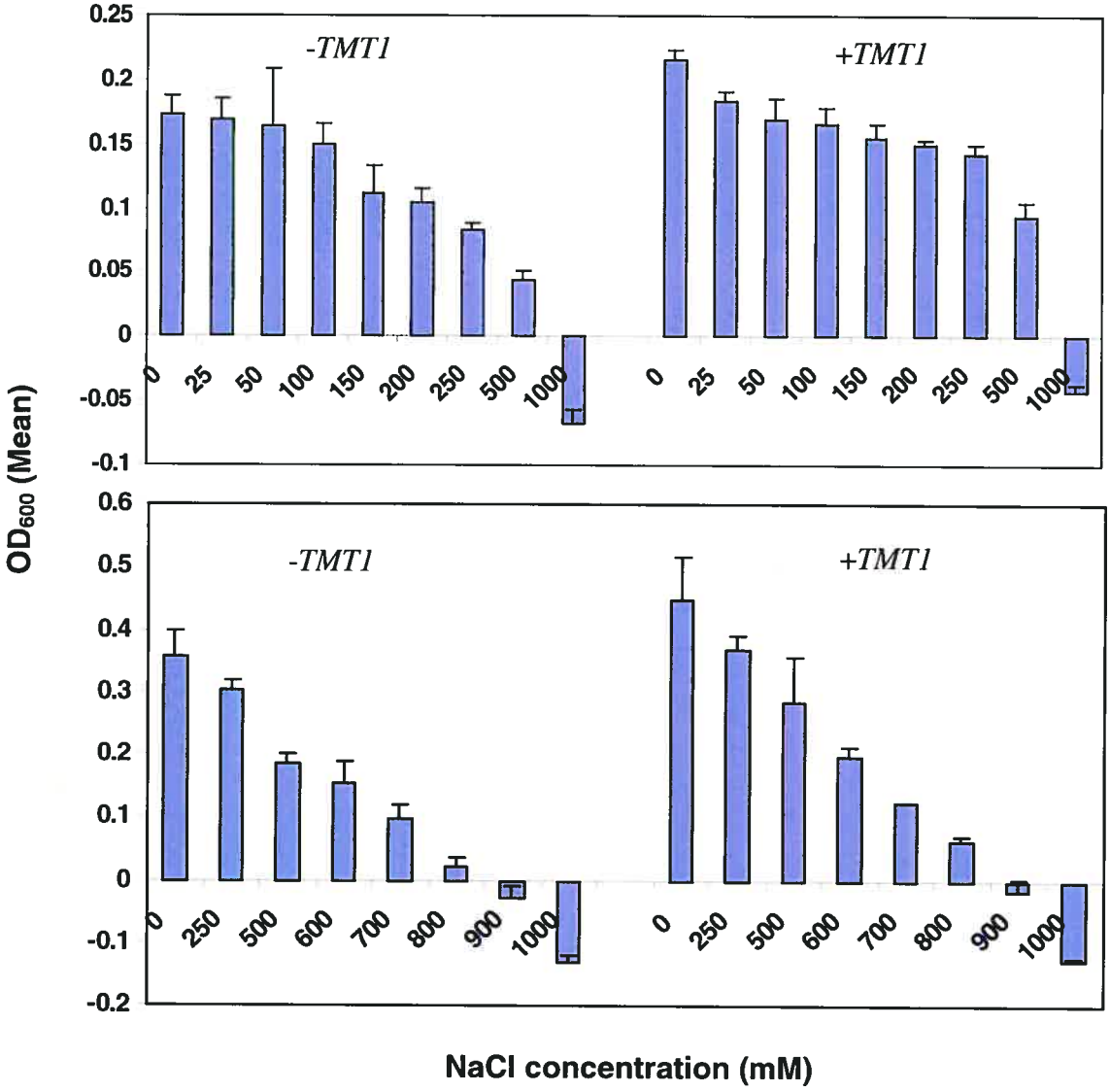


Figure 1. Growth of transgenic bacterial cells under salt stress. After inducing with IPTG an appropriate amount of the culture of *-TMT1* or *+TMT1* bacterial cells was transferred to tubes containing NaCl solution ranging in concentration from 25 mM to 1M. The cells were allowed to grow for an additional 4 hr at 37°C, after which the OD_{600} was measured. Zero value represents the difference between final OD_{600} and OD_{600} of 0.6. Values are the mean ± SD (n = 3).

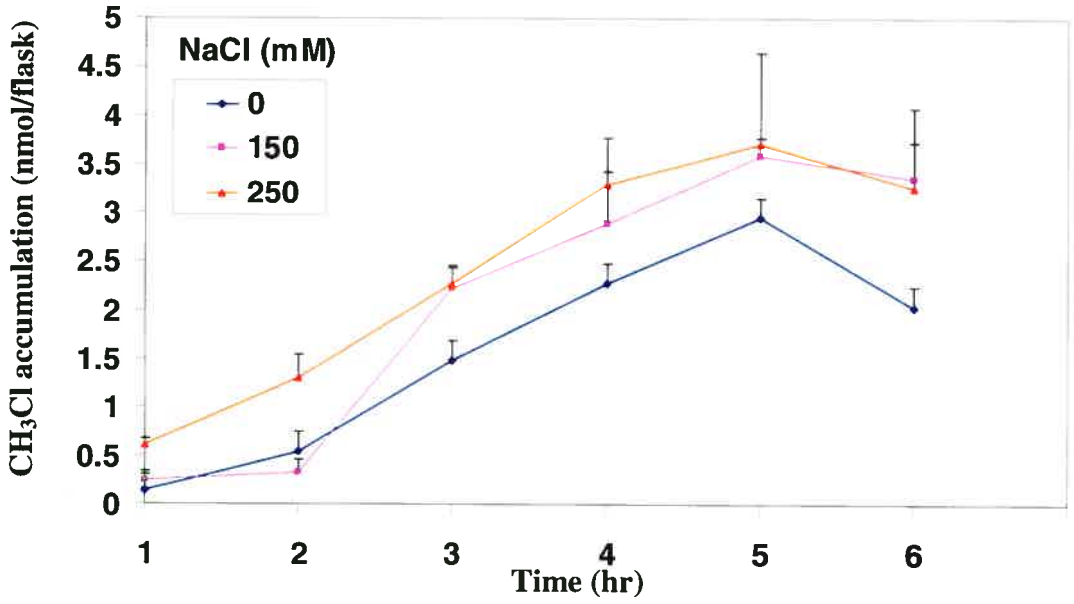


Figure 2. Time course of CH_3Cl production by transformed bacterial cells. The total amount of CH_3Cl accumulated in the headspace of 50 ml Erlenmeyer flasks containing bacterial cultures with 15 ml LB and 100 or 250 mM NaCl was determined at different time points. Values are the mean ± SD (n = 3).

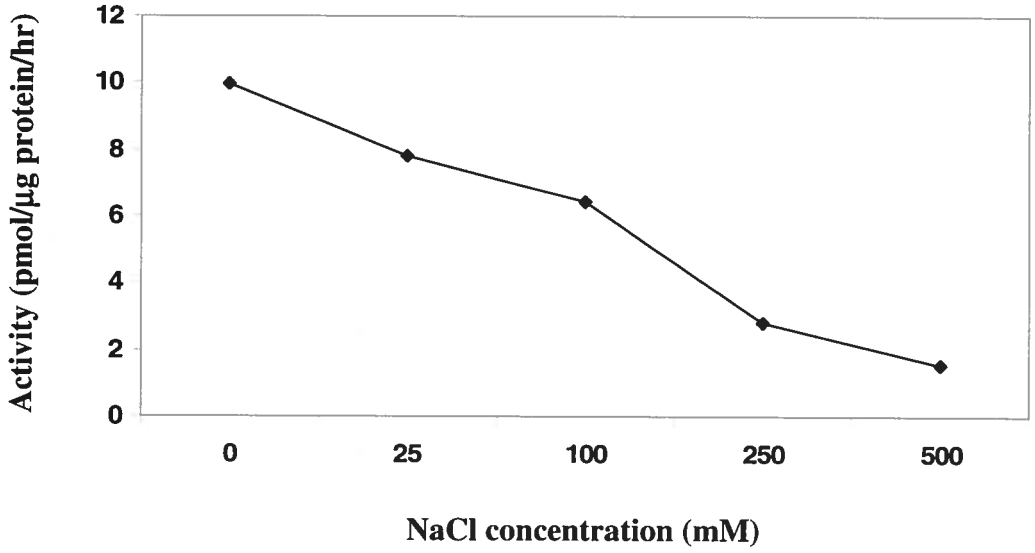


Figure 3. Effect of NaCl concentration in the medium used to grow +*TMT1 E. coli* cells on CH_3Cl production by the lysates of these cells. Cells were grown in 0, 25, 100, 250 and 500 mM NaCl and were then lysed. Fifty µl aliquot of the lysate (enzyme extract) added to total volume of 400 µl of buffer A was mixed with 25 µl of 10 mM AdoMet and 25 µl of 1 M NaCl in a 5 ml vial. CH_3Cl in headspace was determined after incubation for 30 min at the room temperature.

3.2 Effect of *TMT1* insertion on salt tolerance and chloride volatilization ability of root cultures

Twenty wild potato (Table 1) and 7 tobacco (Table 2) root clones were screened in order to select the clones with the highest activity based on CH_3I emission. In parallel, three lines of wild potato and two lines of tobacco that transformed with -*TMT*1 were also screened as experimental control. Finally, four clones of potato +*TMT1*-P1, +*TMT1*-P2, +*TMT1*-P3 and -*TMT1*-P1 and four clones of tobacco +*TMT1*-T1, +*TMT1*-T2, +*TMT1*-T3 and -*TMT1*-T1 were selected for further experiments.

Effect of *TMT1* insertion on growth of potato and tobacco root cultures in response to NaCl

Figure 4 A and B show the effect of NaCl on the growth of potato and tobacco root clones, respectively as measured by root elongation. All the roots of both potato and tobacco were able to grow under different concentration of NaCl. In both species, transgenic +*TMT1* roots were longer compared to -*TMT1* roots even in the absence of NaCl, suggesting that TMT promoted the growth of root cultures. Fifty mM NaCl had a slight positive effect on the growth of tobacco roots compared to those that grew in the absence of NaCl. Higher concentration of NaCl (200 mM) was more toxic to non-transformed roots compare to transformed roots of potato.

Effect of *TMT1* insertion on chloride volatilization ability of root cultures

Ability of transgenic potato roots to volatilize Cl^- to CH_3Cl under different concentrations of NaCl was investigated. All +*TMT1* potato roots were able to produce CH_3Cl in response to different concentrations of NaCl except one line at 200 mM NaCl (Figure 5). The highest CH_3Cl accumulation was observed between 15 to 19 days, after which there was a general decrease in the CH_3Cl emission. In contrast, no detectable amount of CH_3Cl emission was recorded in -*TMT1* roots. All the +*TMT1* roots also grew better compared to -*TMT1* roots under different concentrations of NaCl (Figure 6). Higher concentration of NaCl (200 mM) had a toxic effect on -*TMT1* roots whereas +*TMT1* roots were able to maintain their growth.

Table 1. CH_3I emission from potato root cultures transformed with *TMT1* gene. Roots were incubated with 500 µl of 500 mM KI in 5 ml vial for three hours, and CH_3I emission was quantified by analyzing one ml headspace sample by GC. Each value is the mean ± SD (n = 3).

