Developing Strategies for Homologous/Heterologous Plant Expression System for Physiological Investigations of Respective Target Proteins.

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Dedicated to my beloved Parents

ABSTRACT

Developing Strategies for Homologous/Heterologous Plant Expression System for Physiological Investigations of Respective Target Proteins.

A direct translational control for recombinant gene products in homologous or heterologous plant expression systems is the major constraint for physiological investigations. Especially in the large seed grain legume family, the transformation recalcitrance is drastically limiting the number of independent lines which do not meet the basic requirements for relative expression stability, e.g. the integration of more than one copy of the transgene, which can result in gene silencing. This causes often problems in physiological studies with transgenic plants.

Therefore dicistronic binary vector constructs based on pGreenII vectors were made which allow a direct expression control on cellular and entire plant level. The advantage of this approach is that cap-dependent expression of physically independent *β - glucuronidase* can be monitored by the IRES (internal ribosome binding site) mediated cap-independently co-expressed luciferase, which is located on the same mRNA. As a first proof the functionality of the constructs was shown by using two marker genes coding for a *β*- glucuronidase and a fire fly luciferase behind the IRES elements. The proof of principle for the functionality of the dicistronic constructs in physiological studies was made by overexpressing a sodium antiporter (*AtNHX1*) gene from *Arabidopsis thaliana,* providing improvement of salinity tolerance in transgenic plants. The performance of IRES elements combines absolute transcriptional linkage of two genes on one m-RNA with the translational independence of the genes, resulting in two separate proteins.

As a basic novelty in this work IRES elements were used for the first time to transform plants and plant cells with a gene transferring a functional trait linked to a reporter gene. Through IRES mediated Co-expression of target and reporter gene, instead of a fusion protein it was possible to correlate the functional trait in physiological studies, in terms of cell growth with the activity of the reporter gene in transient and stably transformed cells and leaves. NaCl challenge to *AtNHX1* transgenic vs wild type tobacco suspension cells

showed significant tolerance over wild type up to 150 mM. With the increase in NaCl concentration in the growth medium, increase of cell mass and luciferase expression was observed in transgenic tobacco cells in comparison to wild type cells, the maximum was at 100 mM. Via *Agrobacterium* mediated gene transfer by using the disarmed *EHA* 105 strain, the dicistronic construct MASnhx1/luc was transferred into the pea (*Pisum sativum*) genome. Transgenic T0, T1 and T2 pea plants confirmed by PCR showed luciferase activity, as a first indicator for the *AtNHX I* expression in pea.

Key words: Translation, Internal Ribosome Entry Site (IRES), Co-ordinated expression, Tobamoviruses, Plants and plant cell lines.

ZUSAMMENFASSUNG

Bei physiologischen Untersuchungen stellt die einfache und schnelle Ermittlung der Expression rekombinanter Genprodukte in homologen und heterologen Pflanzenexpressionssystemen ein erhebliches Problem dar. Vor allem bei großsamigen Körnerleguminosen führen niedrige Transformationseffizienzen zu einer sehr begrenzten Anzahl an unabhängigen Transformationslinien, die nicht immer die notwendigen Vorbedingungnen für eine relative Expressionsstabilität erfüllen, wie z.B. die Integration von mehr als einer Kopie des Transgens, was in der Folge zu einem unerwünschten Genesilencing führen kann. Letzteres stellt für physiologische Untersuchungen transgener Pflanzen oft ein Problem dar.

Um dieses Problem zu lösen wurden in dieser Arbeit dicistronische, binäre Vektorkonstrukte hergestellt, welche auf pGreenII Vektoren aufbauen. Unter der Kontrolle eines Promoters befinded sich in den dicistronischen Vektoren hinter dem Targetgen ein zweites Gen, welches über ein IRES-Element (interne Ribosomen indungsstelle) mit dem ersten Gen direkt verknüpft ist und beide Gene so zu einer transkriptionalen Einheit werden. Der Vorteil dieses Ansatzes ist, dass die *cap*-abhängige Expression des ersten Gens, in diesem Fall des *β –Glucuronidasegens,* durch die *cap*-unabhängig co-exprimierte Luciferase detektiert werden kann, welche auf derselben mRNA liegt. Die Funktionalität der Konstrukte wurde zunächst mittels zweier Markergene bewiesen, welche für eine *β* -Glucuronidase und ein Luciferase codieren.

Als prinzipieller Beweis für die Funktionalität dicistronischer Vektoren für das Monitoring der Genexpression wurde ein Salzantiporter aus *A. thaliana* verwendet, welcher erhöhte Salztoleranz in transgenen Pflanzen hervorruft. Die Anwendung von IRES-Elementen führt zur transkriptionellen Einheiter zweier Gene auf derselben mRNA bei gleichzeitiger translationaler Unabhängigkeit der Gene, was entsprechend in zwei getrennten Proteinen resultiert. In der hier vorgestellten Arbeit wurden zum ersten Mal dicistronische Vektoren zur Transformation von Pflanzen wie auch Pflanzenzellen eingesetzt, die ein funktionelles Gen mit einem Reportergen verknüpfen und zu einer Co-Expression ohne Bildung eines Fusionsproteins führen. Hierbei wurde nachgewiesen, daß das funktionelle Merkmal, gemessen am Zellwachstum, mit der gemessenen Aktivität des Reportergens korrelierte. Im Vergleich zu nicht transformierten Suspensionszellen

konnten trangene Suspensionszellen signifikant höhere Salzkonzentrationen, von bis zu 150 mM NaCl, tolerieren. Im Vergleich zu den nicht transgenen Suspensionszellen konnte sowohl die Zunahme der Zellmasse als auch die der Luciferaseaktivität gezeigt werden. Das Maximum für beide Messparameter lag bei 100 mM NaCl im Medium.
An transgenen Erbsen, welche über einen Agrobakterium vermittelten Gentransfer mit dem dicistronischen MASnhx1/luc Konstrukt transformiert wurden, konnte über die Luciferase-Aktiviät ein erster Hinweis auf die rekombinante *AtNHX1* Expression in T0,T1 und T2 Pflanzen gezeigt werden.

Stichworte: Translation, Interne Ribosomenbindungsstelle (IRES), Ko-Expression. Tobamovirus, Pflanzen und Pflanzenzelllinien.

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Zahid Ali

LIST OF ABBREVIATIONS

µl	Micro liter
µM	Micro Mole
A. thaliana	*Arabidopsis thaliana*
A .tumefaciens	*Agrobacterium tumefaciens*
AtNHX1	*Arabidopsis thaliana* Na^+/H^+ exchanger
B5	B5 basal medium
BAP	6-benzyl-amino-purine
Bar	BASTA® (bialaphos) resistance gene
bp	base pair
CaMV	Cauliflower Mosaic Virus
CCLR	Cell Culture Lysis Reagent
CR	Chlorophenol red (pH indicator)
CTAB	Cetyl Tri-methyl Ammonium Bromide
DMSO	Dimethyl sulfoxide (Commercial solvent)
EDTA	Ethylene Diamine Tetra Acetate
g/l	gram per liter
gDNA	genomic Deoxyribo Nucleic Acid
Gus/gusA	β-glucuronidase (enzyme/gene)
IBA	Indole Butyric Acid
Kb	Kilo base pair
LAU	Linear Arbitrary Units
LAR	Luciferase Assay Reagent
LB	Left Border
LB	Lauria Bertani
Luc	*Luciferase*
ml	milli liter
MAS	Manopine Synthase promoter
mg/l	milligram per Liter
MS	Murashige and Skoog medium

MCS	Multiple Cloning Site
mRNA	messenger Ribonucleic Acid
Mug	Fluorometric substrate 4-methylumbelliferyl-β-D-glucuronide
N. benthamiana	*Nicotiana benthamiana*
N. tabacum	*Nicotiana tabacum*
ng	nano gram
NAA	1-naphthyl-acetic acid
Na+/H+	Sodium/proton
PAT	Phosphinothricin acetyltransferase
PCR	Polymerase Chain Reaction
PPT	Phosphinothricin
RB	Right Border
RLU	Relative Light Units
RT	Room temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
T0	First transgenic plant directly induced from callus
T1	First generation of seeds and plants after self-cross on T0 plants
T2	Second generation of seeds and plants after self-cross on T1 plants
T-DNA	Transferred DNA
TE buffer	Buffer solution containing 10 mM Tris and 1 mM EDTA
Ti-Plasmid	Tumor-inducing Plasmid
WT	Wild Type

1 INTRODUCTION

1.1 Overview

Coordinated expression of multiple protein under the control of one promoter may facilitate to influence or alter biosynthetic pathways leading to secondary metabolites or to improve physiological traits and may help to express proteins of therapeutic or diagnostic use. Such a coordinated expression of target genes would open new avenues for functional genomics in transient expression systems as well as in stably transformed tissues or entire plants. Genetic engineering of crop plants can improve drought tolerance as it has been attempted by classical genetic methods with only some successes (Sottosanto *et al*., 2007; Zhang *et al*., 2001; Heranandez *et al*., 2000; Apse *et al*., 1999). Application of agricultural biotechnology to enhance drought and salinity tolerance in plants is still in progress for finding a robust and single translational monitoring system for basic research. For the intervention into the osmo regulative processes the coordinated expression of two or more proteins/enzymes might be necessary. Beside the possibility of gene stacking via crossing of transgenic plants, internal ribosome entry sites (IRES) (Martin *et al*., 2004; Urwin *et al*., 2004; Dorokhov *et al*., 2001) could help to overcome this problem much faster if the second protein is not needed to be of high abundance. One promoter would drive two genes, the first one translated in the cap dependent manner and the second one cap independent by the IRES element since the IRES mediated expression is low (Dirks *et al*.,1993). In agro biotechnology although coordinated expression of two genes is still a problem but seems to be possible by the IRES mediated approach. Therefore dicistronic vectors for the translational control of target genes were made and tested. Three kinds of IRES elements Tobamo IRES, polio IRES and putative *Zea mays* IRES elements were used as intercistronic spacers in dicistronic vector system. Functionality of the vector system was confirmed by marker gene constructs *β-glucuronidase* and firefly *luciferase*. A sodium/proton anti porter gene from *A. thaliana* has been used for the improvement of salt tolerance in plants (Cixin *et al*., 2005; Blumwald, 2000) in place of *GUS* gene as a first cistron in front of a *luciferase* gene which is mediated by an IRES element Comparative investigations were made on the basis of *luciferase* expression in transgenic tobacco plant derived suspension cells by

applying stress against the targeted gene *AtNHX1*. Finally studies were conducted to transform the IRES mediated dicistronic system to pea (*Pisum sativum* L.) by *Agrobacterium* mediated transformation which was used as a leguminous model plant. Studies were conducted on the basis of luciferase expression which can ensure that the first cistron (*AtNHX1*) is also being expressed.

OBJECTIVES

- Development of a dicistronic gene expression system using *β-glucuronidase* and firefly *luciferase* marker genes. This will be based on the performance of IRES elements in which absolute transcriptional linkage of two genes on one mRNA is combined with the physical separation of each of the proteins.

- Cloning of sodium/proton anti porter gene (*AtNHX1*) in a mono and dicistronic vector system for the two reasons
  - As a proof of the principle for the new vectors.
  - For the enhancement of the drought and salt tolerance in legumes.

- *Agrobacterium* mediated plant transformation (Tobacco, Pea) using the dicistronic vector system and selection of transgenic tissues/plants on the basis of expression of the second cistron (*luciferase*) for physiological tests.

1.2 Soil salinity

Among abiotic stresses, salinity is a major environmental problem throughout the world (Blumwald, 2000; Apse *et al*., 1999; Blumwald *et al*., 1985) caused by both biophysical and human factors which limit the crop growth. This problem is going to increase with global warming and thus imposes significant difficulties during plant/crop growth developmental stages (Wang *et al*., 2003) which ultimately reduce the net production and crop yield (Munns, 1993; 2002). The food security of rapidly growing population is dependent on the continuous development and improvement of crop plants with increased salinity and drought tolerance (Denby *et al*., 2005). Plant growth is affected by salinity in various ways e.g. by imposing water deficit, causing ion specific stresses resulting in disturbed K^+/Na^+ ratio and increasing the concentration of Na^+ and Cl^- which are lethal to cells in higher concentrations (Yamaguchi *et al*., 2005).

The detrimental affects of salinity can vary with different growth stages (Adam, 1990). Particularly seed germination and emergence and early developmental stages are sensitive to salinity (Ungar, 1996; Mariko *et al*., 1992).

According to an FAO (2005) survey, more than 800 million hectares of the land are affected by salts and out of this 397 million hectares are affected only by salinity. This problem is going to increase at alarming rates. In Pakistan for example, out of nearly 20 million hectares of cultivable land, about 6 million have been classified as saline or saline sodic and the productive areas are being damaged at an alarming rate of 40,000 hectare per year (Alam *et al*., 2000).

1.2.1 Soil salinity and plant response

For tolerating adverse salinity effects, the plant defense system gets activated and responds in two different ways, either by restricting the uptake of salts and adjusting the osmotic pressure by accumulating the osmolytes for example proline, glycinebetaine and other simple or complex sugars i.e. salt sensitive plants (Chinnusamy *et al*., 2005) or by transporting and sequestering the excessive amount of salts into vacuoles by controlling the concentration of salts in vacuoles and plasma membrane i.e., salt tolerant plants by maintaining high cytosolic K^+/Na^+ ratio in their cells (Glenn *et al*., 1999).

1.2.2 Soil salinity and legumes

Grain legumes are an excellent and inexpensive source of plant protein. Many species are growing well on marginal soils. When eaten in combination with wheat, rice and other cereals they provide a balanced diet for millions of people. Pulses are known as "poor man's meat" in the developing world, while in the developed world they are perceived as "health food". Being leguminous they maintain soil fertility by converting and fixing atmospheric nitrogen in available form through symbiosis with rhizobia (Hafeez *et al*., 1988). Additionally, pulses are also important components of animal feed. Studies have revealed that in grain legumes salt stress causes an alteration of root hair curling, reduction in the number of rhizobia attached to root hairs and decreased nodule size. Also the amount of fixed nitrogen per unit weight of the nodules declines with increased salt stress (Miller *et al*., 1996; Hafeez *et al*., 1988).

1.3 Genetic engineering for stress tolerance

Genetic engineering has provided additional tools for the confirmation of molecular markers in functional genomics or directly to improve the plants. There is broad consensus that climate change continues to occur and that stress from climatic extremes will continue, which are imposing considerable difficulties in plant and crop growth in many parts of the world (Denby and Gehring, 2005).

The technology for transferring genes to plants is already available (Blumwald, 2000; Potrykus, 1991), even for more refractory cereals (Klein *et al*., 1992). However, the major problem with this approach is the isolation of the relevant halo tolerance genes to be transferred. These genes could be components of the normal adaptation of either crop or halophytic plants to drought/salt stress and their constitutive over expression in the transgenic plants which may improve salt tolerance. On the other hand halo tolerance genes could be obtained from non plant resources, in the same manner that the bacterial toxins genes for insect killing are engineered into plants (Vaeck *et al*., 1987). Genes have been isolated from yeast, bacteria and plants which potentially confer drought and salt tolerance in plants. Attempts have already been made to transform model plants like tobacco, *Arabidopsis* or even rice (Grover *et al*., 2003). In 2001, Zhang and Blumwald made an excellent report by developing transgenic tomato plants carrying the *AtNHX1*

(Na^+/H^+ antiporter) gene from *A. thaliana* which could grow and produce fruits in the presence of sea water. Later on Zhang, *et al*., (2001) also transferred *AtNHX1* gene to *Brasscia napus* and reported that the plants over expressing *AtNHX1* were able to grow and produce seeds in the presence of 200 mM sodium chloride. Shi *et al*., (2003) showed the enhancement of salt tolerance in *A. thaliana* using the same gene. Recently Cixin *et al*., (2005) reported the incorporation of *AtNHX1* gene in cotton by showing *AtNHX1* over expressing cotton plants generated more biomass and produced more fibers when grown in the presence of 200 mM NaCl under greenhouse conditions.