Clone	CH_3I Emission (nmol/g/hr)	Selected Clones
+TMT1		
31.1 # 7.4 # 84	5.49 ±1.3	
31.1 # 7.4 #131	6.8 ± 2.4	
31.1 # 7.2 #125	6.56 ± 0.51	
31.1 # 7.2 #127	7.63 ± 1.7	
31.1 # 7.1# 65	10.71 ± 0.59	
31.1 # 4.2 # 58	4.09 ± 0.54	
31.1 # 7.3 # 54	11.8 ± 2.0	
31.1 # 7.8 # 89	6.68 ± 4.9	
31.1 # 18.4 # 32	5.11 ± 0.54	
31.1 # 4.1 # 64	7.12 ± 2.9	
31.1 # 4.1 # 97	7 ± 1.1	
30.1 # 7.4 # 8.5	27.65 ± 15.2	+*TMT*1-P1
26.1 # 15.3 # 20	5.16 ± 2.6	
26.1 # 15.3 # 114	18.05 ± 7.1	+*TMT1*-P2
26.1 # 15.3 # 83	4.72 ± 1.8	
26.1 # 15.3 # 21	13.52 ± 5.4	+*TMT1*-P3
26.1 #18.2 # 28	3.87 ± 1.6	
26.1 # 15.1 # 98	4.13 ±1.5	
26.1 # 18.3 # 24	7.81 ± 3.7	
26.1 # 18.3 # 127	5.4 ± 2.5	
-TMT1		
9.1 # 12	*	-*TMT1*-P4
10.2 # 7	*	
10.3 #37	*	

* There was no detectable amount of CH_3I produced by the -*TMT1* roots.

Table 2. CH_3I emission from tobacco root cultures transformed with *TMT1* gene. Roots were incubated with 500 µl of 500 mM KI in 5 ml vial for three hours, and CH_3I emission was quantified by analyzing one ml headspace sample by GC. Each sample had only one representative.

Clone	CH_3I Emission (nmol/g/hr)	Selected Clones
+TMT1		
26.1 # 10.11	3.29	
26.1 # 15.6	15.91	*+TMT1*-T2
26.1 # 15.11	7.25	
26.1 # 15.37	7.01	
31.1 # 7.5	27.29	*+TMT1*-T1
31.1 # 7.8	5.69	
31.1 # 7.74	13.78	*+TMT1*-T3
-TMT1		
10.1	*	*-TMT1*-T1
10.17	*	

* There was no detectable amount of CH_3I produced by the *-TMT1* roots.

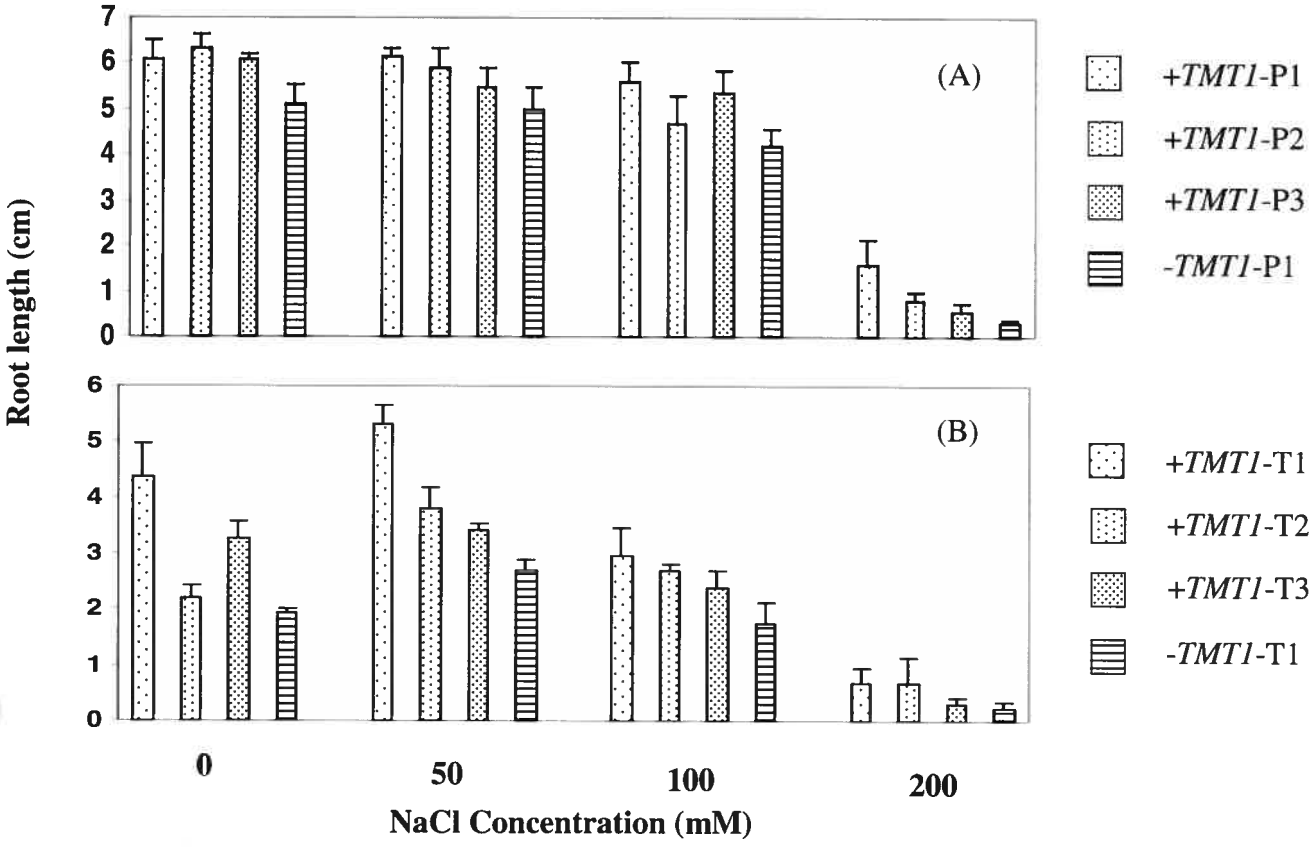


Figure 4. Root growth of potato and tobacco in response to NaCl. Growth of transgenic potato (A) and tobacco (B) roots sub cultured onto Petri plates containing MS and 0.4% (v/v) phytagel supplemented with NaCl ranging from 50 to 200 mM was measured on the 7th day.

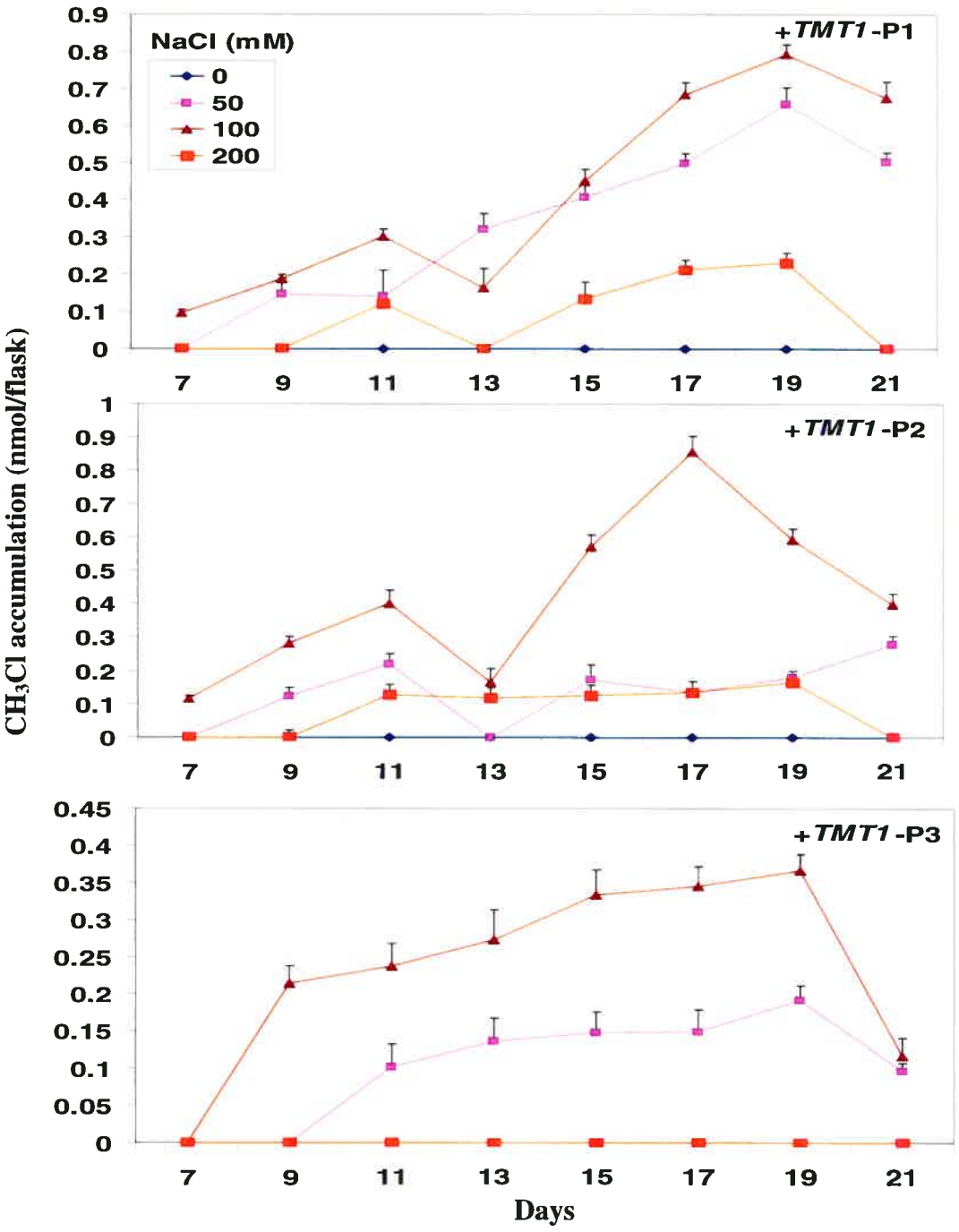


Figure 5. CH_3Cl emission by potato roots under NaCl. The emission from three different +*TMT1* root clones +*TMT1*-P1, +*TMT1*-P2, +*TMT1*-P3, sub cultured on MS supplemented with 0, 50, 100 and 200 mM NaCl was determined at different time intervals. The -*TMT1* roots did not emit CH_3Cl at any NaCl concentration. Values are the mean ± SD (n = 3).

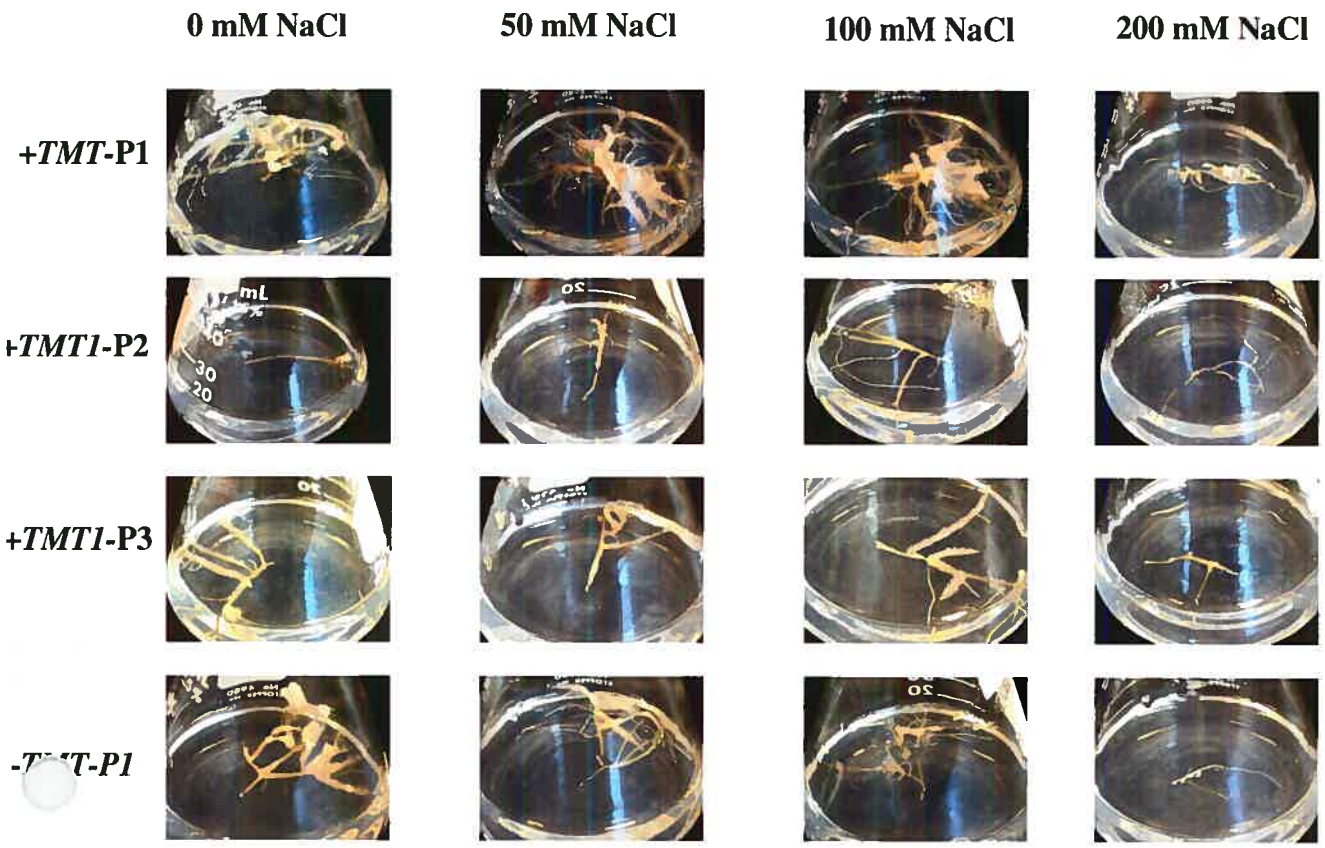


Figure 6. Photographs of potato roots grown under NaCl to estimate the ability of transgenic potato roots to volatilize Cl^- to CH_3Cl. Four clones, +*TMT1*-P1, +*TMT1*-P2, +*TMT1*-P3 and –*TMT1*-P4 of potato were sub-cultured onto 50 ml Erlenmeyer flask containing MS and 0.4% (v/v) phytagel supplemented with 0, 50, 100 and 200 mM NaCl. The roots were photographed on the 21st day.

Figure 7 show the ability of transgenic tobacco root to volatilize Cl^- to CH_3Cl under different concentration of NaCl. All the +*TMT1* lines were able to emit CH_3Cl at all concentration of NaCl. The highest CH_3Cl accumulation was recorded in +*TMT1* roots that grew under 100 mM NaCl. In general, highest accumulation was observed between 23 to 26 days, after which there was a general decrease in the CH_3Cl accumulation. In contrast, no detectable amount of CH_3Cl were recorded from -*TMT1* roots. All these +*TMT1* roots also grew better compared to -*TMT1* roots under different concentrations of NaCl (Figure 8). The +*TMT1* roots were able to maintain their growth even at 200 mM NaCl, whereas -*TMT1* roots were not able to survive. The results found were similar to those with potato roots.

3.3. Effect of *TMT1* insertion on salt tolerance and Cl^- volatilization ability of tobacco plants

3.3.1 Effect of NaCl on germination of tobacco seeds

Figure 9 show the effect of NaCl on germination of tobacco seeds. Most of the seeds of transgenic +*TMT1* and -*TMT1* plants were able to germinate and grow at all concentrations of NaCl. Although NaCl inhibited the germination of both +*TMT1* and -*TMT1* seeds, germination of +*TMT1* seeds was slightly better than that of -*TMT1* seeds. However, 200 mM NaCl severely inhibited the germination of both -*TMT1* and +*TMT1* seeds.

3.3.2 Effect of NaCl on seedling growth of tobacco plants

In order to assess the effect of NaCl on growth of tobacco seedlings, one -*TMT*-T1 and three independent lines of +*TMT* tobacco seedlings, +*TMT*-T1, +*TMT*-T2 and +*TMT*-T3, were grown under different concentrations of NaCl (Figure 10). Based on visual observation, all seedlings showed comparable growth in the absence of NaCl and at 50 mM NaCl. However, at 100 mM NaCl, differences between -*TMT1* and +*TMT1* seedlings were evident. The -*TMT1* seedlings showed growth retardation whereas +*TMT1* transgenic seedlings did not show any sign of inhibition. Distinct growth retardation was found in -*TMT1* seedlings at 200 mM NaCl. All the -*TMT1* seedlings showed chlorosis and drastic reduction in growth, whereas the transgenic lines were able to continue their growth.

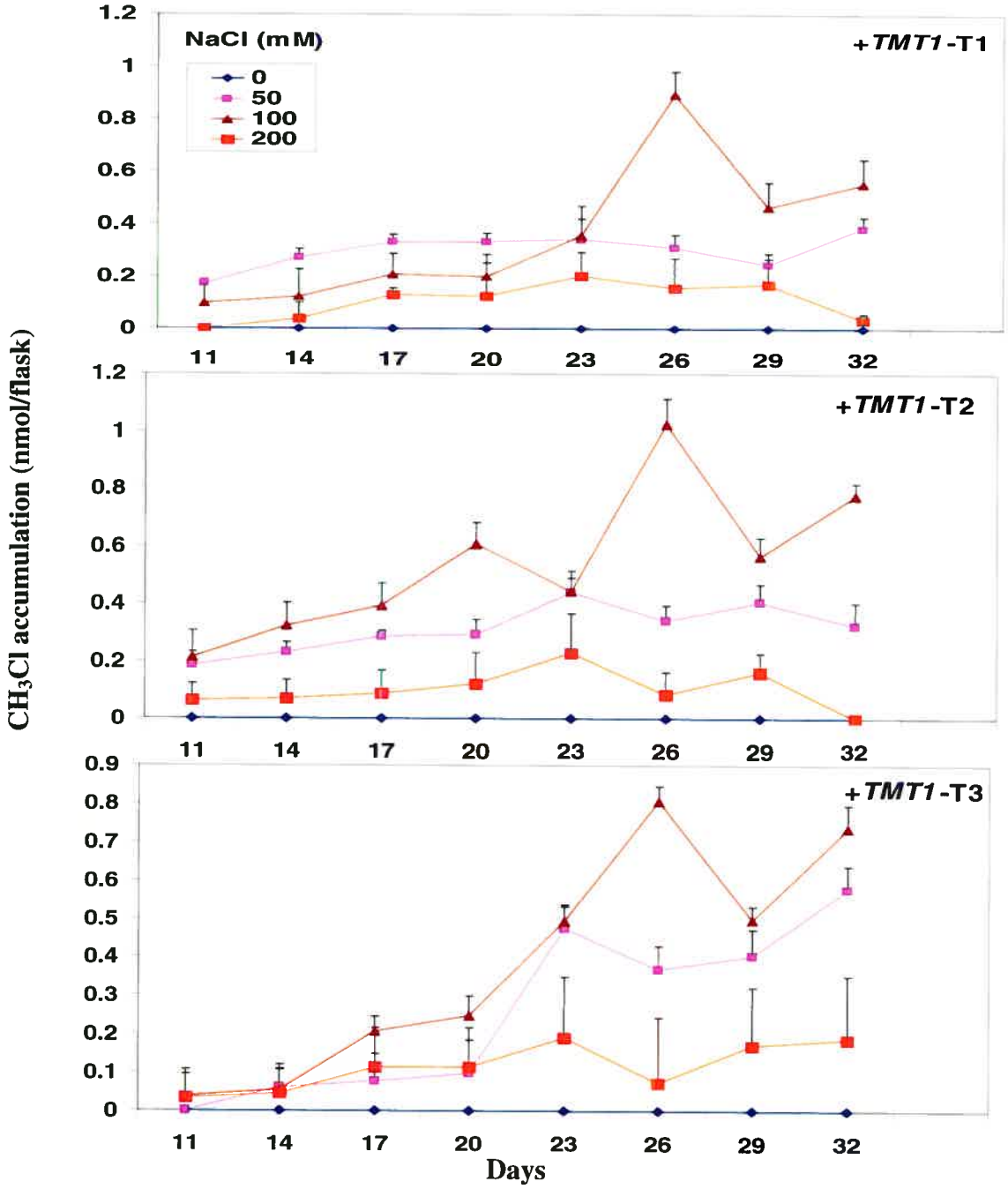


Figure 7. CH_3Cl emission by tobacco roots under NaCl. The emission from three different root clones transformed with *+TMT1* and sub cultured onto MS supplemented with different concentrations of NaCl ranging from 0 to 200 was measured at different time intervals. The *-TMT1* roots did not emit CH_3Cl at any NaCl concentrations. Values are the mean ± SD (n = 3).