Successes have been reported in developing stress (drought/salt) tolerant transgenic plants especially in tobacco, *Arabidopsis*, rice, tomato, canola, cotton etc. (Cixin *et al*., 2005; Shi *et al*., 2003; Zhang *et al*., 2001) but still there are not sound reports proving the applications for agricultural biotechnology to enhance drought and salinity tolerance (Denby *et al*., 2005). All of the approaches which are well established and published are on the basis of single gene transfer used to enhance drought/salt tolerance in transgenic plants. In parallel, there are several technical and financial challenges associated with transforming many crop plants (Yamaguchi *et al*., 2005) especially in monocots (other than rice) and legumes.

1.4 Translation (IRES elements)

In eukaryotic cells translation initiation is recruited by the cap structure present at 5′ end of mRNA (Hershey *et al*., 2000; Salas *et al*., 2001). In addition to cap dependent translation there is another way of translation initiation which is mediated by cis-acting elements. These elements are called internal ribosome entry sites (IRES elements) (Jackson, 2000). The best studied example of the cap-independent mode of translation initiation in eukaryotes is the IRES elements (Eric Jan, 2006; Sachs *et al*., 1997; Pelletier and Sonnenberg, 1988).

With the discovery of Picorna virus RNAs IRES elements, many functional IRES elements have been identified in an increasing number of mRNAs from animal cells and their pathogens (Carter *et al*., 2000).

First discovered in viral DNA, it was later shown that even in eukaryotic translation under stress conditions (e.g. viral infection) cap dependent translation is severely compromised due to many factors for example poor availability of eIF factors (Cuesta *et*

al., 2000; Gradi *et al*., 1998) but IRES mediated translation prevails (Johannes *et al*., 1999; Johannes *et al*., 1998; Macejak *et al*., 1991).

Studies are still in progress for finding IRES elements in plant cells and plant viruses (Salas *et al*., 2001). Naturally many plant viral mRNAs are without a cap structure so their translation initiation can be considered to be *cap* independent (Niepel *et al*., 1999; Skulachev *et al*., 1999).

Using these findings in order to co-express two distinct coding sequences under the control of one promoter is of great interest for the modern biology (Martin *et al.,* 2006).

Gene stacking via crossing of transgenic plants is a conventional breeding practice but can be limited because of many factors, for example the procedure is quite lengthy and time consuming and it is also sensitive for expression instabilities of transgenic in combinatorial plants, since the promoter and other sequence homologies may result in gene silencing (Matzke, 1993; Mette, 2000). The combinatorial plants are highly vulnerable for these negative effects in case identical selectable marker genes or DNA imprinting (Adam, 2000). Although the strategy of crossing transgenic plants has been used successfully (Halpin and Boerjan, 2003) was difficult to obtain the coordinated expression of multiple genes. An alternative strategy to co expressing multiple proteins in plants is by encoding them in two or more open reading frames (ORFs) by IRES elements. In a combined gene expression, IRES elements from polio virus or encephalomyocarditis virus have been used to construct bi or multicistronic expression vectors to co-express various genes from one mRNA (Hennecke *et al*., 2001; Urwin *et al*., 2001). Various other sources have been used for the isolation of IRES elements like Picorna viruses (Pelletier and Sonenberg, 1988; Gan and Rhoads, 1996) or some other animal viruses (for example retroviruses and hepatitis C virus), mammalian and Drosophila RNAs (Reynolds *et al*., 1995; Bernstein *et al.,* 1997; Akiri *et al*., 1998; Negulescu *et al*., 1998; Chappell *et al*., 2000; Henis-Korenblit *et al*., 2000).

The advantage of this IRES mediated approach is that both proteins are under the control of same the promoter (Martin *et al*., 2006) thus the expression of both proteins is linked. Additionally it also limits the usage of multiple promoters in multi genes vector system (Hennecke *et al*., 2001).

1.5 *Agrobacterium* mediated gene transfer

To express plant reporter genes and analyzing structural components of plant promoters, *Agrobacterium* based transient expression system have been successfully used in leaf tissues like *N. benthamiana*, *N. tabacum*, *Phaseolous vulgaris*, *Phaseolous acutifolius*, *Lactuca sativa*, *A. thaliana*, *Pisum sativum* and *Linum usitatissimum* (Cazzonelli *et al*., 2006; Wroblewski *et al*., 2005; Van der Hoorn *et al*., 2000; Kapila *et al*., 1997). The basis of the *Agrobacterium* mediated transient expression system is that the pieces of DNA are transcriptionally competent although these do not integrate into the chromosome (Hellens *et al*., 2005). Furthermore, many different heterologous proteins can be expressed without generating transgenic plants as this might be difficult and time consuming in many plant species (Horn *et al*., 2004; Fisher *et al*., 1999).

For analyzing the functional characteristics of the target proteins, transient gene expression is a fast, flexible and reproducible approach (Cazzonelli *et al*., 2006; Voinnet *et al*., 2003). *Agrobacterium* culture infused into plant leaves, mediates the transfer of transgenes from the T-DNA region of the bacterial Ti plasmid molecules into the plant cells and most of the plant cells in the infiltrated region express the transgene (Kapila *et al*., 1997). For the identification of appropriate candidate genes in transgenic approaches it seems to be essential to test the constructs in the target plant at least at the cellular level. A further essential requirement for an effective transient expression system is a suitable reporter gene assay (Cazzonelli *et al*., 2006). Monitoring transient gene expression in plants by *luciferase* based reporter genes (firefly *luciferase*) is well reported and published. An important advantage of these systems is its respective sensitivity (Cazzonelli *et al*., 2006; Ow *et al*., 1986).

1.6 Legume transformation

Regarding legume transformation many successes have been made and published Köhler *et al*., (1987); Puonti-Kaerlas *et al*., (1989; 1990); De. Kathen and Jacobsen, (1990); Davies *et al*., (1993); Russell *et al*., (1993); Grant *et al*., (1995); Bean *et al*., (1997); Kiesecker (2000); Polowick *et al*., (2000); Ikea *et al*., (2003) Senthil *et al*., 2004; Pniewski and Kapusta, (2005) and Richter *et al*., (2006) by establishing various protocols but still there are species and genotypes which are considered to be more calcitrant for

regeneration and transformation especially chickpea, lentil, bean and also pea. Development of reproducible and efficient regeneration systems enabling and selection of transgenic plants during regeneration is a really critical factor for any successful transformation in monocots and in dicots as well. The bottleneck for legumes (bean, pea, chickpea etc.) transformation is the lacking of transformation compatible regeneration systems, not the transformation itself although transformation efficiency in legumes is also quite poor (0.2% Richter *et al*., 2006; 2.5% Schroder *et al*., 1993).

In addition screening against the target gene for example stress tolerance, needs field components as most of the stress tolerance assays used by basic researchers involve nutrient rich media (Yamaguchi *et al*., 2005) whereas saline soils contain multiple kinds of salts i.e. $CaCl_2$, NaCl, $CaSO_4$, Na_2SO_4 and high amounts of Boron. Each of the salt has its individual and combined effect on plant growth. So those plants which show particular tolerance to salt should eventually be tested on these soils (Yamaguchi *et al*., 2005). Finally there is still lack of availability of reliable gene expression and selection systems for functional level analysis for basic studies. Among legumes, because of high protein contents in the seeds, pea is also becoming more important for the production of high-value recombinant molecules in molecular pharming (Perrin *et al*., 2000; Saalbach *et al*., 2001).

2 MATERIALS AND METHODS

2.1 Vector construction

Mono and dicistronic binary vectors based on pGreenII vectors were made by applying conventional molecular biology techniques (Sambrook and Russell, 2001).

2.1.1 Primers designed for cloning

Following are the nucleotide sequence of all of the primers designed for the cloning of the targeted genes and synthesized by MWG Biotech Company.

Primers	Nucleotide Sequence
AtNHX1(f)	5'-GGC ATG TTG GAT TCT CTA GTG TC -3'
AtNHX1(r)	5'- GCG TTA CCC TCA AGC CTT AC -3'
Tr*cla*I(f)	5′-ATATCGATGATTTGGTGTATCGAGATTGGTTATG -3`
uidA*Hin*dIII(r)	5′-GATAAGCTTCATTGTTTGCCTCCCTGCTG-3′
cp148*Hin*d III(f)	5′-CAGAAGCTTCAGTTCGGTTGCAGCATTTAAAG-3′
cp148*Not*I(r)	5′-TTCGCGGCCGCTTTCTTCTTTCAAATTAAACGAATCAGG-3′
luc*Not*I(f)	5′-CTTGCGGCCGCATGGAAGACGCCAAAAACATAAAGAA-3′
Sac.term(r)	5′-ATCGAGCTCTGGATTTTAGTACTGGATTTTGGTTTTAG-3′
ZmIRES*Xma* I (f)	5′-CAGAAGCTTGTAGACTCCCGGCGAACACTCC-3′
ZmIRES*Hin*d III(r)	5′-AGGCGGCCGCTGCTTCTCGGTCCTCAGTC-3′
luc*Xma*I (f)	5`-AAACCCGGGATGGCCAAACCTTTTCTATC-3′
luc *Sac*I(r)	5′-TTTAAGCTTTCAAAGTTCATCCTTCTCATTC-3′
cp148r.c*Hin*dIII (f)	5′-TAAAAGCTTTTTCTTCTTTCAAATTAAACG-3′
cp148 r.c *Not*I (r)	5′-ATAGCGGCCGCCGATTCGGTTGCAGC-3′
nhx1*Xma*I(f)	5′-ATTCCCGGGATGTTGGATTCTCTAGTGTCGAAACTG-3′
nhx1 *Hin*dIII(r)	5′-AATAAGCTTCAAGCCTTACTAAGATCAGGAGGG-3′

2.1.2 Specific primers designed for confirmation of cloned gene fragment.

For the confirmation of integrated genes in transgenic suspension cells and entire tobacco and pea plants specific primers were designed against each targeted gene by Operon (molecules for life) Company as below.

Primer name	Nucleotide sequences
Bar 382	5`-AGCCCGATGACAGCGACCAC-3´
Bar 149	5`-GCAGGAACCCGAGTGGA-3´
At-nhx (f) 138	5`-ATAGATGGATGAACGAAT-3´
At-nhx (r) 437	5`-AGTCAAAGGTTCCAATGT-3´
Luc 837 (f)	5`-CCTTCCGCATAGAACTGCCT-3´
Luc 837 (r)	5`-TCCAAAACAACAACGGCG-3´
nhx 808 (f)	5`-GACATTGGAACCTTTGACTTGG-3´
nhx 808 (r)	5`-CGGCCCTTGTAAACTTGTTGTA-3´

2.1.3 Proof reading High Fidelitiy (HF) PCR Mixture for cloning of target genes.

Reagents and concentrarions	Probe
10x HF buffer + $MgCl_2$	5 µl
10 mM dNTPs	1 µl
10 pmol primer (F)	1 µl
10 pmol primer (R)	1 µl
Immulase polymerase	0.5 µl
Double dist H_2O	40.5 µl
Plasmaid DNA (1:100 dilution)	1 µl
Total	50 µl

2.1.4 PCR Programme.

For all of the PCR based cloning steps following PCR profile was used.

For High fidelity (HF) proof reading polymerase

PCR steps	Temperature (°C)	Time (s)	No. of cycles
Initial denaturation	94	300	1
Denaturation	94	40	35
Annealing	57	40	
Extension	72	120	
Final extension	72	600	
Cooling and storage	4		

2.1.5 Purification of PCR product

For all of the PCR based cloning experiments, the amplified PCR products were purified by using the Qiagen MinElute PCR purification kit protocol as below:

5 volumes of Buffer PB were added to 1 volume of PCR reaction mixture (before the amplified PCR product was confirmed by running on agarose gel) and mixed gently. Then MinElute column provided in 2 ml collection tube was put in a suitable rack and applied the sample to the MinElute column. Centrifuged for 1 min, discarded the flow through and placed the MinElute column back into the same tube. For washing 750 µl of buffer PE was added to the MinElute column and again centrifuged for 1 min. The flow through was discarded and put the column back into the tube. The column was centrifuged again for 1 min at maximum speed to remove the residual ethanol completely. After washing the column was put into new 1.5 ml micro centrifuge tube and elution of the DNA was done by adding 10 µl buffer EB directly on the center of the

column. Let the column stand for 1 min and centrifuged again for 1 min to collect the DNA in micro centrifuge tube. The purified product was used for further cloning steps.

2.1.6 Monocistronic vectors

Monocistronic vectors were constructed using Cauliflower mosaic virus (CaMV35S) and Mannopine synthase (MAS) promoter.

2.1.6.1 Isolation of *AtNHX1* from *A. thaliana*

The *AtNHX1* gene was isolated from *A. thaliana*. PCR amplification of the target gene was based on specific primers designed against the sequence information available with the Gene Bank accession (NM122597).

A restriction map, shown in Fig.1 was prepared using computer software Vector NTI Advance™ 10.

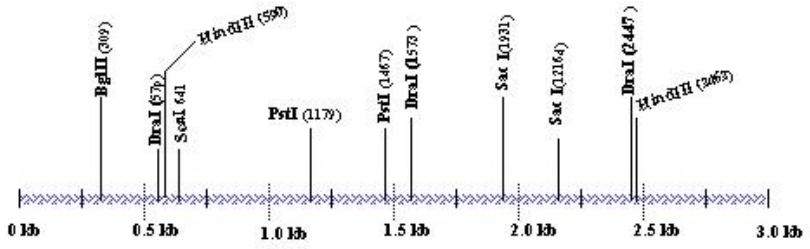

Fig. 1: Restriction map of AtNHX1 (3016 bp) from A. thaliana.

Forward primer (23 mer) 5'-GGC ATG TTG GAT TCT CTA GTG TC -3'
Reverse primer (20 mer) 5'-GCG TTA CCC TCA AGC CTT AC -3'

AtNHX1 gene was confirmed by compatible restriction enzymes, subcloned into the TA cloning vector pTZ57R. Then the most authentic confirmation was made by sequencing of the PCR product.

2.1.6.2 Sub cloning of *AtNHX1* into pGreen vector

Upon confirmations made by restriction analysis and finally by sequencing, the *AtNHX1* gene was sub cloned into the pGreen vector under the control of Cauliflower mosaic virus promoter (CaMV35S) naming pGIIMH35S*AtNHX1*.vector (monocistronic).

2.1.7 Dicistronic vectors

A series of plasmid constructs were made based on the pGreen vector pGII0229 using Cauliflower mosaic virus 35S promoter, and Mannopine synthase (MAS) promoter down stream marker genes *β-glucuronidase* (Jefferson *et al*., 1987) and firefly *luciferase* gene (promega). Three kinds of IRES elements, tobacco mosaic virus derived IRES element TMVIREScp148 (Dorokhov *et al*.,2002), polio virus derived IRES (Dirks *et al.,* 1993) and *Zea mays* IRES (Dinkova *et al.,* 2004) elements were used as inter cistronic sequences. Intermediate constructs were made by using pSBCI vector (Dirks *et al*., 1993). Brief description of the vectors construction is explained below.