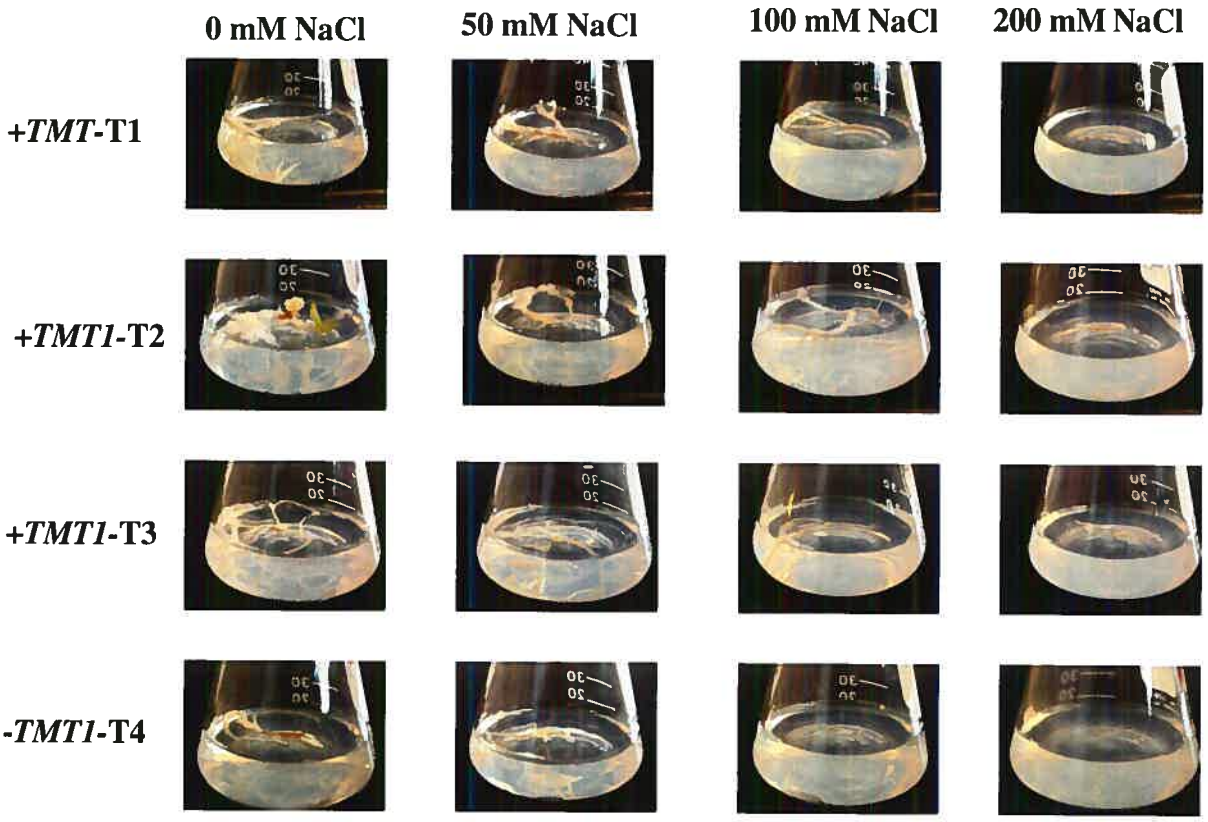


Figure 8. Photographs of tobacco roots grown under NaCl to check the ability of tobacco roots to volatilize Cl^- to CH_3Cl. Four clones, +*TMT1*-T1, +*TMT1*-T2, +*TMT1*-T3, and -*TMT1*-T1, of tobacco were sub-cultured onto 50 ml Erlenmeyer flasks containing MS and 0.4% phytagel supplemented with 0, 50, 100 and 200 mM NaCl. The roots were photographed on the 32nd day.

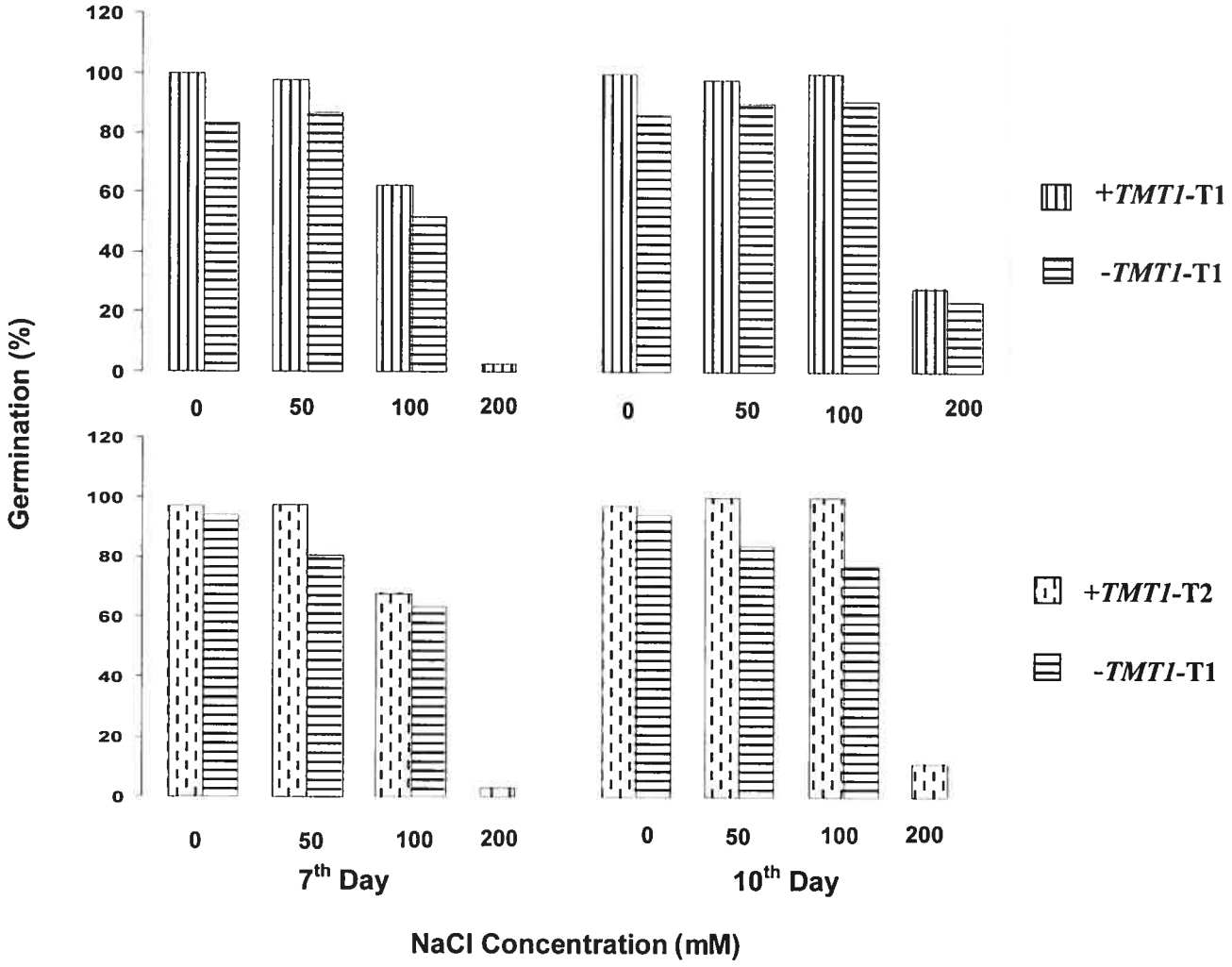


Figure 9. Effect of NaCl on seed germination of transgenic tobacco. Seeds of *-TMT1*-T1 plants and two independent lines of transgenic *+TMT1* plants- *+TMT1*-T1, and *+TMT1*-T2 were sown on agar plates containing MS medium, and their germination was recorded on the 7^{th} and 10^{th} day.

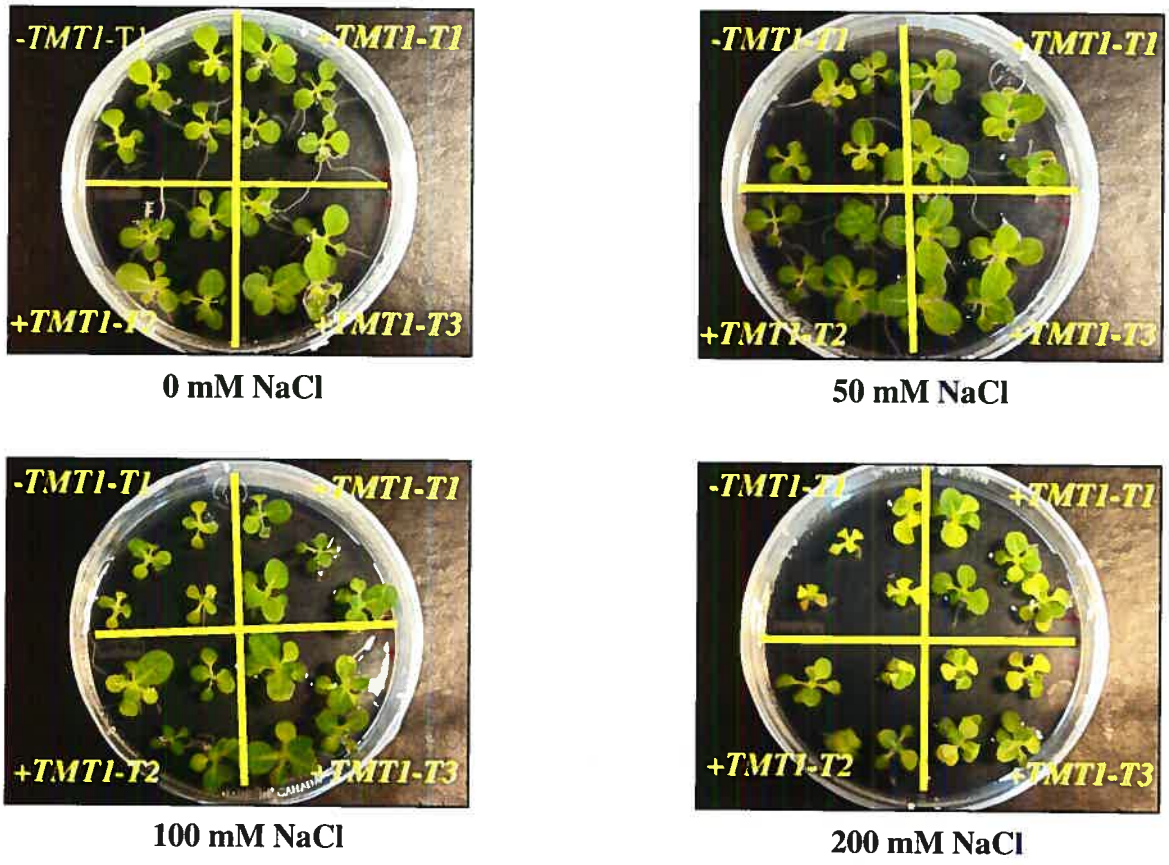


Figure 10. Effect of NaCl on seedling growth of transgenic tobacco. Seeds of *-TMT1*-T1 plants and three independent lines of transgenic plants- *+TMT1*-T1, and *+TMT1*-T2 and *+TMT1*-T3 were sown on agar plates containing MS medium and the growth of resulting seedlings was monitored.

3.3.3 Effect of *TMT1* insertion on the ability of transgenic tobacco plants to volatilize Cl^- to CH_3Cl and influence growth

To test the effect of *TMT1* insertion on the ability of transgenic tobacco plants to volatilized Cl^- under different concentrations of NaCl, 3 different lines of +*TMT*, +*TMT*-T1, +*TMT*-T2 and +*TMT*-T3 and -*TMT1*-T1 plants were used. All the transgenic lines were able to produce CH_3Cl under different concentrations of NaCl; CH_3Cl emission increased with an increase in NaCl concentration (Figure 11). However, in one transgenic line emission at 200 mM NaCl was less than that at 100 mM. In contrast, there was no detectable amount of CH_3Cl emitted from non-transgenic plants. All the +*TMT1* transgenic lines also showed better growth compared to -*TMT1* plants (Figure 12). However, there was a decrease in the growth of all the lines under stress as the concentration of NaCl increased. The results obtained with these plants were similar to those obtained with transgenic tobacco seedlings.

3.3.4 Growth of transgenic tobacco plants under different concentrations of NaCl under greenhouse conditions

In order to assess the tolerance of transgenic plants towards salinity stress, 2 week old plants were grown in pots containing perlite under different concentrations of NaCl (Figure 13). No major difference was found in the height of transgenic and wild type plants in the absence of NaCl. As the concentration on NaCl increased, an apparent reduction in the growth of both types of plants was found. However, the growth reduction due to salinity was more severe in all the -*TMT1* plants compared to transgenic plants subjected to the same stress treatment. No major difference was found in the number of leaves in both types of plants. However, an increase in total leaf surface area was recorded in +*TMT1* plants compared to -*TMT1* plants in the absence of NaCl, and the salt treatments affected the leaf area of -*TMT1* plants to a much greater extent (Table 3). NaCl caused dramatic decline in weight but more in -*TMT1* plants than in +*TMT1* plants. At 50 and 100 mM NaCl, the difference between -*TMT1* and +*TMT1* was ~ 2-fold. All the -*TMT1* plants showed chlorosis and growth reduction and ultimately died when subjected to 200 mM NaCl whereas transgenic plants were able to survive and continue to grow.

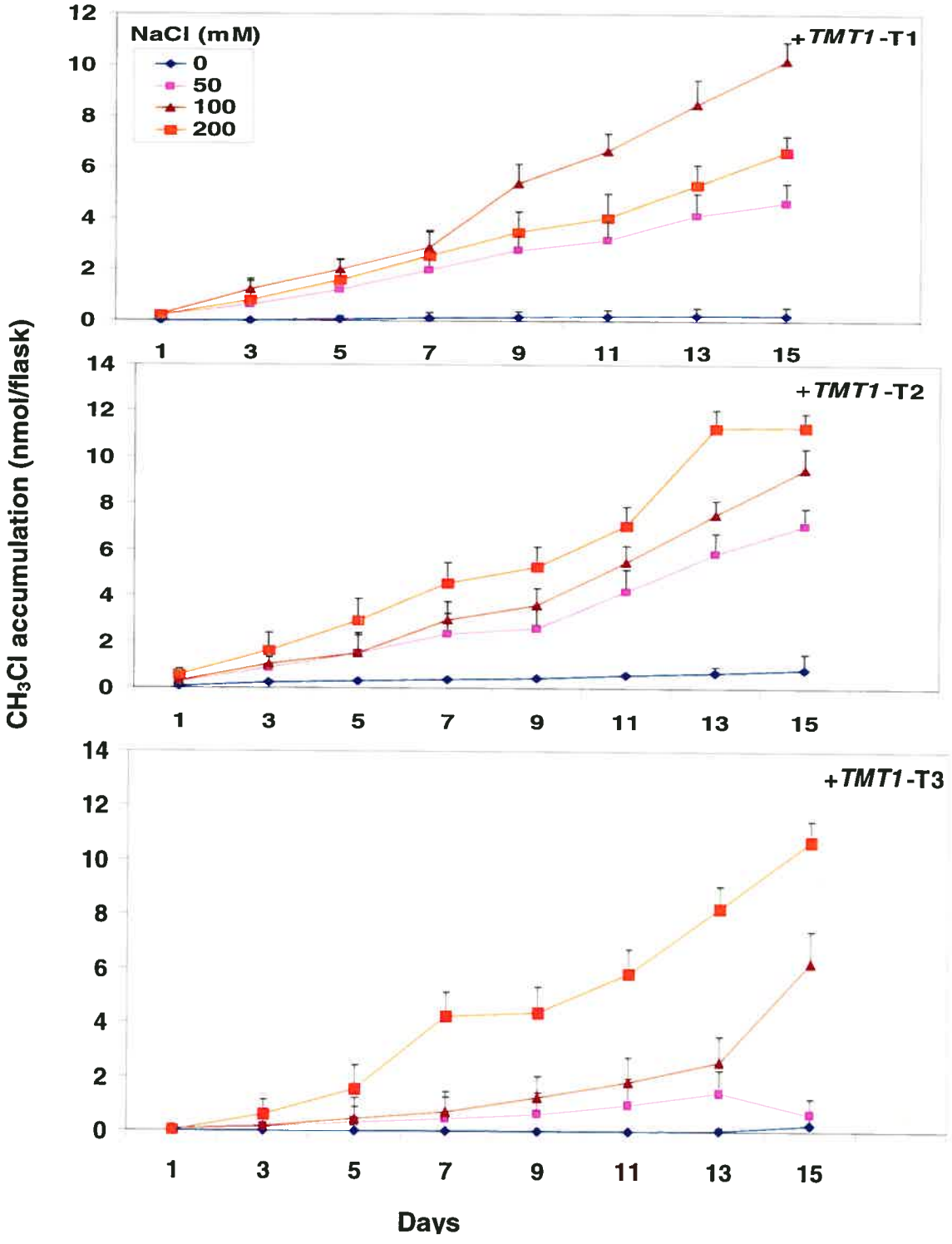


Figure 11. CH_3Cl emission by tobacco plants grown under NaCl. Three different independent lines of tobacco plants +*TMT1*-T1, and +*TMT1*-T2 and +*TMT1*-T3 were sub-cultured on 50 ml Erlenmeyer flask containing MS medium supplemented with 0 to 200 mM NaCl. CH_3Cl emissions from these plants were measured at different times. Values are the mean ± SD (n = 3).

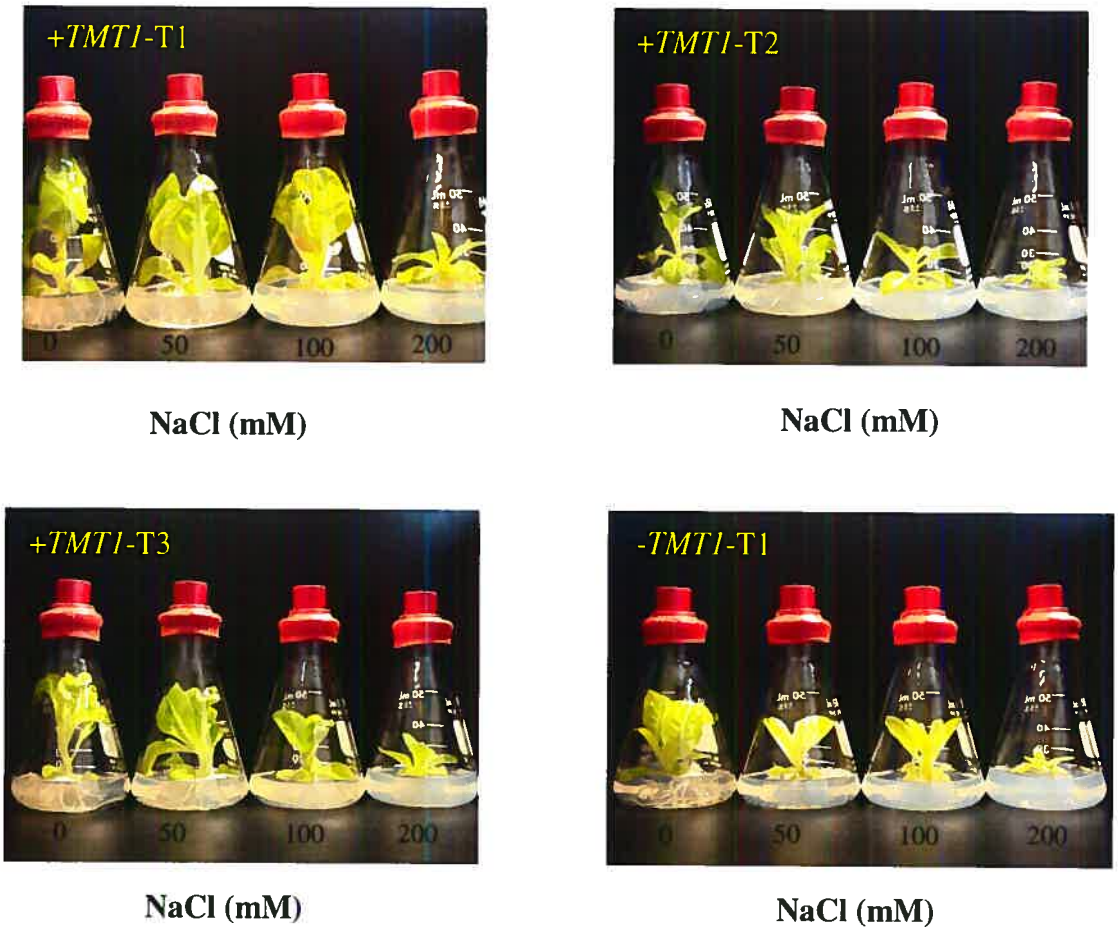


Figure 12. Photographs of tobacco plants grown under NaCl. Seeds of -*TMT1*-T1 plants and three independent lines of transgenic plants +*TMT1*-T1, and +*TMT1*-T2 and +*TMT1*-T3 were sown on 50 ml Erlenmeyer flasks containing MS medium supplemented with different concentrations of NaCl. The plants were photographed on the 21st day.

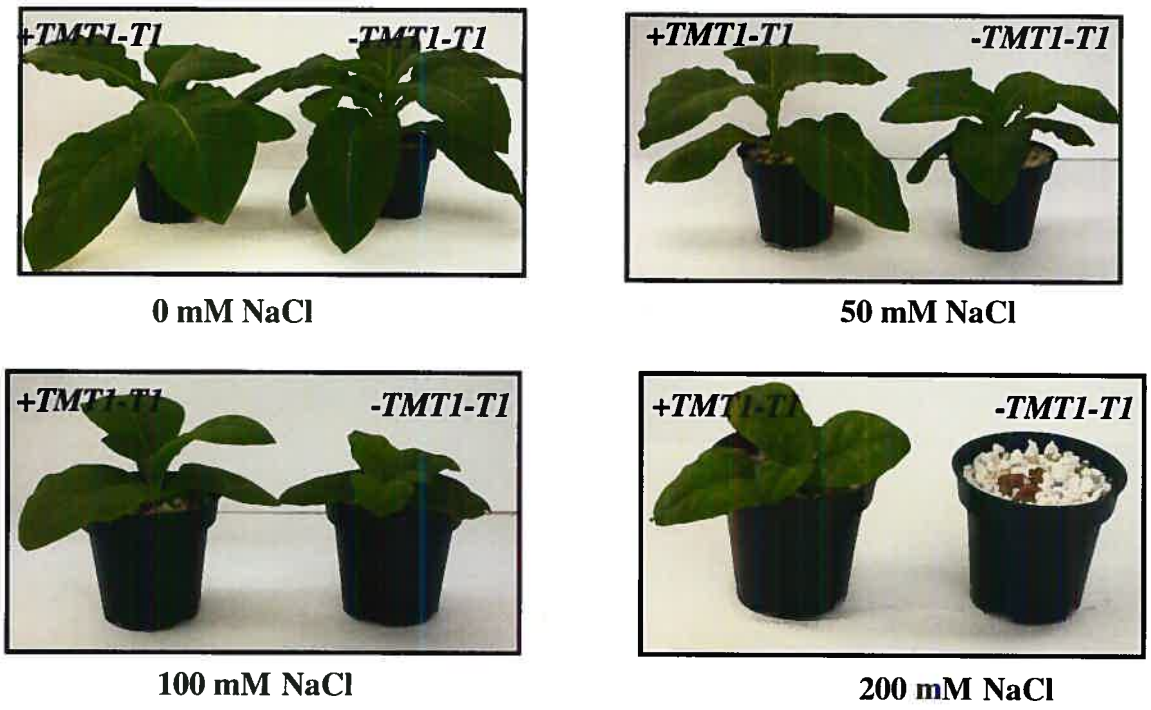


Figure 13. Effect of NaCl on growth of transgenic tobacco plants. Two week old seedlings were transferred to small pots containing perlite soaked with half strength MS solution. NaCl, in MS solution, was applied when plants had reached an age of 3 weeks and treatment lasted 4 weeks. Each treatment had 3 replications.