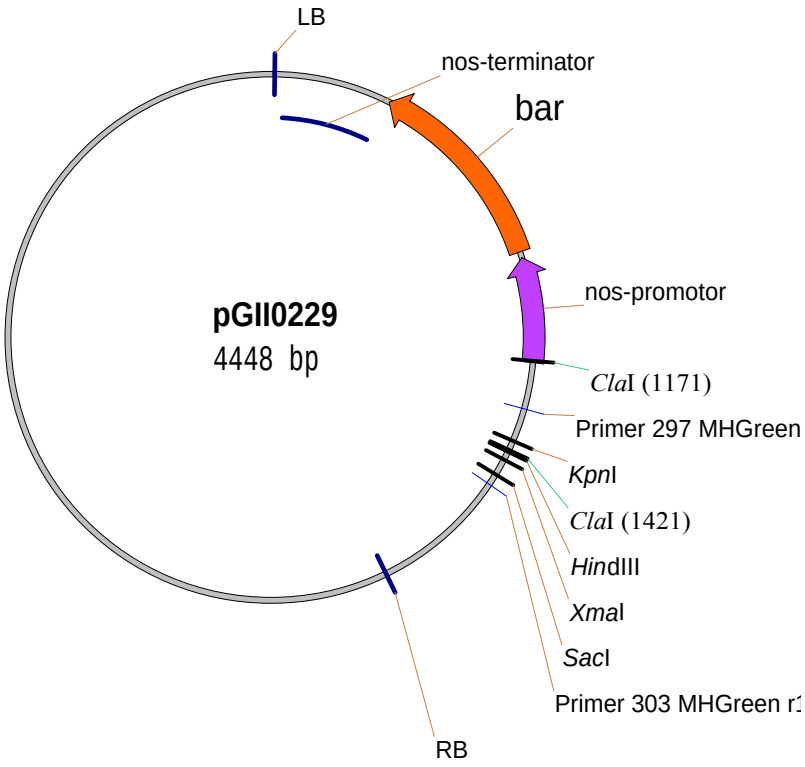

Fig. 2: Map of plasmid pGII0229 (basic vector)

2.1.7.1 pGII0229MASguscp148luc (control vector)

In the first approach, based on the pGreenII vector *β-glucuronidase*(*GUS*) gene (Jefferson *et al*., 1987) was cloned under the control of Mannopine synthase(MAS) promoter (Fox *et al*., 1992) as a first cistron downstream firefly *luciferase* gene as a second cistron behind Tobamo TMVcp148 IRES elements (Dorokhov *et al*., 2002) as

intercistronic sequences under the control of same promoter. The *β-glucuronidase* gene along with MAS promoter was amplified from the intermediate vector PSBC1MASgus using forward primer,

TR *cla*I(f) 5′-ATATCGATGATTTGGTGTATCGAGATTGGTTATG -3` and reverse primer, uidA*Hin*dIII(r) 5′-GATAAGCTTCATTGTTTGCCTCCCTGCTG3′-. The 2336 bp fragment was sub cloned into the multiple cloning region of the binary plasmid vector pGII0229 via C*la*I and *Hin*dIII restriction enzymes as a first cistron. 140 bp Tobacco mosaic virus derived IRES elements TMVIREScp148 were amplified using forward primers: cp148*Hin*dIII(f) 5′-CAGAAGCTTCAGTTCGGTTGCAGCATTTAAAG-3′ and reverse primer.

cp148*Not*I(r) 5′-TTCGCGGCCGCTTTCTTCTTTCAAATTAAACGAATCAGG-3′ from the yy367 vector (Accession: AB086436) and sub cloned behind *GUS* gene using *Hin*dIII and *Not*I restriction enzymes. *luciferase* gene along with CaMV terminator was merged with *Not*I and *Sac*I restriction sites from the vector pGII1579 (intermediate vector) by using forward primer.

luc*Not*I(f) 5′-CTTGCGGCCGCATGGAAGACGCCAAAAACATAAAGAA-3′ and reverse primer

Sac.term(r) 5′-ATCGAGCTCTGGATTTTAGTACTGGATTTTGGTTTTAG-3′, and sub-cloned by using *Not*I and *Sac*I restriction enzymes as a second cistron as shown in Fig. 3.

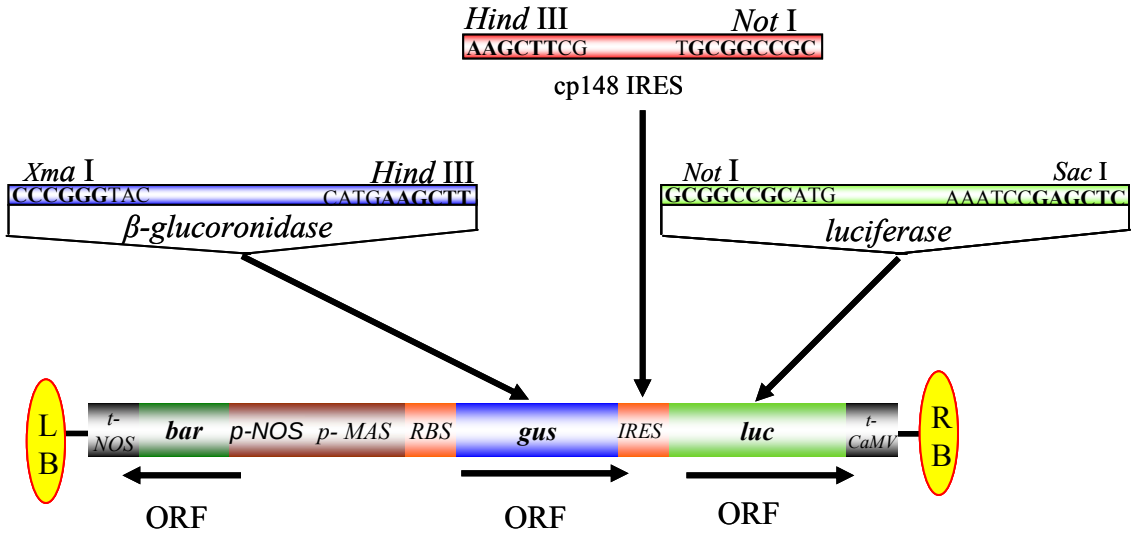

Fig. 3: Cloning of target genes in pG0229 basic vector.

In second approach *Zea mays* IRES elements were cloned in place of TMV IRES using forward primers

ZmIRES *Xma*I (f) 5′-CAGAAGCTTGTAGACTCCCGGCGAACACTCC-3′ and a reverse primer ZmIRES *Hin*dIII (r) 5′-AGGCGGCCGCTGCTTCTCGGTCCTCAGTC-3′. In third approach Polio IRES elements were cloned into dicistronic vector system in place of Tobamo IRES elements for comparative studies by merging *Hin*dIII and *Not*I restriction on their 5′ and 3′ ends, respectively.

2.1.7.2 pGII0229MASluc

The *luciferase* gene was cloned directly behind MAS promoter (monocistronic system) naming pGII0229MASluc for the reason that the translation of the cap dependent reading frame could be used as an internal standard for the determination of the IRES dependent translation of the down stream reading frame (Hennecke *et al*., 2001). The *luciferase* gene was amplified by using forward primer luc*Xma*I (f) 5`-AAACCCGGGATGGCCAAACCTTTTCTATC-3′ and reverse, luc*Sac*I(r) 5′-TTTAAGCTTTCAAAGTTCATCCTTCTCATTC-3′ primer and sub cloned by using *Xma*I and *Hin*dIII restriction enzymes into pGII0229 vector as shown in Fig. 4.

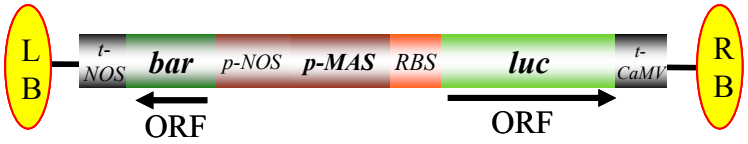

Fig. 4: Subcloning of the luciferase gene (monocistronic).

2.1.7.3 pGII0229MASguscp148(antisense)luc

For comparative studies TMVcp148 IRES were cloned in antisense orientation. These IRES elements were amplified by using forward primer

cp148 r.c *Hin*dIII (f) 5′-TAAAAGCTTTTTCTTCTTTCAAATTAAACG-3′ and reverse primers cp148 r.c *Not*I (r) 5′-ATAGCGGCCGCCGATTCGGTTGCAGC-3′ and religated by using *Hin*dIII and *Not*I restriction enzymes in the vector pGII0229 as shown in Fig. 5.

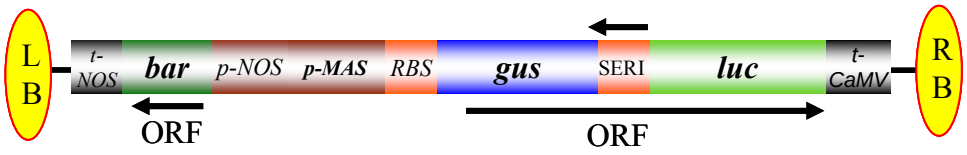

Fig. 5: TMV cp148 IRES in antisense orientation.

2.1.7.4 pGII0229MAS nhx1/luc

As a proof of the principle, sodium/proton antipoter gene *AtNHX1* have been chosen (from *A. thaliana)*. First *AtNHX1 gene* was transferred into *N. tabacum* plants. From the transgenic T1 tobacco plants cDNA of *AtNHX1* was constructed. The PCR amplified cDNA by forward primers

nhx1*Xma*I(f) 5′-ATTCCCGGGATGTTGGATTCTCTAGTGTCGAAACTG-3′ and reverse primer

nhx1 *Hin*dIII(r) 5′-AATAAGCTTCAAGCCTTACTAAGATCAGGAGGG-3′ was merged into a dicistronic expression system as a first cistron in place of *gus* gene (Fig. 6) and then confirmed again by sequencing.

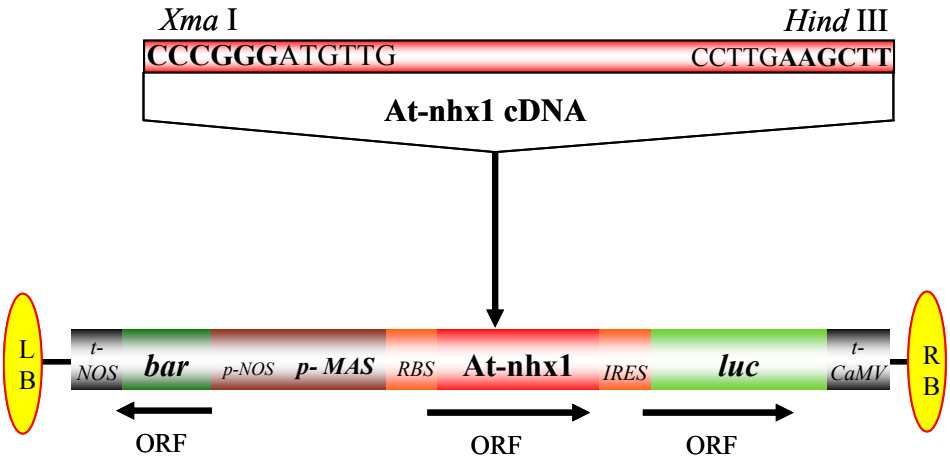

Fig. 6: Subcloning of AtNHX1 in dicistronic vector system in place of β-glucuronidase gene.

2.1.8 Confirmation of the cloned gene fragments.

The target genes which were cloned into mono or dicistronic vectors were confirmed either by the compatible restriction enzymes or by Immulase PCR amplification using the

specific primer designed against the target sequences. Following PCR mixture and programme were used.

2.1.8.1 PCR Mixture (Immulase PCR)

Reagents and concentrations	Probe
10x Immulase buffer	5 µl
50 mM $MgCl_2$	2 µl
100 mM dNTPs	1 µl
Plasmaid DNA (1:100 dilution)	1 µl
10 pmol primer (F)	1 µl
10 pmol primer (R)	1 µl
Immulase polymerase	0.5 µl
Double dist H_2O	38.5 µl
Total	50 µl

2.1.8.2 PCR programme using Immulase polymerase for confirming cloned genes

PCR steps	Temperature (°C)	Time (s)	No. of cycles
Initial denaturation	95	420	1
Denaturation	94	40	35
Annealing	57	60	
Extension	72	120	
Final extension	72	600	
Cooling and storage	4	∞	

2.2 *E. coli* competent cells preparation for heat shock transformation

Many of the species of bacteria including *E. coli* take up DNA in a limited amount under normal conditions. For efficient transformation of these species these bacteria have to pass through chemical and physical treatments to enhance their ability to take up DNA. Such cells which pass through such kind of treatments are called competent cells (Brown, 2006). To prepare *E. coli* competent cells (Tang *et al*., 1994; Nakata *et al*., 1997), *E. coli* strain (2163) was grown overnight in 1-5 ml of LB medium (appendix I) at 37°C (without antibiotics) to the stationary phase. The overnight culture was diluted in fresh LB 1:50 and grown at 37°C until an $O.D_{600}$ was reached of ~0.4. The cells were harvested by centrifugation at 4°C, 4400 rpm, and re-suspended in 1/2 volume ice-cold 100 mM $CaCl_2$ and centrifuged again. The supernatant was discarded and the pellet was re-suspended in 1/2 volume of ice-cold 100 mM $CaCl_2$. Upon centrifugation pelleted cells were re-

suspended in 1/10 volume of cold 100 mM $CaCl_2$ and incubated on ice for 1 hour. The cells were used immediately for heat shock transformation. For storage, 86% sterile glycerol was added to a final concentration of 15% and then aliquots of 100 µl were made in 1.5 ml tubes, the tubes were transferred immediately in liquid nitrogen and stored at -80°C for further processes.

2.2.1 *E. coli* heat shock transformation

Competent *E. coli* cells (from-80°C) were kept on ice to thaw, 50 ng of ligation mixture or plasmid DNA were gently mixed with competent cells (in 1.5 ml Eppendorf tubes). Incubated on ice for 20 min upon incubation, a temperature shock at 42°C was given to competent cells (containing ligase mixture or plasmid DNA) for 40 seconds, immediately returned back the tube on ice for 2 minutes to release heat stress. 900 µl of pre-cooled SOC medium without antibiotics was added to reduce damage of *E.coli* cells. The tube was incubated on a shaker at 250 rpm for 90 min at 37°C. 50 µl, 100 µl and 200 µl of the resulting culture was spread independently on LB plates containing appropriate antibiotic and grown overnight at 37°C. The colony growth was observed after 12-16 hours.

2.3 Preparation of *Agrobacterium tumefaciens* EHA105pSoup competent cells for electroporation (Hood *et al*., 1993)

Agrobacterium tumefaciens strain EHA105 was co transformed with the pSoup helper plasmid according to the pGreenII system (pGreen website: Hellens *et al*., 2000). An overnight seed culture of 25 ml YEB supplemented with 5 mg/l tetracycline was incubated with 250 µl of glycerol stock of EHA105pSoup at 28°C on a shaker. 2 ml of bacterial suspension (overnight seed culture) were added to 50 ml LB agro (appendix II) supplemented with kanamycin antibiotic and grown for 2-5 hours until $O.D_{600}$ reached ~0.4-0.5. Bacteria were pelleted by centrifugation at 4400 rpm and 4°C for 10 min, re-suspended twice in 25 ml ice-cold 10 % glycerol. The pellet was then re-suspended twice in 2.5 ml ice-cold 10 % glycerol after centrifugation at 4400 rpm at 4°C for 10 min. Finally, the pellet was re-suspended in 1 ml ice-cold 10 % glycerol. Aliquots of 100 µl were split in 2 ml eppendorf tubes and transferred immediately into liquid nitrogen and stored at -80°C.

2.3.1 *Agrobacterium* transformation through electroporation

Agrobacterium (EHA-105-pSoup) competent cells were thawed on ice. Gently mixed 50 ng of plasmid DNA with 50 µl competent cells in a 1.5 ml tube (eppendorf). Transferred mixture to a pre-cooled cuvette (gap 0.2 cm) and electroporated in a BioRad electroporator at 25 µF capacitor, 200 Ω resistance and 2.5 KV. With the field strength between 6.25 – 12 kV/cm for 4-8 sec. 950 µl of pre-cooled SOC medium (without antibiotic) were added immediately afterwards, then transferred the mixture to a new 2 ml tube. The tubes were incubated for 3 hours at 28°C while shaking (250 rpm). The resulting culture was spread on LB agro plates containing specific antibiotic (Kanamycin) in four dilutions I e 1:1, 1:10, 1:100 and 1:1000 and grown overnight at 28 °C. The colony growth was observed after 24- 48 hours.

2.3.2 Preparation of glycerol stocks of bacteria

Single colony were picked from the master plate and dissolved in 2 ml YEB or LB medium. Inoculated for 2-3 hours on a shaker at 250 rpm, then transferred to 25 ml YEB or LB medium containing the specific antibiotics and incubated on a shaker at 250 rpm, 28 ºC or 37°C in the dark for 15 h until the O.D 0.8-1.0 for *E coli* and 1.0-1.2 for agrobacteria. The stock solution was prepared using 350 µl glycerol (86%) and 650 µl of growing bacterial-suspension in 2 ml cryogenic vials (Cryoware-Nalgene, Rochester, USA) and stored at -80°C for future use.

2.4 Plasmid DNA Isolation

2.4.1 Requirements

Solution A.

15 mM Tris-HCl, pH 8.0

10 mM EDTA

50 mM Glucose

2 mg/ml fresh lysozyme

Solution B

0.2 M NaOH,

1 % SDS (Lysis buffer)

Solution C

3 M NaOAc, pH 4.8. (Neutrilizing buffer)

Solution D

0.1 M NaOAc, pH 7.0

0.05 M Tris-HCl pH 8.0

2.4.2 Procedure

2 ml of bacteria suspension were centrifuged at 14,000 rpm for 5 min. The supernatant was discarded completely. For agrobacteria this step was repeated. The pellet was carefully re-suspended in 200 µl of sol. A. The pellet was vortex till pellet dissolved completely and incubated for 15 min at RT. 400 µl of sol. B was added and mixed very gently to transparent color and then immediately 300 µl of sol. C was added, mixed gently by inverting the tubes 5-6 times, followed by incubation for 15 min on ice. The mixture was centrifuged twice for 10 min and the transparent supernatant (800 µl) was transferred into a new 1.5 ml eppendorf-cap. Then 600 µl cold isopropanol (-20°C) were added and gently mixed till the DNA started precipitating. After centrifugation for 10 min at 14,000 rpm, the supernatant was discarded. The DNA pellet was re-dissolved in 200 µl of sol. D, and incubated for 5 min at RT. Then 400 µl $EtOH_{abs}$ was added and mixed, centrifuged for 10 min. The supernatant was discarded and the pellet was air dried for 30-60 min at RT. The pellet (plasmid DNA) was dissolved in 50 µl TE buffer (48 µl TE +2 µl RNase). The quality of the DNA was checked by running on gel whereas the quantity was measured by spectrophotometer by taking O.D of dsDNA at 260nm.

2.5 Plant Transformation

2.5.1 *N. benthamiana* leaf infiltration for transient studies

N. benthamiana seeds were germinated in soil. 4 to 5 weeks old (4-6 leaf stage) plants were selected for leaf infiltration. Two experiments were conducted for the leaf infiltration transient assay (Cazzonelli *et al*., 2006). In first experiments the functionality of IRES elements was analyzed using different vector constructs i.e. pGII0229TRluc (monocistronic), pGII0229MASguscp148luc, pGII0229MASguscp148(antisense)luc, pGII0229MASguspolio IRESluc and pGII0229MASguszeamaizeIRESluc. In a second

series of experiments transient comparative study was conducted with and without salt stress using pGII0229MASguscp148luc and pGII0229MASnhx1cp148luc vectors.

Agrobacterium strain EHA-105 harboring the specific plasmid construct were grown overnight prior to leaf infiltration. Overnight grown cultures (OD_{600} 0.8-1.0) were independently centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was discarded and the pallet was dissolved in the same volume of infiltration medium MMA (appendix III). The suspended agrobacteria pellet was incubated at room temperature for 2 hours. When all the dead cells settled down, the upper 10-15 ml of media was collected; OD_{600} was set to 0.9-1.0, and mechanically infused by pressing the tip of the syringe against the lower surface of the leaf and applying the gentle pressure on the plunger as shown in Fig.7. The infusion of the bacteria was monitored visually by observing a spread of the capacity in the leaf as the bacterial suspension fills air space. Fully infused leaves were marked. In the controls only the infiltration medium was infused. Plants were watered after leaf infiltration equally. For salt stress studies the EHA105 strain harboring the vector constructs pGII0229MASguscp148luc and pGII0229MASnhx1cp148luc were grown over night and the same procedure was repeated as explained above but after infusing agrobacteria into tobacco leaves, plants were watered with normal tap water containing 100 mM NaCl. As control, plants were watered with the same volume of tap water but without any additional NaCl. Plants were kept to grow at 20°C in dark for 60-72 hours.

Fig. 7: Infusion of the agrobacteria harboring specific plasmid construct into tobacco leaves for transient expression.

Fully infused leaf areas were marked and selected for crude protein extraction.

2.5.2 Tobacco transformation

Transgenic T0 tobacco plants were recovered from *Agrobacterium* mediated leaf disc transformation (Horsch *et al*., 1985) with some modifications. Leaf discs were taken from in vitro grown tobacco plants (SR1). The over night culture of *A. tumefaciens* harbouring specific plasmid construct was diluted as 50 ml MS-liquid (appendix V) + 500 µl bacteria suspension. Soaked the leaf discs for maximum 30 min in an overnight culture of *A. tumefaciens*, blotted dry and placed upside-down culture plates containing MS salts including B5 vitamins (Duchefa), MS-1 medium (appendix VI). After 3 days of co culture, the discs were washed with MS liquid containing 300 mg/l Tic. and transferred to the same medium containing 300 mg/l of ticarcillin in dim light condition at 24°C.for one week. Then further subculture on the same MS-1 medium containing 200 mg/l Tic and 5 mg/l ppt. and again after two weeks on MS-1 medium with reducing Tic (100 mg/l) and ppt 5 mg/l. From the regenerating calli shoots were selected and subcultured on the MS-2 medium (appendix VII) Tic (100 mg/l) and ppt 5 mg/l in baby jars. Then subculture the transgenic tobacco plants on MS-0 (appendix VIII) containing Tic 100 mg/l and ppt 5 mg/l.

2.5.2 Generation of suspension cells from transgenic tobacco plants

From the transgenic *N. tabacum* T0 and wild type tobacco plants, calli were induced on 4x Medium (appendix IV) (MS, containing 5 mg/l ppt). After callus induction and confirming the transgenic nature of calli by PCR, cell suspensions were established in 4x liquid medium from one of those T0 plant derived calli which showed *luciferase* expression. Suspension cultures were maintained in 300 ml Erlenmeyer flasks containing 100 ml suspension under continuous light at 24°C and shaken on a gyratory shaker at a speed of 100 rotations per minute. Routine subculture was done on a weekly basis by dilution of 50 ml suspension with 50 ml fresh 4X medium.

2.5.3 Pea (*Pisum sativum* L.) transformation

2.5.3.1 Seed selection and surface sterilization

Pea seeds (cultivar Sponsor) were surface sterilized by soaking in 70% ethanol (EtOH) (v/v) for 1 min followed by 6% sodium hypochlorite (NaOCl) for 5-10 min, with

agitation. Seeds were washed for 5-6 times with sterile de-ionized water and embedded in water overnight.

2.5.3.2 Preparation of explants and *Agrobacterium* inoculation

Agrobacterium mediated pea transformation was carried out according to the protocol of Schroeder *et al*., (1993) and Bean *et al*., (1997) with some modifications. Sterilized seeds were split open, cotyledons were removed, radical tips were cut and the remaining embryonic axis were sliced longitudinally with the help of razor blade (dipped into desired agrobacteria culture) passing from the plumule to the embryonic stem into three to five segments. The sliced embryos were inoculated with *Agrobacterium* suspension supplemented with 100 µM acetosyringon and 5 µM TDZ for 40-50 min, while shaking at 28°C with 60-70 rpm. Explants were blotted dry for 3-4 min on sterile filter paper and plated on B5hT (appendix X) co cultivation medium for three days in the dark at 22±2°C. After co cultivation, explants (white and white greenish color) were washed 3-4 times in sterile distilled water until the wash out water become clear, the final wash was supplemented with 100 mg/l Ticarcillin and incubated for 15 min on a shaker to remove the agrobacteria. Then the explants were blotted dry on sterile filter paper and cultured on shoot regeneration MST medium (appendix XI) for 10 days while covering the plates with tissue paper to protect the embryos from high light stress, then subculture to MST medium for another 10 days in light. Thereafter, the explants were sub-cultured on selection medium P2 (appendix XII) and the healthy green shoots were sub-cultured every three week to P2 fresh medium with increased concentrations of ppt to 2.5 mg/l, 5 mg/l, 7.5 mg/l, 10 mg/l, 12.5 mg/l, and 15 mg/l.

2.5.3.3 Brief summary of pea transformation

1) Excision of mature embryo in slices.
2) Inoculation the sliced embryos with *Agrobacterium* suspension for 40 to50 min at 28°C while shaking at 60 rpm.
3) Drying the agro inoculated embryos on sterile filter paper and incubation the agrobacteria inoculated embryos for three days in the dark
   (B5hT medium+TDZ) (co culture).

4) First subculture for one week in semi-dark condition (MST+TDZ+NAA+Ticarcillin).
5) Second subculture for one week in light (MST+TDZ+NAA+Ticarcillin).
6) First selection for 2 to 3 week (P2+BAP+NAA+2.5 mg/l PPT).
7) Second selection 2 week (P2+BAP+NAA+5 mg/l PPT).
8) Further subcultures to fresh media in three to four week interval for selection and multiplication.
9) In vitro grafting of shoots surviving the selection at 7.5 mg/l PPT.
10) Successful grafted shoots (after 8-15 days) transferred to greenhouse.
11) T0 seeds harvested after 30-45 days post grafting.

2.6 Functional analysis assays

2.6.1 Semiquantitative luciferase assay

Luciferase expression was observed by using Promega luciferase assay kit. For tobacco leaves infused with desired agrobacteria, after incubation for 72 hours, Quick froze the leaf tissues in liquid nitrogen; ground the frozen tissues to powder and re suspended in 1X lysis (CCLR) reagent by homogenization at RT. The homogenized material was incubated at 4°C for one hour. Pelleted debris was removed by brief centrifugation and supernatant was transferred to a new tube. 20 µl of cell lysate was mixed with 100 µl of Luciferase Assay Reagent (LAR) and measured by luminometer Lumat L B 9501 by Berthold Luminisence meter.

The suspension cells inoculated with agrobacteria have been washed 2-3 times with plant media containing ticarcilline (150 mg/l). 100 mg cells were taken and dried on filter paper. 300 µl 1X CCLR were added to 100 mg cells and cells were re suspended completely by vortexing for 1-2 min. The cells were incubated at 4°C for one hour. Then the cells centrifuged at 14,000 rpm at room temperature for 10 min. The supernatant was collected 20 µl of this supernatant was mixed with 100 µl LAR and the RLU (relative light units) values measured immediately by luminescence meter as explained above.

2.6.2 Luc imaging

Leaves were washed with tween (5%), rinsed with water and dried on filter paper. Luciferin sodium salt was dissolved in water to a 1 mM concentration and sprayed over the surface of the prepared leaves. In vitro callus material was sprayed with luciferin salt without pretreatment. The images were taken with a Fuji LAS imager 2000 and processed with the Aida® quantification software.

2.6.3 Fluorimetric MUG Assay

Because of highly increased sensitivity and a wide dynamic range, fluorometry is preferred over spectrophotometry. for GUS activity measurement. GUS activity in solution is usually measured with the fluorometric substrate 4-methyllumbelliferyl-β-D-glucuronide (MUG).

20 µl of the extract (see 2.6.1) was added to 1 mM of MUG-buffer, (50 mM $NaPO_4$, pH 7.0, 10 mM dithiothreitol (DTT), 1 mM Na_2EDTA, 0.1% Sodium Lauryl Sarcosine, 0.1 % Triton X 100, 1 mM 4-methyllumbelliferyl-β-D-glucuronide (MUG). A kinetic of recombinant glucuronidase activity was assayed in a semi quantitative way in three replicates by measuring emission 465 nm after excitation at 360 nm at the temperature range 35°C-38°C with a number of 20 replicate cycles 20 with a 15 minutes interval for each and shaking before each measurement for 10 seconds. Measurement was performed in a TECAN Genios device and analyzed with Magellan® software.

2.6.4 Chlorophenol Red (CR) assay

Plant cells on the specific medium supplemented with chlorophenol red (CR) induce a color shift by decreasing the pH (from 6.0 to 5.0) if they are live and growing. The magnitude of the pH decrease relates to growth rate of the cells. The CR assay was performed according to protocol of Kramer *et al.,* (1993) with some modifications for callus, suspension cells and embryos. Phosphinothricin resistant calli and suspensions cells were incubated on the media containing 5 mg/l ppt and 50 mg/l CR solidified with 1.0% sea-plaque agarose in the 24 well microtiter plate (each well contained 500 µl media) under the fluorescent light with a 16/8 light/dark period. The pH of the medium was adjusted to 6.0 without any buffering chemical which causes the medium color to be red. Non transgenic calli and tobacco suspension cells were also grown for comparative

studies. For pea embryos and calli small petri dishes were used instead of micro titer plates. The color shift in the medium was observed after 4 to 12 days of growth.