Table 3. Effect of NaCl on various growth parameters of *+TMT1* or *-TMT1* tobacco plants. Two week old seedlings were transferred to small pots containing perlite soaked with half strength MS solution. NaCl, in MS solution, was applied to 3-week old plants and lasted for 4 weeks, after which the data were recorded. Each value is the mean ± SD (n = 3).

Plants (T1) transformed with *+TMT1*				
Concentration (NaCl)	**0 mM**	**50 mM**	**100 mM**	**200 mM**
Height (cm)	5.83 ± 0.76	4.33 ± 0.58	2.77 ± 0.15	1.27 ± 0.55
Number of Leaves	11 ± 0	8 ± 0.58	7 ± 1.15	5 ± 0.58
Total Leaf Surface Area (μm^2)	47314 ± 8969	24103 ± 4022	10708 ± 2342	4695 ± 1793
Fresh Weight (g)	11 ± 1.6	6.2 ± 1.8	2.5 ± 0.79	1.4 ± 0.83
Plants transformed with empty vector (*-TMT1*)				
Concentration (NaCl)	**0 mM**	**50 mM**	**100 mM**	**200 mM**
Height (cm)	4.8 ± 1.8	2.0 ± 0.21	1.1 ± 0.26	*
Number of Leaves	11 ± 1.15	7 ± 0.58	5 ± 1	*
Total Leaf Surface Area (μm^2)	38584 ± 22712	14238 ± 5916	5922 ± 1280	*
Fresh Weight (g)	9.9 ± 7	3.5 ± 1.4	1.2 ± 0.2	*

* All the plants were dead at 200 mM NaCl.

3.3.5 Effect of NaCl on growth of transgenic tobacco plants throughout the life cycle

In order to assess the tolerance of transgenic plants towards salinity throughout their life cycle, stressed plants were grown under different concentrations of NaCl (Figure 14). Different growth parameters such as height, diameter of stem, and number of leaves were recorded (Table 3). There was no significant difference found in overall growth between -*TMT1* and +*TMT1* plants in the absence of NaCl. At higher concentration of salinity (50 and 100 mM) both -*TMT1* and +*TMT1* plants suffered a growth reduction. However, all the +*TMT1* lines showed a better performance (down to 42.2% of control) than -*TMT1* plants (down to 52.2% of control) when subjected to stress treatment. At 200 mM NaCl, -*TMT1* plants were already dead; whereas +*TMT1* transgenic plants tolerated this degree of salinity, continued to grow, flowered and set viable seeds (Figure 14). The flowering time was delayed in the plants growing under salinity. Non-stressed plants flowered a week earlier compared to stressed plants based on visual observation.

3.4. Effect of *TMT* gene on the salt tolerance and chloride volatilization ability of *Arabidopsis* plants

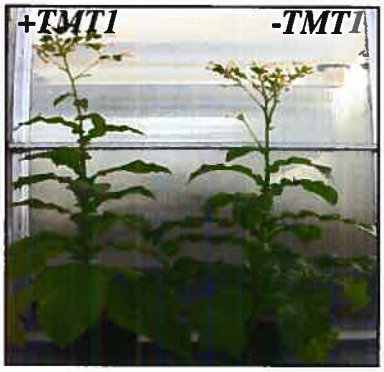
Effect of NaCl on growth of *Arabidopsis* plants and their ability to volatilize Cl^- to CH_3Cl under different concentration of NaCl were investigated. Wild type plants which naturally contain a *TMT1* homologue, plants over expressing *TMT1* and knock-out mutants grew to a similar extent in the absence of NaCl (Figure 15). As the concentration of NaCl increased, the three plant types showed growth retardation. Higher concentration of NaCl had toxic impact on all three types of plants and all plants ultimately died.

The wild type plants and plants over-expressing *TMT1* gene were able to emit CH_3Cl under all the concentrations of NaCl (Figure 16). The *TMT1* over-expressing plants emitted the highest amount of CH_3Cl at the NaCl concentration of 100 mM whereas the highest emission from the wild type plants was recorded at 50 mM of NaCl. In both types of plants, emission severely decreased at 200 mM of NaCl. In contrast, no detectable amount of CH_3Cl was recorded by knock-out mutant plants.

Figure 17 shows the visual impact of NaCl on overall growth of *Arabidopsis* plants. Plant height, number of leaves per plant, number of stems, number of flowers, and

fresh weight were recorded at the end of experiment (Table 4). All lines responded to salinity to a similar extent with respect to height. Wild type plants had many more (~ 30%) leaves than did either +*TMT1* or mutants. Mutants had fewer branches and inflorescences than the other two categories. However, the number of siliques was significantly greater in plants over-expressing *TMT1* gene compared to wild type plants and knock-out mutants. The difference persisted under NaCl, but +*TMT*1 plants apparently suffered greater reduction in the number of leaves (down to 20% of control, compared to 36% for WT and 45% for mutants). All the three lines became severely inhibited and ultimately died when treated with 200 mM of NaCl. Wild type plants had slightly greater fresh weight compared to the other two categories.

0 mM NaCl



50 mM NaCl

100 mM NaCl

200 mM NaCl

Figure 14. Effect of NaCl on growth of transgenic tobacco plants grown to maturity. Seven week old plants were transferred to pots containing soil. Salinity treatment was applied twice a week and fertilized once a week until the end of the experiment. Each treatment had 3 replicates. The plants were photographed on the 140^{th} day.

Table 4. Effect of salinity on different growth parameters of *+TMT1* and *-TMT1* tobacco plants grown to maturity under greenhouse conditions. Seven week old plants were transferred to pots containing soil. Salinity treatment was applied twice a week and pots were fertilized once a week until the end of the experiment. Each value is the mean ± SD (n = 3).

Plants (T1) transformed with *+TMT1*

Concentration	0 mM	50 mM	100 mM	200 mM
Height (cm)	139 ± 1	104.3 ± 3.8	80.3 ± 4.7	51.7 ± 6.8
Numbers of Leaves	44 ± 7	36 ± 1	24 ± 1	22 ± 1.5
Diameter (cm)	2.03 ± 0.058	1.94 ± 0.058	1.66 ± 0.058	1.33 ± 0.058

Plants transformed with *-TMT1*

Concentration	0 mM	50 mM	100 mM	200 mM
Height (cm)	32.6 ± 4.7	92.3 ± 3.8	63.3 ± 3.5	*
Numbers of Leaves	43 ± 7.5	30 ± 3	15 ± 3.2	*
Diameter (cm)	2 ± 0.058	1.88 ± .115	1.34 ±0.058	*

* All the plants were dead at 200 mM NaCl.

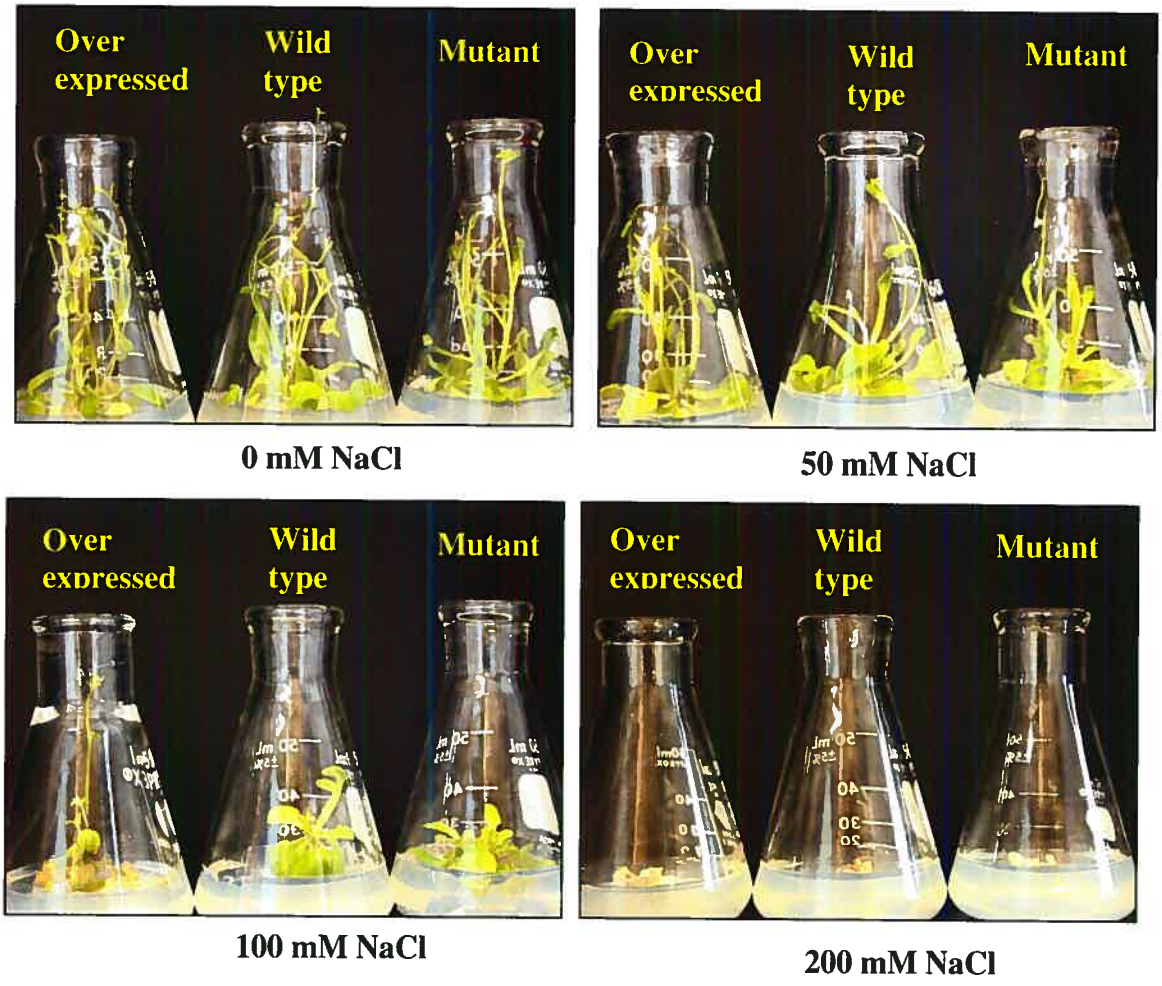


Figure 15. Photographs of wild type, *TMT1* over-expressing and TMT-mutated *Arabidopsis* plants grown under NaCl. Sixteen day old seedlings were transferred to 50 ml flasks containing MS and 0.4% phytagel supplemented with 0, 50, 100 and 200 mM NaCl. Each treatment had 3 replicates. The plants were photographed on the 14th day.