2.7 Genomic DNA isolation

For the characterization of transgenic plants and transgenic calli, the isolation of pure and good quality DNA is an important tool for the identification of transgenes. In the present study isolation of gDNA was done by two methods using the Invitrogen charge switch gDNA plant kit and the CTAB method of Doyle and Doyle, 1990. First, for the PCR screening, small scale (100-200 mg leaf material) DNA isolation was performed and on the basis of results large scale (1-2 g leaf materials) DNA isolation was carried out. Both protocols are described as:

2.7.1 Invitrogen charge switch gDNA plant kit protocol

Plant tissues were frozen in liquid nitrogen and the frozen tissues ground to powder using a mortar and a pestle (for soft and non fibrous plant tissues: cut the tissues into small pieces). 1 ml of charge switch lysis buffer and 2 µl RNase was added to ground tissues at room temperature. The lysate was prepared by homogenizing the piece of soft tissues with a tissue homogenizer or by vortexing. Then 100 µl of 10% SDS was added to the plant lysate and incubated at RT for 5 min. 400 µl of charge switch precipitation buffer was added to the lysate, mixed by vortexing for 10 sec until the precipitate was formed. The lysate was centrifuged at maximum speed (14,000 rpm) for 5 min at RT. The clear lysate was transferred to new sterile 1.5 ml microcentrifuge tubes. The tubes containing the magnetic beads were thoroughly vortexed to fully resuspend the beads in the storage buffer. Then, from the resuspended beads 100 µl were added to the approximate 1.2 ml lysate. Now 40 µl ChargeSwitch magnetic beads were added and mixed gently by pipetting. The mixture was incubated at RT for 1 min. The tubes were placed on the MagnaRack to form a tight pellet. Without removing the tubes from the magnet, the supernatant was carefully discarded without disturbing the pellet. Finally the pellet was washed three times with 1 ml ChargeSwitch washing buffer. After washing, 150 µl ChargeSwitch elution buffer was added and the pellet dispersed by pipetting 15-30 times up and down (pipette volume adjusted to 100 µl). Then the resuspended beads were incubated for 1 min at RT. Now the DNA free magnetic beads were precipitated again by

placing the tube back to the MagnaRack. When the DNA containing supernatant was clear it was transferred to a new sterile micro centrifuge tube and used for further processes.

2.7.2 CTAB method of genomic DNA isolation

CTAB-buffer

3 % CTAB (added after autoclaving and stirred overnight

1.4 M NaCl

0.2 % ß-Mercaptoethanol (added directly before using)

20 mM EDTA

100 mM Tris-HCl pH 8.0 (base)

0.5 % PVP-40 polyvinyl pyrolidone (soluble)

24:1 CI-Mix

24 part Chloroform +1 part Isoamylalcohol

TE-buffer + RNase A

10 mM Tris-HCl, pH 8.0

1 mM EDTA

10 µg/ml RNase A

Wash buffer (WB). 76 % Ethanol abs.

10 mM Ammonium acetate

7.5 M NH_4-Acetate

0.5 M EDTA (pH 8)

RNase A

(10 µg/µl Stock solution in ddH_2O)

2.7.2.1 Isolation of genomic DNA for PCR (mini isolation)

100 mg leaf material was harvested, crumbled to powder in liquid nitrogen using a pre-cooled mortar and pestle. The ground material was transferred to 2 ml reaction tubes. 800

µl of pre-heated (60°C) CTAB-buffer was added (containing 0.2 % β-Mercaptoethanol) followed by vigorous vortexing under a fume hood. The tubes were incubated for 30 min at 60°C. After incubation 800 µl CI-mix was added and tubes were gently mixed to avoid shearing of genomic DNA by inverting the tube for 4-5 times. The mixture was centrifuged at room temperature for 10 min at 10,000 g. Two phases were developed. The aqueous phase (~800 µl) was transferred into a fresh 1.5 ml tube (the step was repeated to get a clear sample). 2/3 volume (550 µl) of pre-cooled (-20°C) isopropanol was added and gently mixed to allow precipitation of gDNA. The tubes were centrifuged for 10 min at 14,000 rpm to precipitate the gDNA. The supernatant was discarded and the DNA pellet was washed with 200 µl wash buffer until the pellet floats. Washing buffer was carefully removed and the pellet was re-suspended in 200 µl TE buffer supplemented with RNase A, incubated for 30 min at 37°C, and then 100 µl 7.5 M NH_4-acetate and 750 µl $EtOH_{abs}$ was added and gently mixed. The mixture was centrifuged at maximum speed for 10 min at room-temperature. The supernatant was discarded completely and the pellet let to dry for 40-50 min at 37°C. After drying the pellet was re-suspended in 100-250 µl ddH_2O or 100 µl TE buffer (for better solving and storing) and stored at 4°C over night.

2.7.2.2 Isolation of genomic DNA for Southern blot (maxi isolation)

2 g leaf material was harvested and pulverized in liquid nitrogen in a pre-cooled mortar and pestle. The resulting powder was transferred into a 50 ml fresh tube. 3-5 ml of preheated (60°C) CTAB-buffer (containing 0.2 % β-Mercaptoethanol) was added followed by vortexing under the fume hood. The solution was incubated for 30 min at 60°C. 1 vol. of CI-Mix (3-5 ml) was added and gently mixed by inverting the tubes to avoid shearing of genomic DNA. The mixture was centrifuged for 10 min (6400 rpm) at RT and the clear aqueous phase transferred into a fresh tube (3-5 ml). For precipitation of the gDNA, 2/3 volume of pre cooled (-20°C) isopropanol (2-3 ml) was added and gently mixed. Precipitated DNA was collected by centrifugation for 10 min at 4000 rpm at RT; the resulting pellet was washed with 1-2 ml WB until the pellet floats. The washing-buffer was carefully removed and the pellet resuspended in 0.5-1 ml TE buffer supplemented with RNase A and incubated for 30 min at 37°C. 1/2 vol of 7.5 M NH_4-acetate and 2.5 vol. of $EtOH_{abs}$ were added and gently mixed. Then the mixture was centrifuged for 10 min at 4600 rpm at room-temperature. The supernatant was completely

discarded and the pellet dried at 37°C for 60 min, the pellet was resuspended in 200-400 µl TE buffer at 4°C overnight to allow dissolving of the gDNA. Samples were heated for 5 min at 60-65°C before observing DNA quality on an agarose gel.

2.8 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For the transcription analysis of the introduced genes RT-PCR was applied. RNA was isolated from transformed and non-transformed plants. cDNA was synthesized by reverse transcriptase (MMLV-RT). Then normal PCR was performed with the cDNA as template. RNA was isolated from young tobacco leaves using NucleoSpin RNA plant kit (Machery-Nagel, Germany) following the manufacturer's instructions or using Plant RNA Reagent (Invitrogen, Canada).

2.8.1 Isolation of RNA

100 mg of plant material was quickly frozen in liquid nitrogen and ground using a cold mortar and pestle. The plant powder was transferred to 1.5 ml caps and 500 µl cooled (4°C) Plant RNA Reagent was added and mixed by vortexing. The mixture was incubated for 5 min at room-temperature and then the solution clarified by centrifugation (2 min at 12,000xg at RT). The supernatant was transferred to fresh 1.5 ml tubes and 100 µl of 5 M NaCl was added and tubes were tapped to mix. Finally 300 µl of chloroform was added and mixed thoroughly by inversion and the mixture centrifuged at 4°C for 10 min at 12,000 g. The aqueous phase was transferred to a fresh tube (~ 500 µl) and an equal volume of isopropanol was added and mixed by inversion and let stand at RT for 10 min. The samples were centrifuged at 4°C for 10 min at 12,000 g. The supernatant was discarded and the pellet washed with 1000 µl Ethanol abs. followed by centrifugation for 1 min at 12,000 g. The liquid was carefully decanted by taking care not to loose the pellet. Then it was briefly centrifuged to collect the residual liquid and the liquid was removed with a pipette. The pellet was re dissolved in 30 µl RNase free water and the RNA concentration was measured by spectrophotometer.

2.8.2 Measuring RNA concentration

The RNA concentration was quantified using a spectrophotometer. The RNA was diluted 1:200 (199 µl H_2O + 1 µl RNA), and respective RNA-concentration was calculated as follows:

RNA concentration µg/ml = (OD_{260} x Dilution factor x 40)

The purity of the RNA was determined using the ratio of OD_{260}:OD_{280}, which should be between 1.9 and 2.0 for pure RNA.

2.9 Southern blot by DIG labeled probe

To confirm integration patterns of TDNA and to determine the copy number of the integrated transgenes, Southern blotting was performed according to Southern, (1975). Genomic DNA was prepared from transgenic and non transgenic plants by the large scale DNA preparation method. Non radioactive detection methods and DIG labelled PCR products for the different genes were used.

2.9.1 Buffers and solutions

Pre-hybridization solution:	Dig Easy Hyb. (Roche Diagnostics, Mannheim, Germany)
Hybridization solution:	45 µl probe + 33 ml Dig Easy Hyb.
Blocking Solution:	1 % blocking solution (Roche) in maleic acid buffer
Antibody solution:	(Anti-Digoxigenin-alkaline phosphatase conjugate Fab Fragments) (Roche Diagnostics) 1:10,000 (75 mU/ml in blocking solution.
Depurinizing solution	0.25 M HCl
Neutralization Solution pH 7.5	0.5 M Tris-base, (pH 7.5), 3 M NaCl
SDS	10 % (Filter sterilized)

Maleic acid buffer pH 7.5 (autoclaved)	0.1 M maleic acid, 0.15 M NaCl
Detection buffer pH 9.5	100 mM Tris-HCl, 100 mM NaCl
Denaturation Solution pH 9.5	0.5 N NaOH, 1.5 M NaCl
20x SSC buffer pH 7	3 M NaCl, 0.3 M sodium citrate
Washing buffer (not autoclaved)	0.1 M Maleic acid buffer, 0.15 M NaCl 0.3 % Tween 20
Stripping buffer	0.2 M NaOH, 0.1 % SDS
DEA buffer pH 9.8	0.1 M DEA, 1 mM $MgCl_2$
Developing solution	1**:** 3.5 dil. of Roentogen developer (Tetenal) Photowerk GmbH, Norderstedt, Germany)
Fixation solution	1:4 dilution of Roentogen Superfix (Tetental Photowerk GmbH, Norderstedt, Germany).
NBT	18.8 mg/ml (in 67%DMSO)
BCIP	9.4 mg/ml (in 67%DMSO)
Substrate buffer pH 9.5	100 mM Tris/HCl + 100 mM NaCl +5 mM $MgCl_2$
Substarte for detection	40 ml substarte buffer +264 µl NBT + 272 µl BCIP

2.9.2 DIG labeling probe preparation by PCR

2.9.2.1 PCR mixture

Compounds and concentrations for Probe and Control

Reagents and concentrations	Probe	Control
10x Immulase buffer	5 µl	2.5 µl
50 mM $MgCl_2$	2 µl	1 µl
100 mM dNTPs	-----	1 µl
PCR Dig Probe synthesis	5 µl	-----
Plasmaid DNA (1:100 dilution)	1 µl	1 µl
10 pmol primer (F)	1 µl	1 µl
10 pmol primer (R)	1 µl	1 µl
Immulase polymerase	0.5 µl	0.5 µl
Double dist H_2O	34.5 µl	17 µl
Total	50 µl	25 µl

To make Dig labeled probe PCR was performed for *AtNHX1* and *luciferase* gene with plasmid DNA 0229MASnhx1/luc by using forward and reverse primers, 808 for *AtNHX1* and 837 for luciferase. After confirming the amplified PCR product by loading 5 µl on agarose gel, the amplified product was heated up to 95° C for 5 min and mixed with hybridization buffer. Used directly for hybridization or stored at –20°C.

2.9.2.2 PCR Programme.

	Temperature (°C)	Time (s)	No. of cycles
Initial denaturation	95	420	1
Denaturation	94	40	20
Annealing	58	60	
Extension	72	40	
Denaturation	94	40	30
Annealing	58	60	
Extension	72	40	
Final extension	72	600	
Cooling and storage	4	∞	

The probe quality was observed by running 5 µl on a 0.8 % agarose gel and comparing with the control sample.

2.9.3 Restriction digest of gDNA for Southern blot

25 µg of gDNA isolated from 0229MASnhx1/luc tobacco suspension cells, was digested by *Apa*I and *Stu*I restriction enzymes and positive control (plasmid DNA 0229MASnhx1/luc) with *Kpn*I and *Stu*I in the respective buffer at 37°C over night followed by heat inactivation for 15 min at 65°C.

2.9.3.1 Precipitation of the digest

To precipitate the digested gDNA, 1 volume of 7.5 M NH_4-acetate (100 µl) and 7.5 vol. $EtOH_{abs}$ (750 µl) were added. After mixing gently, centrifugation at maximum speed (14,000 rpm) for 10 min at RT followed. The supernatant was discarded completely and the pellet was re-dissolved in 100 µl TE buffer. The digest was precipitated by adding 100 µl $EtOH_{abs}$ to remove salts. The mixture was centrifuged for 10 min and the pellet

was dried for 1 h. at 37°C. Finally it was re-dissolved in 40 µl TE buffer and incubated for 1 hour at RT.

2.9.3.2 Electrophoresis

Next day 8 µl of 6x loading buffer were added to the restriction digest (40 µl). The digested gDNA, 40 µl of 1:1000 dilution of restricted plasmid DNA and 3 µl of DIG-labelled-DNA Molecular Weight Marker II (Roche) were loaded on 0.8 % agarose gel containing 0.5 µg/ml EtBr in 1x TAE buffer. The gel was run overnight at 0.6 V/cm (15-20 V). The DNA was visualized under a UV Trans illuminator and then the gel was rinsed in ddH_2O. The gel was then incubated in 250 ml of depurinizing sol. (0.25 M HCl) for 10 min to nick the DNA and facilitating the transfer of large fragments. The gel was rinsed again in ddH_2O to remove the acid followed by submerging it in denaturation solution for 2 x 15 min at room temperature on a shaker. Prior to neutralization the gel was rinsed in ddH_2O and was neutralized at room temperature in neutralization solution for 2 x 15 min

2.9.3.3 Capillary Southern-transfer

20 x SSC solutions were put in a tray where filter paper bridges were built on a glass plate. A layer of filter paper was soaked in 20 x SSC solutions, and then placed on the top of the bridge (avoiding any air bubbles under the paper) in such a way that the edges of the filter paper were dipped in 20x SSC solutions. The gel was put on the filter paper and plastic paper was placed under the edges of gel. A piece of positively charged nylon membrane (Roche) was first wetted in ddH_2O, then in 20X SSC and placed on top of the gel. Another 3 layers of filter paper were soaked in 20x SSC solutions, and then placed on the membrane to avoid air bubbles. Tissue paper stacks were loaded onto the filter papers and a glass plate centered on top of the paper towels. A 500 ml bottle full of water was placed in the center of the glass plate to distribute the weight evenly across the gel, the papers and the membrane. Transfer by capillary force took place over night. When the transfer was completed, the membrane was rinsed 3x in 2X SSC and then air dried. The membrane was either UV exposed (254 nm) for 10-15 min for covalently cross-linking the DNA to the membrane or placed between two filter papers for 30 min at 120°C in the oven, then covered with foil and stored at RT.

2.9.3.4 Pre-hybridization and hybridization

After drying the dry blot was placed in an autoclaved hybridization tube and 50 ml of pre hybridization solution was added and the tube incubated for 40-60min at 40 °C. Then a preheated (68°C) probe was added and incubated overnight at 40 °C. The membrane was washed as follow,

2 x 5 min in 2 x SSC + 0.1 % SDS at 42°C

1 x 15 min in preheated (65°C) 0.5 x SSC + 0.1% SDS at 65°C

1 x 15 min in 0.1 X SSC + 0.1 % SDS at 65 °C

1 min in maleic acid buffer at RT

Incubated in blocking solution for 30 min at RT

Incubation with antibody solution again for 30 min at RT

Afterwards the blot was rinsed in washing buffer for 2 x 15 min at RT.