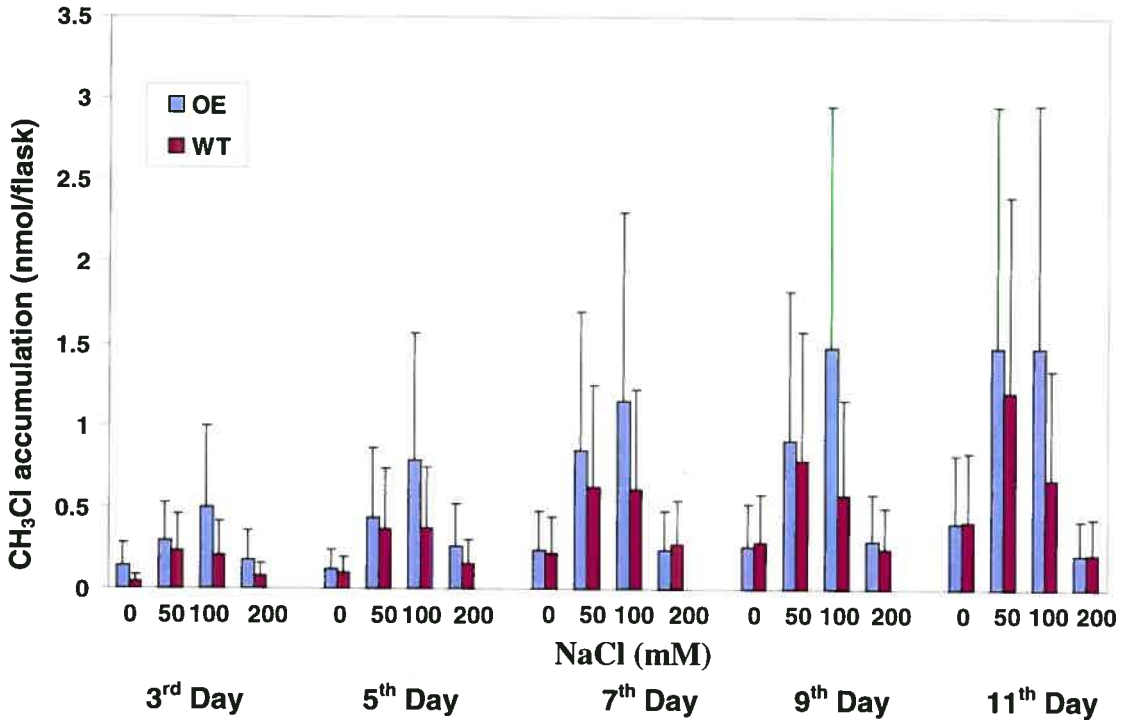
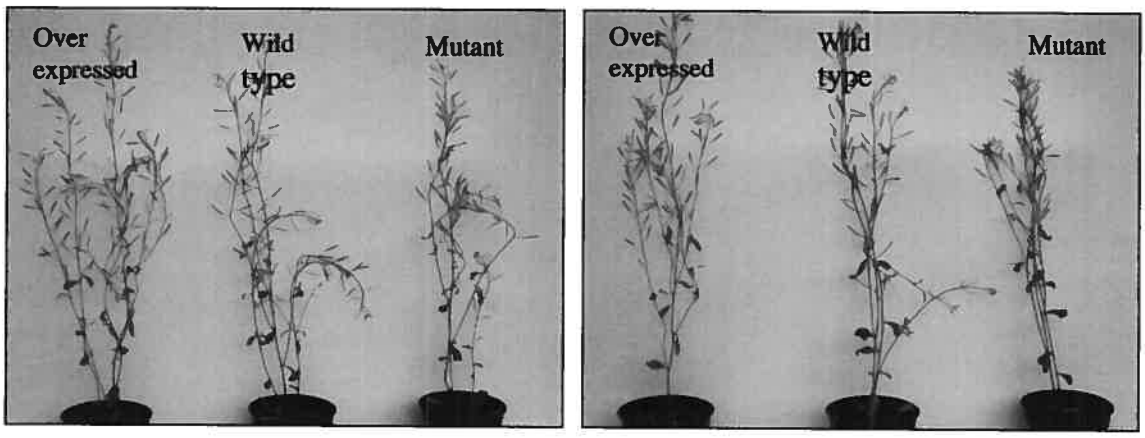
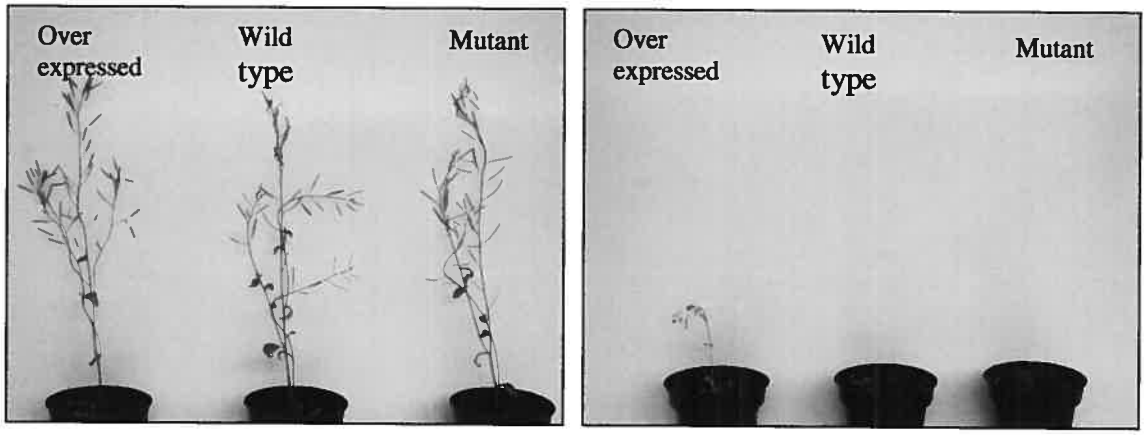


Figure 16. CH_3Cl emission from wild type and *TMT1* over-expressing *Arabidopsis* plants. *Arabidopsis* seedlings were transferred to 50 ml flasks containing MS and 0.4% phytagel supplemented with 0, 50, 100 and 200 mM NaCl in 50 ml Erlenmeyer flasks. CH_3Cl concentration in 1ml headspace samples taken from the flasks was determined with GC at different time points. Values are the mean ± SD (n = 3).



0 mM NaCl **50 mM NaCl**



100 mM NaCl **200 mM NaCl**

Figure 17. Effect of NaCl on the growth of wild type, *TMT1* over-expressing and TMT-mutated *Arabidopsis* plants grown to maturity. Seeds of wild type, knock-out mutants and plants over-expressing with *TMT1* gene were grown on soil contained in pots. Salinity treatment was applied to 2 weeks old plants and lasted until the end of the experiment. NaCl concentrations were 50, 100 and 200 mM. Each treatment had 3 replicates.

Table 5. Different growth parameters of *Arabidopsis* plants grown to maturity under saline conditions in a greenhouse. Seed of wild type, knock-out mutants and plants over expressing *TMT1* gene were grown on pots containing soil. Salinity treatment was applied to two week old plants and lasted until the end of the experiment. NaCl concentrations were 50, 100 and 200 mM. Each value is the mean ± SD (n = 3).

Concentration (NaCl mM)	0			50			100		
	+TMT1	Wild type	Mutant	*+TMT1*	Wild type	Mutant	*+TMT1*	Wild type	Mutant
Height (cm)	33.8 ± 2.4	34 ± 3.1	32 ± 2.5	29.3 ± 4.8	32 ± 0.95	29.2 ± 1.3	28.9 ± 3.0	28 ± 0.61	26 ± 0.85
Number of leaves	20 ± 5.5	33 ± 9.3	22 ± 4.5	7 ± 4	22 ± 4.9	14 ± 3.5	4 ± 1.5	12 ± 4	10 ± 3.6
Number of branches	10 ± 0.58	9 ± 0.58	6 ± 0.58	7 ± 0.58	7 ± 0.58	4 ± 0.58	5 ± 0.58	3 ± 0.58	3 ± 0.58
Number of siliques	133 ± 9.1	90 ± 9	80 ± 24	90 ± 13.8	79 ± 12.3	65 ± 20.1	45 ± 6.9	57 ± 19	47 ± 20.2
Number of Inflorescen	16 ± 1.5	16 ± 1.5	9 ± 4	5 ± 1.5	10 ± 3.1	6 ± 3.2	3 ± 1.5	4 ± 2	5 ± 1
Fresh weight (g)	1.2 ± 0.58	1.2 ± 0.28	0.91 ± 0.16	0.99 ± 0.19	1 ± 0.13	0.89 ± 0.16	0.39 ± 0.02	0.41 ± 0.03	0.31 ± 0.01

4. DISCUSSION

CH_3Cl, the most abundant chlorine-containing gas in the atmosphere, has received great attention due to the facts that it is the largest natural source of chlorine in the atmosphere, and because of its involvement in stratospheric ozone depletion [35]. In recent years, there has been renewed interest in identifying and quantifying the principal sources and sinks of atmospheric CH_3Cl along with numerous attempts to understand the mechanisms of its biosynthesis and degradation in nature.

In 1990, Wuosmaa and Hager [80] reported that a marine alga *E. muricata,* a wood-rotting fungus *P. pomaceus*, and a halophytic plant *M. crystallinum* could synthesize CH_3Cl by an enzymatic methyl transfer reaction. MCT catalyzes the conversion of Cl^- to CH_3Cl by using AdoMet as the methyl donor. The methyltransferase enzyme is found in several organisms, such as marine algae, fungi, and halophytic plants. Harper suggests that this enzyme could potentially be used in engineering of transgenic crops leading to enhancement of salt tolerance by Cl^- volatilization [33]. This enzyme has been characterized from *Batis maritima* [56]. Furthermore, presence of this enzyme activity has been interpreted as a possible mechanism for chloride detoxification via its volatilization in certain organisms that live in saline habitats.

This is the first detailed experimental report on the effect of engineering a *TMT1* gene, encoding a TMT enzyme and isolated from red cabbage [7] in various species, and assessment of salt tolerance in different transgenic organisms. This enzyme can methylate a variety of substrates including chloride. In addition, the novelty of this study is that it provides the first experimental data on the correlation between the ability of TMT to volatilize Cl^- to CH_3Cl and the influence salt tolerance. This was achieved by engineering a *TMT1* gene into *E. coli* cells, root cultures of potato and tobacco, as well as in tobacco and *Arabidopsis* plants.

Next, the data presented in this study on bacterial cells demonstrates that the presence of TMT promotes the growth of bacterial cells in the presence of NaCl. However it does not improve their tolerance to NaCl salinity despite the fact that their production of CH_3Cl from Cl^- containing medium increases at higher rate (Figure 2).

Moreover, there is a wide spectrum of salinity tolerance among higher plants. For instance, glycophytes are extremely sensitive to salt, and on the contrary halophytes can survive in environments containing large amounts of salt. Further, plants that tolerate and grow in saline environments have evolved a number of strategies to survive under saline conditions. An important strategy of plants in high salinity conditions involves compartmentalization of ions into the plant's vacuoles or exclusion and sequestration of the excess salt from the cells [18]. Additionally, exclusion of ions can be achieved through the passive exclusion of ions by a permeable membrane, the active expelling of ions by ion pumps, or by dilution of ions in the tissue of plant [4]. Na^+ and Cl^- induce both osmotic and ionic effects when administered at high concentrations. Although it is hard to completely differentiate between the effects caused by each ion, many studies have concentrated on the effects on Na^+. There are some species such as citrus, barley, and grape in which Cl^- contributes a large part to toxicity than Na^+ [49]. For example, around 50 mM NaCl of soil solution, salt-sensitive rootstocks of citrus take up larger amounts of Cl^- than Na^+ [11]. According to Levit [45] accumulation of Cl^- in the aerial parts of rice is the main reason of toxicity of the NaCl. Maize seems to accumulate the same amount of Cl^- and Na^+ [40]. Accumulation of Cl^- in the leaves is the major cause of foliar injury of *Glycine max* [79]. Similarly, Luo et al. [46] reported that Cl^- was more toxic than Na^+ to seedlings of *Glycine max* under salt stress. Seedlings of two G. max cultivars- salt-tolerant Nannong 1138-2, and Zhongzihuangdou-yi, were damaged much more heavily in the solution of Cl^- than in that of Na^+ and their leaves were found to be more sensitive to Cl^- than to Na^+. The findings from the previous results that TMT could efficiently methylate Cl^- to CH_3Cl, led to speculation that TMT might protect plants against high internal Cl^- concentration through its volatilization. I have conducted studies to determine whether expression of *TMT1* enables the transgenic species to survive under salinity conditions.

Furthermore, the results obtained with potato and tobacco roots indicate that all the +*TMT1* roots grew better than -*TMT1* roots (Figure 4) and were able to volatilize Cl^- to CH_3Cl (Figure 6 & 8). The data presented here, show that TMT efficiently volatilized chloride from the cells and thus plays a key role in increased growth.

Next, studies that were undertaken to determine the salt tolerance of tobacco plants throughout their life cycle, started with experiments at germination level. Germination of

seeds of +*TMT1* plants was similar to -*TMT1* plants in absence of NaCl. Although there was a reduction in the germination of seeds as the concentration of NaCl increased, no major differences were observed between +*TMT1* and -*TMT1* seedlings (Figure 9). Salt tolerance at one stage of plant development is often not correlated with tolerance at other stages, for example, tolerance at germination does not correlate with tolerance at later growth stages [24, 25]. However, the germination rate is the least likely process to predict the ability of plants to grow under saline environments [55]. It has been established that there is little or no relationship between salt tolerance at germination and that of the seedlings, for example in durum wheat, bread wheat, and barley [5, 44, 47]. Similarly, it has been recognized that rice plants show high salt tolerance during seed germination but are extremely sensitive to it during growth stages [3]. In tomato, the ability of the plants to germinate under conditions of high salinity is not always correlated with the ability of the plant to grow under salt stress because both are controlled by different mechanisms [25].

Damaging effects of salt accumulation on growth are not confined to the first few days as they are evident even weeks after salt stress is imposed [52]. Thus further experiments were undertaken at different growth stages of tobacco plants. It was noted that during early growth stage, all +*TMT1* and -*TMT1* seedlings experienced similar growth in the absence of NaCl. A degree of salt tolerance was observed at higher concentrations of salt. Hence, these results clearly indicated that the inhibition of growth was less pronounced in all the +*TMT1* plants as they were able to methylate Cl^- to CH_3Cl (Figure 11 & 12). On the contrary, it was noticed that growth of all the -*TMT1* plants under salinity stress was severely undermined and no detectable production of CH_3Cl was recorded. Although, the growth of +*TMT1* plants was negatively impacted with an increase in NaCl, they were still able to maintain their growth and volatilized Cl^- at much higher levels as compared to -*TMT1* plants. Therefore, these findings are of great significance demonstrating that TMT plays a key role in volatilization of Cl^- leading to enhancement in their tolerance towards salt.

In addition, a few more experiments were conducted in the greenhouse in order to assess stress tolerance of transgenic plants at older age and its effects on the ability to produce flowers and seeds. As a result when these plants were grown without any exposure to NaCl, no notable differences in the overall performance of +*TMT1* plants were found in comparison with the -*TMT1* plants (Figure 13 & 14). The +*TMT1* and -*TMT1* plants

experienced comparable growth in all aspects of the analysis when grown without NaCl. (Table 2 & 3). Contrary to findings of bacterial and root level experiments, it was indicated that during later stages of plant life TMT has minimal or no effect on their growth. However, there is a possibility that TMT's impact on plant growth may vary depending on whether the response times are of a short-term nature or long term. Furthermore, when exposed to moderate levels of salt stress, both *+TMT1* and *-TMT1* plants were able to survive, flowered and set viable seeds. However, *+TMT1* plants performed better in all aspects of growth as compared to *-TMT1* plants. Next, when both *+TMT1*, and *-TMT1* plants were exposed to higher levels of salt stress (200 mM), it was noticed that *+TMT1* plants were able to flower and set viable seeds, whereas *-TMT1* plants could not tolerate this concentration and ultimately died. Hence, these results strongly indicate that when TMT introduced into a species that does not contain its normal metabolic context, it can play a role in enhancing plant growth when they are subject to high salinity conditions.

There are also numerous examples where compartmentalization of Na^+ in the vacuole increased the salt tolerance of most plant species, which depend on Na^+/H^+ antiporters as well as V-type H^+-ATPases and H^+-PPases. Over-expression of vacuolar H^+-PPases- AVP1 in *Arabidopsis* enhanced sequestration of Na^+ into the vacuole and showed increased tolerance to salt stress than that of wild type [27]. Similarly, the over expression of AtNHX1, a vacuolar Na^+/H^+ antiport, in Arabidopsis plants allowed the transgenic plants to grow in 200 mM NaCl [6]. Zhang *et al* show that transgenic Brassica napus plants over-expressing AtNHX1, a vacuolar Na^+/H^+ antiport were able to grow, flower, and produce seeds in the presence of 200 mM NaCl [87]. Similar results have been reported for transgenic tomato over-expressing AtNHX1 [86]. These tomato transgenics were able to grow, flower and produce fruits in the presence of 200 mM NaCl.

Sanan-Mishra *et al.* characterized a functional DNA helicase from pea, Pea DNA helicase 45 (PDH45) [63]. Its over expression in tobacco plants confers high salinity tolerance. The T1 transgenics were able to grow to maturity and set normal viable seeds under salinity stress without affecting yield. Recently, over-expression of trehalose biosynthetic gene has also contributed toward development of abiotic stress-tolerant genotypes in rice [26]. The Yeast HAL1 genes reported to improve salt tolerance in

transgenic tomato by maintaining a high internal K^+ concentration and decreasing intracellular Na+ during salt stress [29].

Considerable work has been carried out to genetically engineer overproduction of compatible osmolytes in transgenic plants such as *Arabidopsis*, rice, wheat, and tobacco. For example, over-expression of enzymes leading to increased production of glycine betaine in tobacco [38], proline in tobacco [39], trehalose synthesis in rice [26], mannitol in tobacco [70], wheat [1] and *Arabidopsis* [72], D-ononitol production in tobacco [65], and fructan synthesis in tobacco [58], conferred increased tolerance to salt stress.

There are many different model systems such as tobacco, ice plant (*M. crystallinum),* tomato, and *Arabidopsis*. However, *Arabidopsis* has emerged as an excellent model system to study salt tolerance [88], and the fact that it naturally contains TMT, *Arabidopsis* plants were used for the further assessment of the role of TMT in salt tolerance. Various studies were conducted to determine whether over-expression of *TMT1* or knock-out mutants enables the *Arabidopsis* plants to survive better under salinity conditions (Figure 15 & 17). Experiments were carried out on the three lines – wild type, knock-out mutants, and plants over-expressing *TMT1*. Additionally, the data in the present study indicated that there were no differences found between the three lines in terms of their responses towards salinity stress (Table 4). It was further noted that at 200 mM NaCl all three lines were severely inhibited and ultimately died. *Arabidopsis* naturally contains TMT, where it is involved in the methylation of thiol compounds. Also, it has been shown that the most preferred substrate for TMT is ^-SCN, based on its low k_m value and its ability to inhibit the methylation of iodide [9]. Thus, TMT does not efficiently methylate Cl^- when natural thiol substrates, with much greater affinity for the enzyme's active site, are present. Hence, when *TMT* gene is mutated out of the plants or over expressed in the plants, there was no change in the plant's salt tolerance.

The results presented in this study indicate a correlation between the ability of TMT to volatilize Cl^- and enhancement in salt tolerance. Taken together, the results showed that:

- All the engineered species acquired an ability to efficiently transform Cl^- to CH_3Cl.
- The *+TMT1* plants developed a high degree of tolerance to salt concentrations that were toxic to *-TMT1* counterparts.

- Volatilization of chloride is a detoxification event that can contribute to the plant's ability to withstand salinity stress.

Significance and future prospects:

Several papers have presented characterizations of numerous biochemical pathways associated with stress tolerance in different plants, and numerous genes involved in these processes have been identified. Also many compatible solutes such as carbohydrates, amino acids, and quaternary N-compounds have been proposed to play a role in osmoregulation under stress [53]. Moreover, much of the effort towards improving salt tolerance of crop cultivars assumed that salt tolerance would be achieved only after pyramiding several characteristics in a single genotype. However, various authors have claimed that single-gene transfers have led to the enhancement of salt tolerance of plants [6, 29, 63]. Similar to these results, data in the presented study suggest that modification of a single trait significantly improved the salinity tolerance of tobacco plants.

Thus these results together with the data presented here clearly demonstrate TMT containing organisms could tolerate high concentration of salt by detoxifying them through methylation. These results demonstrate a major role in developing salt tolerant plants by means of introducing the *TMT* gene and suggest possibilities for engineering a chloride detoxification capability into a high value crop to improve tolerance against chloride ion toxicity under saline conditions. This gene might also be use for desalinization of soil.

It has been known that CH_3Cl affect the integrity of the stratospheric ozone layer [35]. So, aside from understanding to contribution of the environmentally important CH_3Cl gas, accurate prediction of the impact of CH_3Cl emission on the atmospheric budget, will allow to use this new pathway for enhancement of salt tolerance.

Two different mechanisms for stress tolerance have been discussed previously which are: mechanisms controlling the osmotic effect of salt and mechanisms controlling the salt specific effect of salt. It appears that the ability of *TMT* to volatilized Cl^- comes under the second category; where it detoxifies excess Cl^-. However, it is still unclear that up to what extent *TMT is* able to regulate their internal Cl^- ion concentration. We have to yet determine how this mechanism operates at cellular level.

In summary, thiol methyltransferase can detoxify intracellular chloride when engineer into an appropriate species, when excessive salt is present in the environment.

5. Conclusion

This study provides the first detailed experimental data on the effect of engineering a novel thiol methyltransferase (*TMT*) gene into various species such as *E. coli* cells, root culture of potato and tobacco and tobacco and *Arabidopsis* plants, on their tolerance to sodium chloride stress. This enzyme can methylate Cl^- ions to CH_3Cl, in addition to its natural thiol substrates. Detailed physiological studies were performed to examine the relationship between salt tolerance and chloride volatilizing capacity of transgenic organisms.

All the engineered species acquired an ability to efficiently transform chloride ions to chloromethane. Parallel with this, the transformed plants developed a high degree of tolerance to NaCl salinity, which was toxic to the untransformed counterparts. The results convincingly demonstrate that volatilization of chloride is a detoxification event that can contribute to the plant's ability to withstand salinity stress. Conversely, when *TMT* gene knock-out or over-expressed in *Arabidopsis* there was no change in the plant's salt tolerance. (For *Arabidopsis* mutants, only one individual T-DNA insertional line was used). This is presumably because *Arabidopsis* naturally contains the *TMT* gene, where it is involved in the methylation of thiol compounds. Thus, TMT does not efficiently methylate Cl^- when natural thiol substrates, with much greater affinity for the enzyme's active site, are present.

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