Equilibration for 2 min in detection buffer at RT

2.9.3.5 Non-radioactive detection

The substrate was prepared by mixing 40 ml substrate buffer, 264 µl of from NBT stock and 272 µl from BCIP stock solution. The substrate was dropped by pipetting on the membrane while lying in the plastic transparent chamber at RT. Incubated the membrane in the substrate for 1-2 hours for the probe development.

2.9.3.6 Stripping of the membrane

After usage, the membrane can be stored in 2x SSC buffer for a second hybridization. The membrane was rinsed in sterile H_2O and incubated twice for 15 min in stripping buffer at 37°C in the hybridization tube followed by rinsing in ddH_2O. The membrane could be stored in 2x SSC buffer without SDS at 4°C.

2.10 Fresh / dry weight measurement of the calli

After a specific growth period, transgenic as well as wild type cells fresh weight measurement were carried out by collecting the cells growing on the MS agar medium in the pre weighted caps by avoiding any agar along with the cells. Fresh weight was recorded by using the analytical balance. To record the dry weight the cells containing caps were put in an oven at 60°C for 72 hours and dry weight was calculated by

subtracting the weight of cells containing caps after drying from the weight before drying.

2.11 Statistical analysis

For the fresh and dry weight of transgenic vs wild type suspension cells Statistical data nalysis was made with SigmaStat® 3.1. All pair wise multiple comparison procedures were made according to the Holm-Sidak method: Overall significance level = 0.05. Power of the performed tests: alpha = 0.050: 1.00. The box whisker plots were generated by SigmaPlot® 9.0 software in which the boxes mark the 25% and 75% percentile as well as the median. The whiskers mark the 5% and 95% percentile and the dots show the outliers.

3 RESULTS

3.1 Vector construction

3.1.1 Isolation of *AtNHX1*

The *AtNHX1* gene, coding for a sodium antiporter in *A. thaliana* was cloned from *Arabidopsis* genomic DNA with provided sequence data from NCBI database (AT Database seq. At5g27150).

3.1.1.1 PCR based cloning of the *AtNHX1* gene

PCR amplification of the target gene was based on specific primers designed against the sequence information available from the Gene Bank data base. The *AtNHX1* gene (3016 bp) was amplified from the genomic DNA of six different plants of *A. thaliana*.

Confirmation of amplified fragment

First the size of the amplified product was found to be of correct size as shown in Fig. 8. Left lane1: 1 kb ladder.

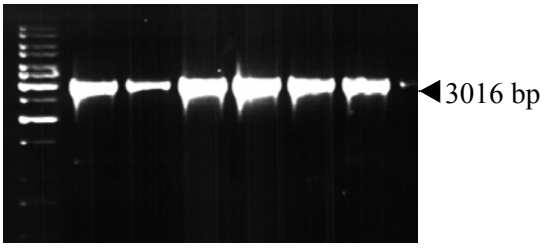

Fig. 8: PCR amplification of AtNHX1 from six Arabidopsis plants.

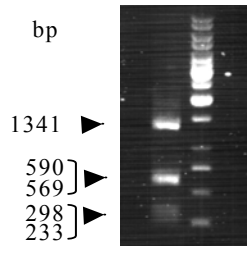

***Fig. 9: PCR amplified product restricted with Hin*dIII *and Sac*I.**

For further confirmation, the amplified fragment was digested with *Hin*dIII and *Sac*I restriction enzymes. As seen from the restriction map shown in Fig. 1 the amplified product should produce five fragments each of 1341, 590, 569, 298 and 233 bp. Fig. 9 shows the results of double digestions of the amplified fragment with *Hin*dIII and *Sac*I. A band of 1341 bp is clearly visible while that of 590 and 569 appeared as one due to size similarity. Similarly the two bands of 298 and 233 appeared together at approximately the same position. This double digestion confirmed that the amplified fragment is the same gene, which was being targeted. In order to further confirm the amplified fragment, this was purified from the gel and was digested only with *Hin*dIII, which has two sites in the targeted region. Upon digestion with *Hin*dIII, the targeted fragment should produce three fragments each of 1872, 590 and 569 bp. Fig. 10 shows that correct sized fragment of 1872 bp was produced while two fragments of 569 and 590 bp appeared together on the gel due to size similarity.

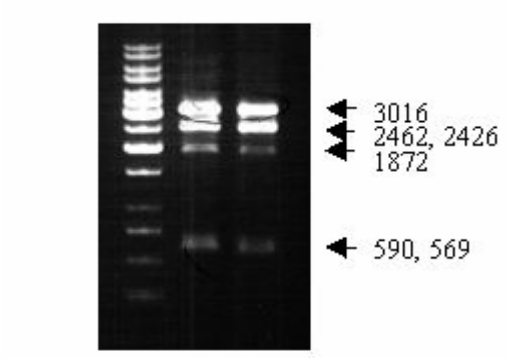

Fig. 10: Confirmation of the AtNHX1 with Hin*dIII. *Left lane is 1kb DNA marker

Fig. 10 also shows the undigested fragment of 3016 bp and the production of band(s) of 2462 or 2426 bp because of partial digestion at one of the two *Hin*dIII sites. This also indicated that the amplified fragment is the same which was targeted.

3.1.1.2 Cloning of *AtNHX1* gene in T/A Cloning vector

The 3016 bp amplified fragment was cloned in T/A cloning vector named pTZ57R (2886 bp) as shown in Fig. 11 at the *Eco*RV site (in the MCS).

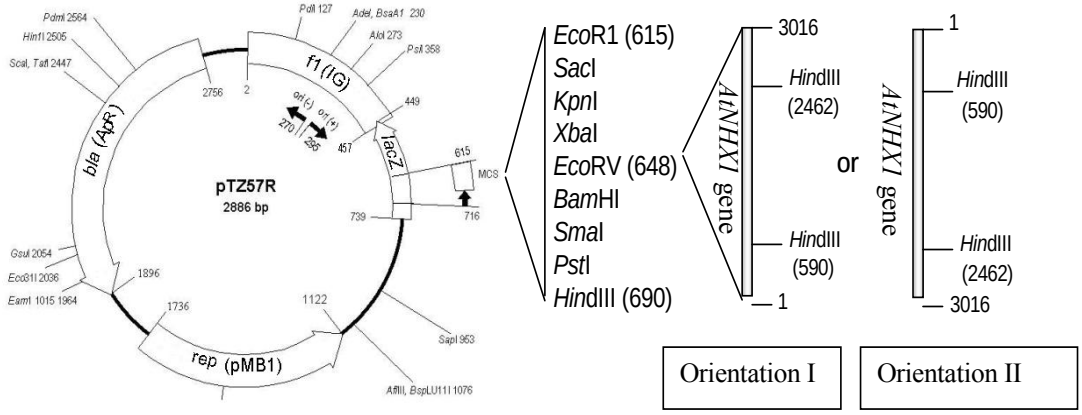

Fig. 11: Cloning of AtNHX1 gene in T/A cloning vector (pTZ57R).

After ligation, the cloning of the target gene in pTZ57R was confirmed with restriction digestion. The fragment can have two orientations either orientation I or orientation II as shown in Fig. (11). Upon digestion with *Hin*dIII, the resultant vector(s) should produce three fragments as shown in Fig. 12. In case of orientation I, the fragment sizes should be 3398, 1872 and 632 bp, respectively whereas orientation II should result in fragment sizes of 3434, 1872 and 595 bp, respectively. Therefore the orientation I was confirmed via *Sac*I digest which gave a ~900 bp fragment whereas the opposite orientation would have resulted in a ~2000 bp fragment (data not shown).

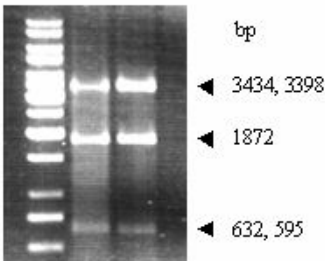

***Fig. 12: Confirmation of the cloned gene fragment through restriction analysis of pTZ57R with Hin*dIII**

3.1.1.3 Confirmation of the cloned fragment by sequencing

The final and most authentic confirmation of the PCR amplified fragment was made through its nucleotide sequence. The sequencing results indicated that there is 100% similarity between the reported sequence and the sequence data of our cloned fragment.

3.1.1.4 Sub cloning of *AtNHX1* into pGreenII binary vector (Monocistronic)

AtNHX1 gene from pTZ57R was sub cloned into pGreenII vector under the control of double 35S promoter by using *Bam*H1 and *Xba*1 restriction enzymes, named pGII MH 2-35S-*AtNHX1* as shown in Fig. 13.

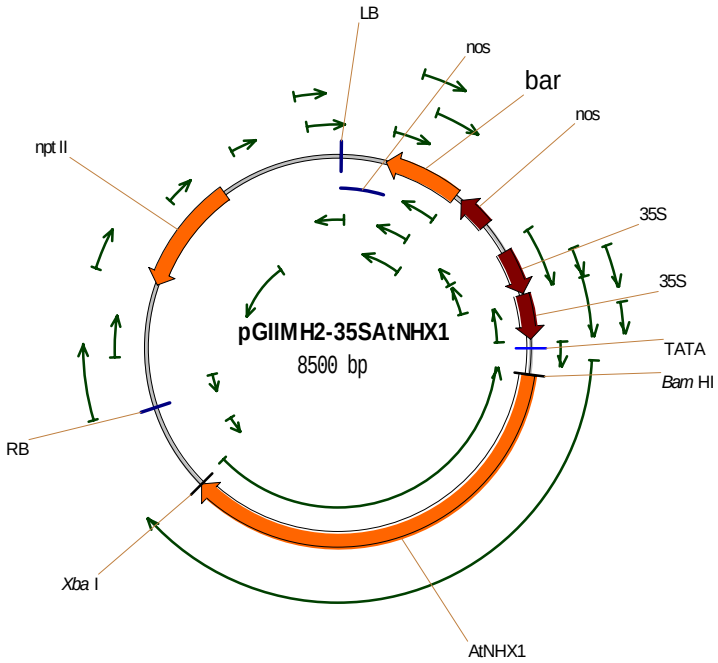

Fig. 13: Subcloning of AtNHX1 in pGreen vector under the control of double 35S promoter.

3.1.1.5 Functionality of *AtNHX1* (monocistronic)

The functionality of *AtNHX1* gene was observed in *N. tabacum* plants transformed by the *A. tumefecience* strain EHA105 harboring the vector pGIIMH2-35S*AtNHX1*. NaCl stress first 100 mM and later on 200 mM was applied to *AtNHX1* transgenic T1 tobacco plants for 3 to 4 week but no difference in growth could be seen between transgenic and wild type tobacco plants. Further functional investigations were made at the cellular level. Calli were induced from transgenic T1 tobacco plants. The growth rate of transgenic and wild type cells were observed under different salt (NaCl) concentrations ranging from 0 to 150 mM. After 4 week, reduction in growth was observed in wild type but not in *AtNHX1* transgenic calli upto 100 mM NaCl stress as shown in Fig. 14(b).

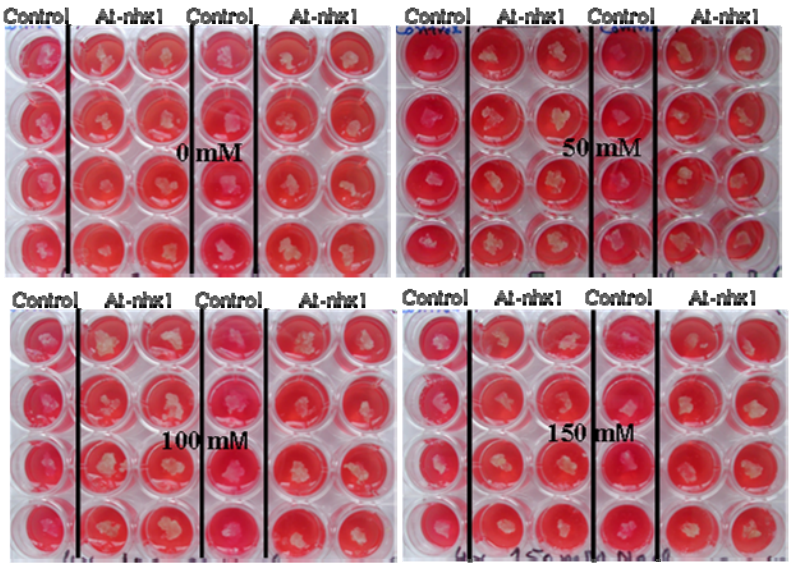

Fig. 14(a): WT vs AtNHX1 transgenic calli (monocistronic) on Gamborg B5) growth medium having CR pH indicator (first day of growth).

Growth under different NaCl concentations,

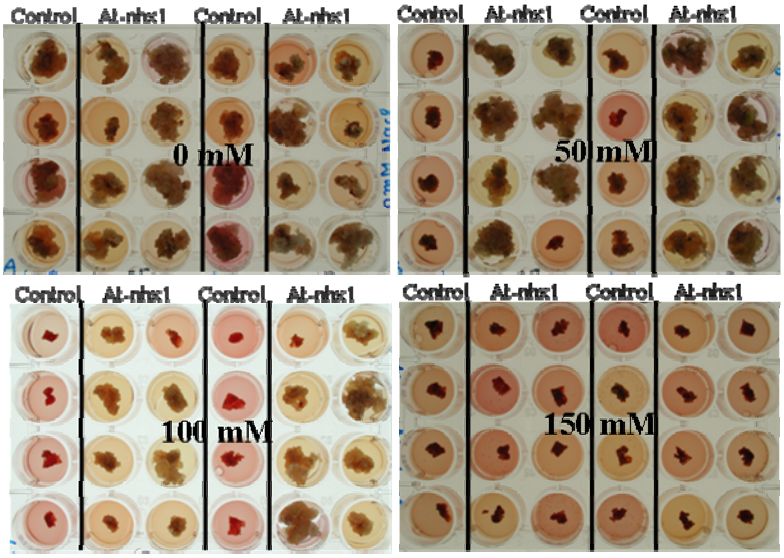

Fig. 14(b): Functionalitiy of AtNHX1 (monocistronic) transgenic vs wild type calli after 4 weeks of growth.

Growth on Gamborg B5 medium (4x) containing different NaCl conc. and chlorophenol red pH indicator.
Transgenic cells survived and changed medium color

The dry weight of transgenic and wild type calli was recorded after 4 weeks of growth. As shown in Fig 15 statistically significant difference (P =<0.01) among all treatment group of cultures of *AtNHX1* cell lines.

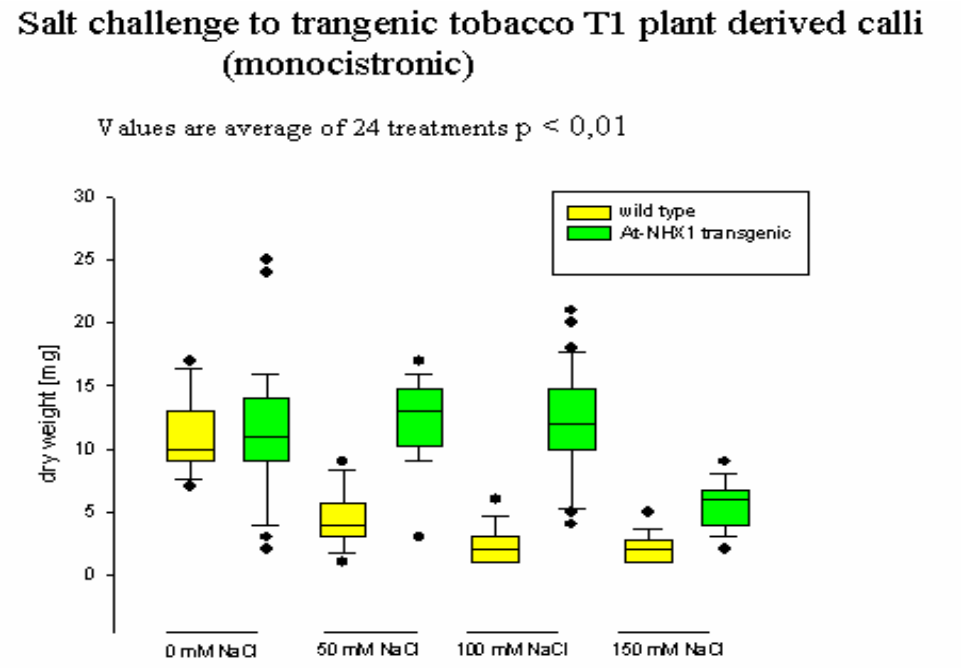

Fig. 15: Dry weight of transgenic AtNHX1 (monocistronic) vs wild type tobacco calli growing on Gamborg B5 medium .

Dry weight was measured after 4 weeks of growth.

3.1.2 Dicistronic vector constructs

Three kinds of IRES elements, namely polio virus derived, the *Zea mays* IRES and tobacco mosaic virus derived IRES (TMVcp148 IRES) elements have been tested. Series of Dicistronic constructs were made using these three IRES elements as inter cistronic sequences. In case of TMVcp148 IRES the two open reading frames (ORFs) were separated by 148 bp, in the polio virus IRES elements 625 bp and in *Zea mays* IRES elements both ORFs were separated by 205bp.

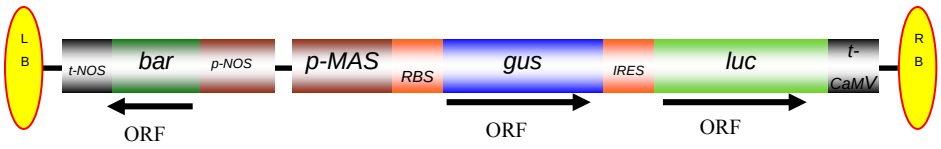

***Fig. 16: Dicistronic vector construct,* pGII0229MASgus/luc.**

3.1.2.1 Confirmation of the dicistronic vector construct pGII0229MASguscp148luc

After cloning of the target genes in the vector pGII0229 the resultant vector pG0229MASguscp148luc was confirmed by restriction enzymes (Fig. 17). Upon restriction by *Kpn*I and *Xba*I there should be two fragments 522 bp and 8275 bp (lane 2). As *Kpn*I site is infront of MAS promoter and *Xba*I site is behind the promoter, therefore production of 522 bp fragment showed the presence of MAS promoter in the vector construct. The *β-glucuronidase* gene was confirmed by restriction enzymes *Xba*I and *Hin*dIII enzymes. The resultant construct should produce two fragments (1840 bp and 6967 bp) as the *Xba*I site lies in front of the *GUS* gene and *Hin*dIII behind therefore the production of 1840 bp fragment confirmed the presence of the *GUS* gene (lane 3). The presence of *luciferase* gene along with TMV cp148 IRES and CaMV terminator was confirmed by restriction with *Hin*dIII and *SacI* enzymes. As the *Hin*dIII site lies in front of IRES elemets and *Sac*I behind the CaMV terminator. By restriction digest with these enzymes the 2099 bp fragment confirmed the presence of *luciferase* being along with TMVcp148 IRES and CaMV terminator (lane 4).

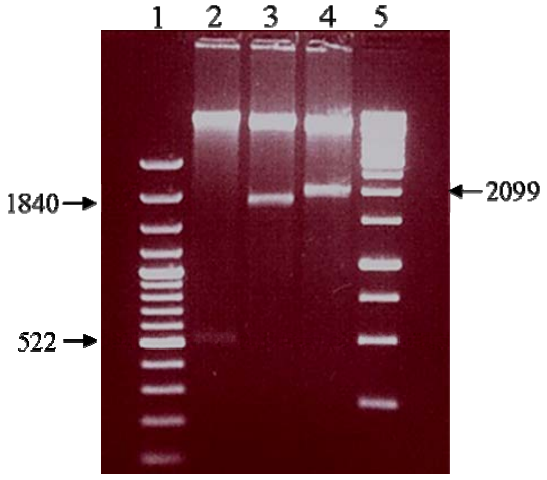

Fig. 17:.Confirmation of the cloned genes in dicistronic vector pG0229MASguscp148luc

lane1: 100 bp DNA marker and lane 5: 1 kb DNA marker.

3.1.2.2 Functionality of Vector constructs

From the tobacco leaf infiltration experiments crude protein was extracted from 100 mg leaf material and Photon emission (RLU) were observed under a luminometer. Fig. 18 shows the transcriptional units of the expression plasmids encoding the *luciferase* gene in mono and dicistronic configurations. The expression of the *luciferase* gene as the first cistron translated by cap dependent manner was clearly detectable, whereas *luciferase* expression as second cistron translated by cap independent manner could detected only in significant quantities when TMV IRES (cp148 IRES) was present as inter cistronic sequence. No significant expression could be detected with polio and maize IRES elements.

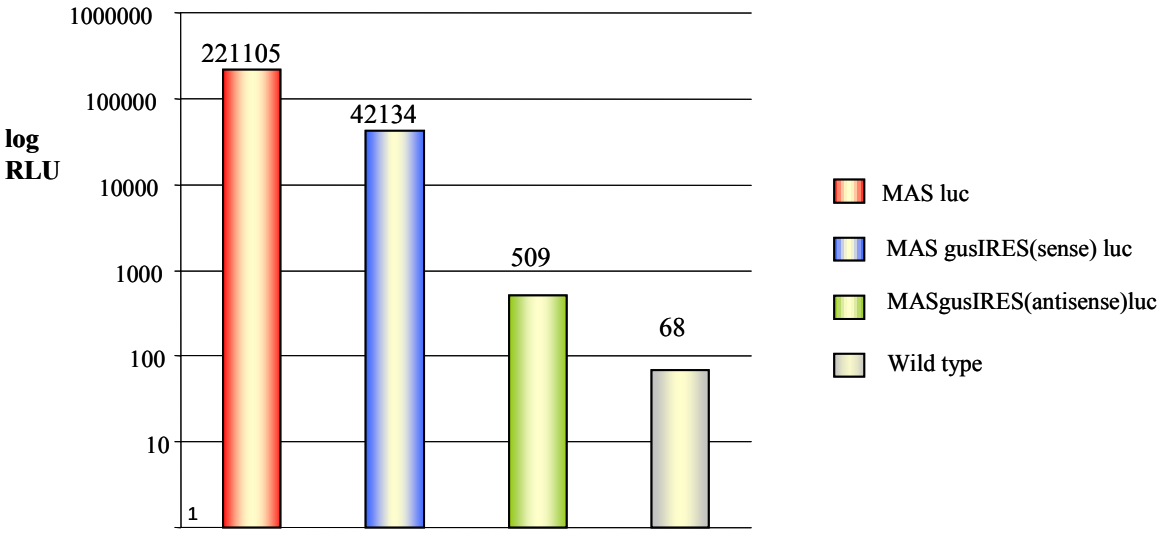

Fig. 18: Luciferase activity in transient expression after 3 days of leaf infiltration.

A series of leaf infiltration experiments were conducted and each time the same significant expression level of luciferase was detected only with TMVcp148 IRES elements. The luciferase expression in the monocistronic vector was ~5-7 folds higher as compared to the expression found in plants transformed with the dicistronic vector. To prove that luciferase expression in a cap independent manner was due to cp148 IRES elements, these IRES elements were cloned in antisense orientation (see Fig. 5). In this case only slight expression of luciferase which was magnitudes lower than for the "sense" orientation construct was detected as expected (Fig. 18).

The relative efficiency of *GUS* gene expression was also examined in all dicistronic constructs containing TMVcp148 IRES elements in sense and anti sense orientation in

transient studies. Activity of the *β-glucuronidase* as the first cistron is shown in the three kinetics in Fig. 19. Remarkably the *β-glucuronidase* activity in the MAS gus/luc antisense construct (Fig. 19) infiltrated leaf is higher than in the MAS gus/luc infiltrated.

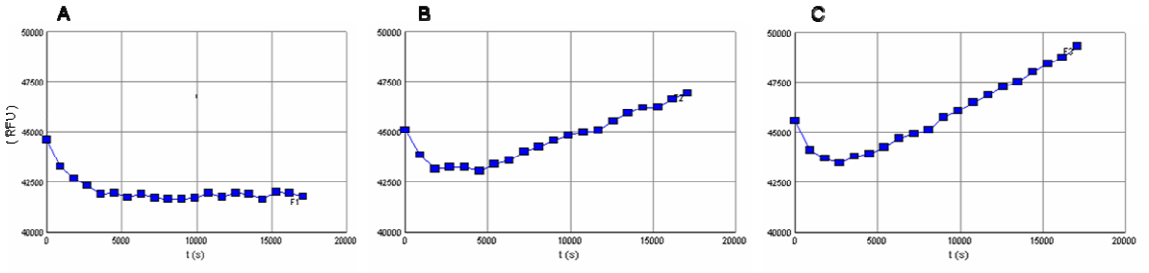

Fig. 19: Kinetics of β-glucuronidase activity after transient expression in N. bethamiana leaves

A: Non inoculated plant; B: MAS gus/luc, C: MAS gus/luc_antisense

3.1.3 Sub cloning of *AtNHX1* in dicistronic vector system

AtNHX1 cDNA was synthesized from transgenic T1 tobacco plants

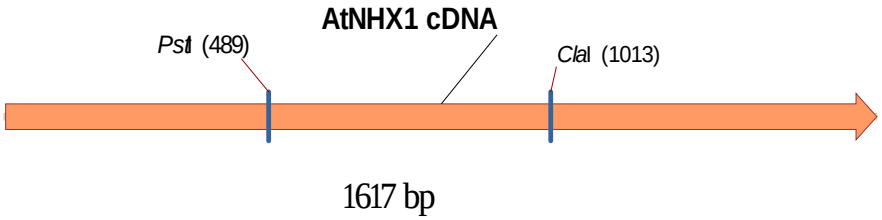

Fig. 20: Synthesis of AtNHX1 cDNA.

3.1.3.1 Confirmation of *AtNHX1* cDNA

From transgenic *N. tabacum* T1 plants *nhx*1 cDNA (1617 bp) was synthesized by RT-PCR (lane no.7). The identity of the cDNA was analysed by *Cla*I (lane no.3) and *Sac*I (lane no.5) restriction enzymes. No.1 and 9 are 100 bp markers (Fig. 21).

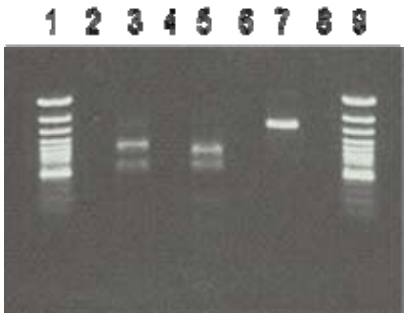

Fig. 21: Confirmation of AtNHX1 cDNA by ClaI and SacI restriction enzymes

After sequencing, 100% sequence homology was found with the NCBI accession (NM122597).

3.1.3.2 Functionality of 0229MAS nhx1/luc

After synthesizing *AtNHX1* cDNA and subcloning into dicistronic vector construct 0229MASguscp148luc, comparative tobacco leaf infiltration experiments were performed with 0229MASguscp148 (model system) and 0229MASnhx1/luc to proof the functionality of vectors. Observations were made on basis of luciferase expression. Higher luciferase expression was observed when the *luciferase* gene was in combination with the *AtNHX1* gene (Fig. 22)

Transient *luciferase* expression 3 days after leaf infiltration

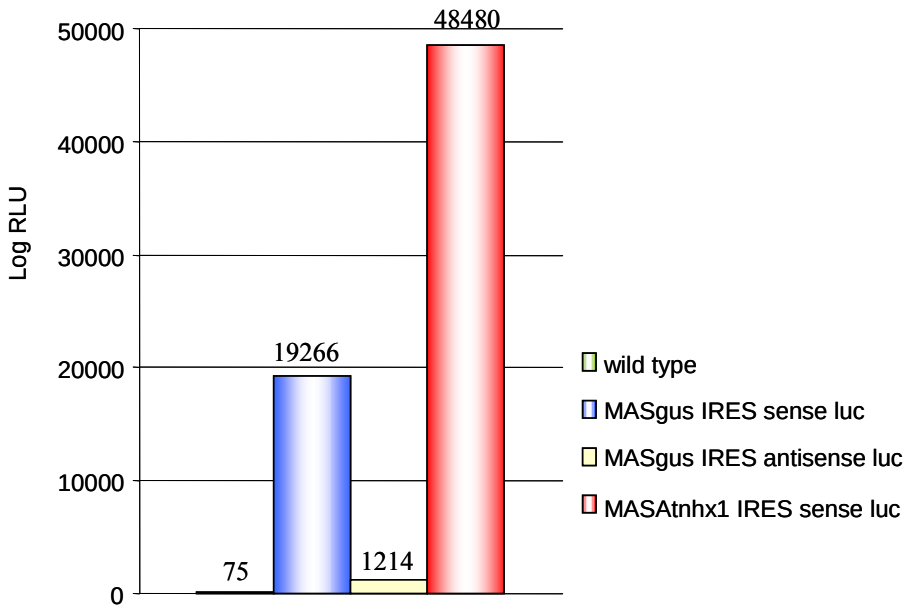

Fig. 22: Functionality of MASgus/luc and MASnhx1/luc vectors.

Transient expression of luciferase. Luciferase in combination with *AtNHX1* showed higher expression (red bar)

By confirming the functionality of our dicistronic vector system along with TMVcp148 IRES elements, series of comparative leaf infiltration experiments were performed by using 0229MASguscp148 (model vector) and 0229MASnhx1cp148luc under different salt (NaCl) stresses and the luciferase expression was observed in both vectors with and without salt stress. Different salt levels of NaCl i.e. 50 mM, 100 mM, 200 mM, 300 mM and 500 mM were applied and achieved one standard lavel i.e 100 mM. Under salt

challenge even higher luciferase expression was observed in MASnhx1/luc as compare to MASgus/luc vector as shown in Fig. 23.

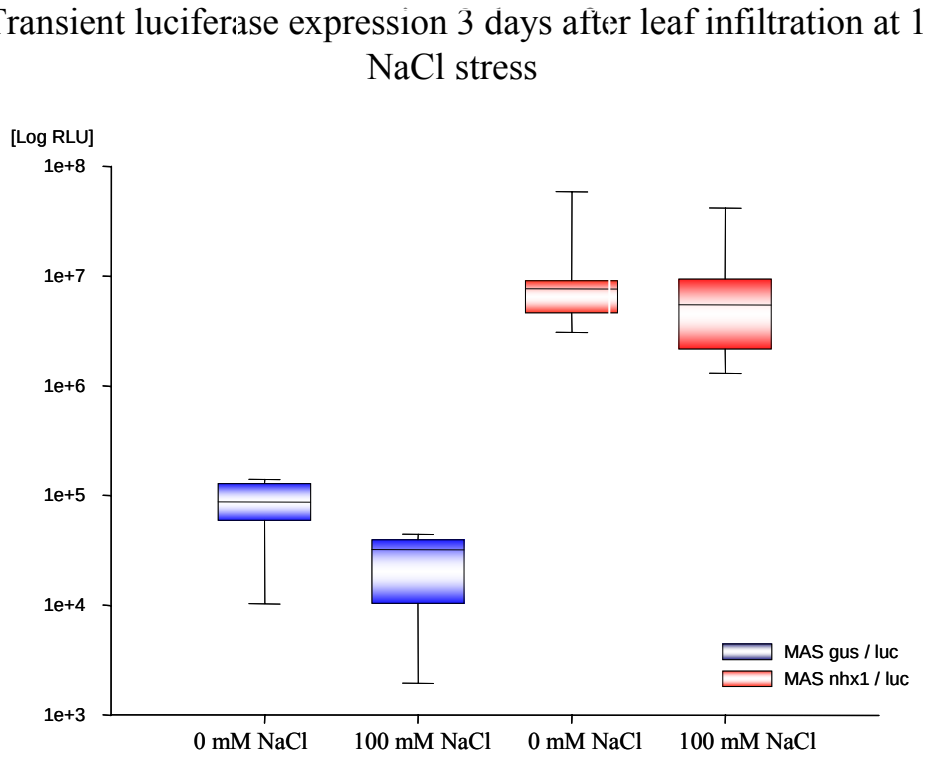

Fig. 23: Transient luciferase expression of MASgus/luc and MASnhx/luc in infiltrated tobacco leaves under salt challenge.

Salt challenge was given along with irrigation water after leaf infiltration.

3.2 Stable transformation of dicistronic vectors (0229MASgus / luc and 0229MASnhx1/luc)

The functionality of dicistronic vectors along with *AtNHX1* gene was confirmed in stably transformed tobacco plants as a model system before moving to legumes or other crop plants, as it takes much more time to transform in target plant (pea). Methodologically the mode of action of recombinant *nhx1* gene can only be investigated when positive effects can be expected. Hence the major interest is the development of a reliable monitoring system for expression studies. From the transgenic T1 tobacco plants calli were derived and expression of glucuronidase in MASgus/luc was quantified by Mug assay where as

luciferase expression was observed in MASgus/luc and MASnhx1/luc by usind Promega luciferase assay kit in luminometer as shown in Figs. 24 and 25.

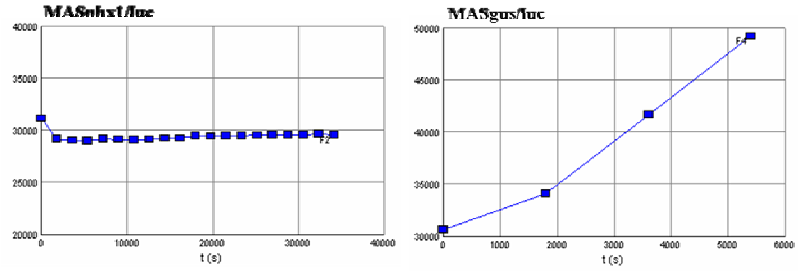

Fig. 24: Kinetics of β-glucuronidase activity in MASgus/luc tobacco cells.

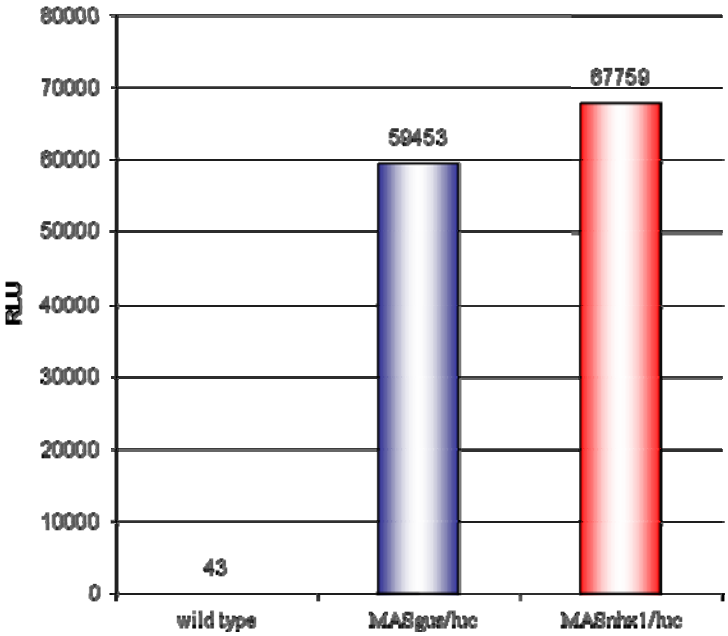

Fig. 25: Luciferase expression in transgenic MASgus/luc and MASnhx1/luc plant derived suspension cells.

Further investigations were made with MASnhx1/luc transgenic calli. Investigations were made under salt challenge ranging from 0 to 150 mM. After 4 weeks of calli growth significant increase in cell mass was seen in MASnhx/luc transgenic calli over wild type. Chlorophenol red (CR) pH indicator was also used in medium. Under salt challenge viable calli could show the colour shift in medium as shown in Figs. 26 and 27.

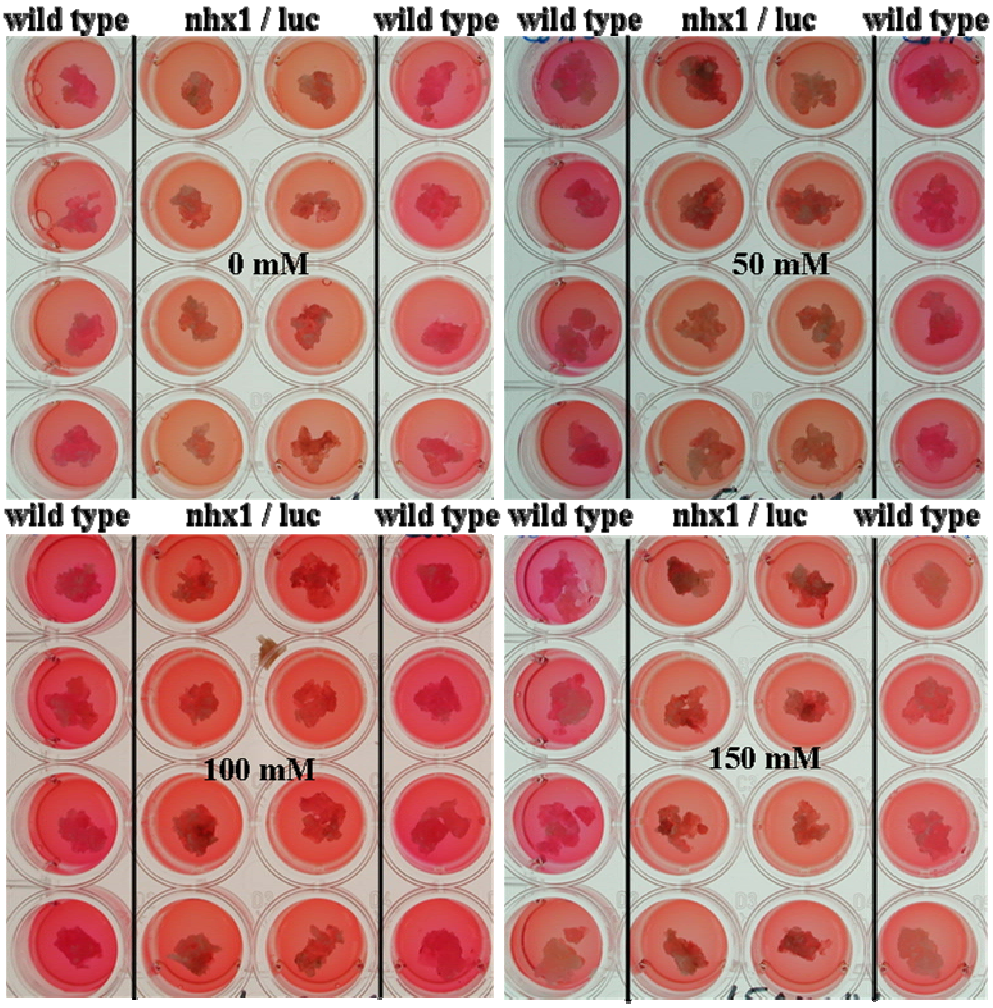

Fig. 26: Wild type vs MASnhx1/luc transgenic tobacco calli growing on Gamborg B5 (4x) medium containing CR pH indicator at different NaCl concentrations (2nd day of growth).

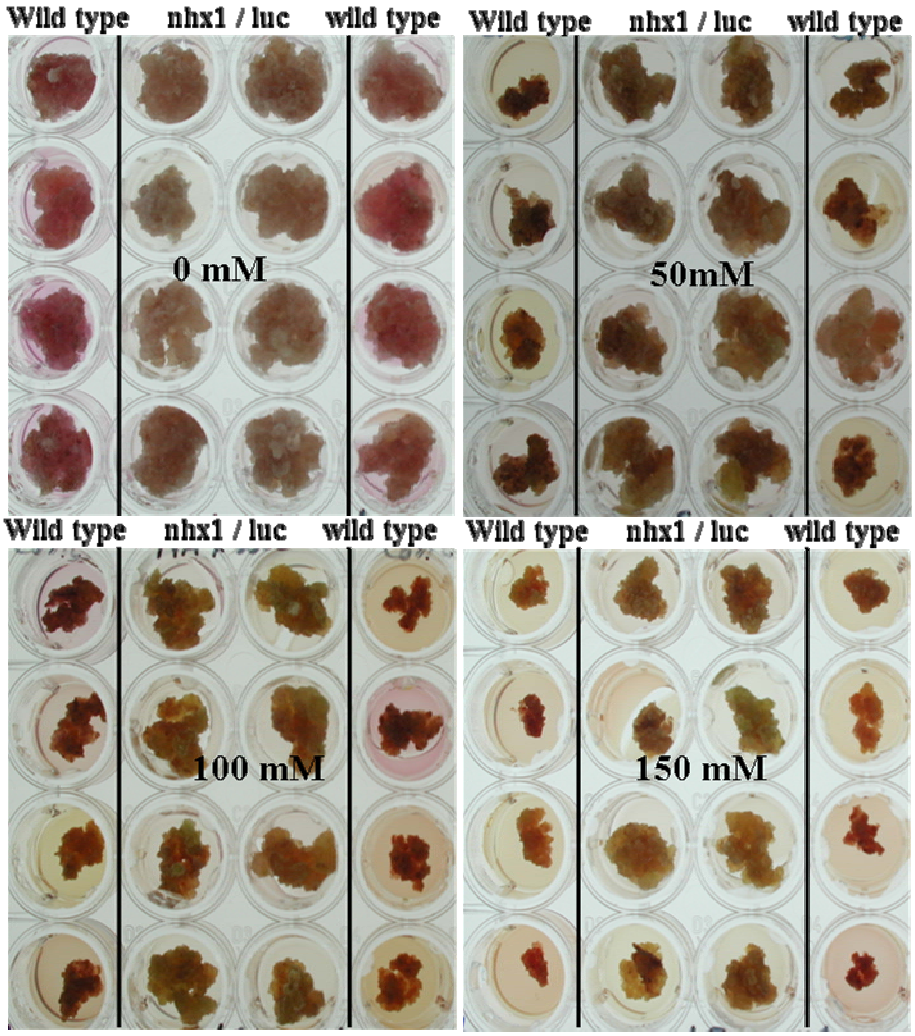

Fig. 27: Wild type vs MASnhx/luc transgenic tobacco calli growing on Gamborg B5 (4x) medium containing CR pH indicator at different NaCl concentrations (after 4 weeks of growth).

Dry weight of MASnhx1/luc transgenic and wild type calli was recorded after 4 week of growth. All transgenic calli showed higher cell masses over wild type cells as shown in Fig. 28.

The box wisker plots were generated by SigmaPlot® 9.0 software and the statistical data analysis was made with SigmaStat® 3.1.

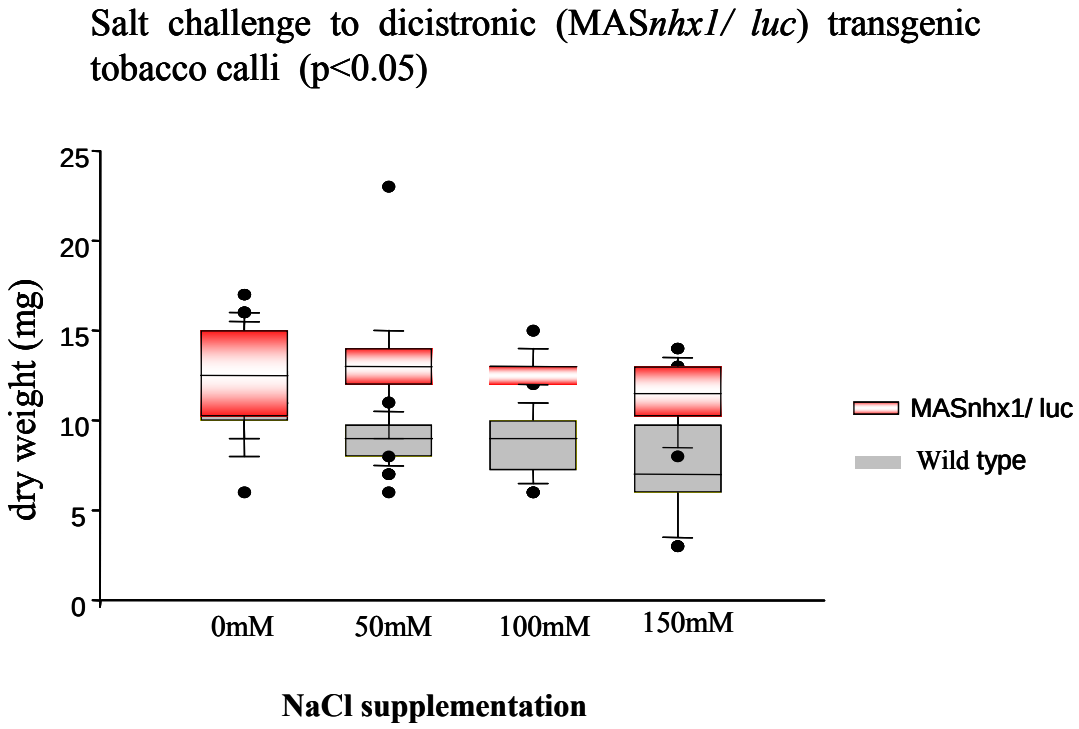

Fig. 28: Dry wt of* MASnhx1/luc *transgenic and wild type calli after 4 weeks of growth under different NaCl concentrations.

3.3 Generation of suspension cells from MASnhx1/luc transgenic calli and selection on the basis of luciferase expression

Suspension cells were generated from dicistronic MASnhx/luc transgenic tobacco calli. Equal amounts of cell material (fresh weight) were grown on the Gamborg B5 (appendix IV) medium containing again cholorophenol red pH indicator and different NaCl concentrations. The viability of the cells was observed on the basis of *luciferase*

expression. Luciferin was applied over the growing cells and light emission was observed under a Fuji LS 3000 imager at weekly intervals. In Fig. 29 chlorophenol red pH indicator also showed the viability of cells as the transgenic cells which grew at 0 mM, 50 mM, 100 mM and even at 150 mM NaCl selection could change the color of the medium from red to light yellow.

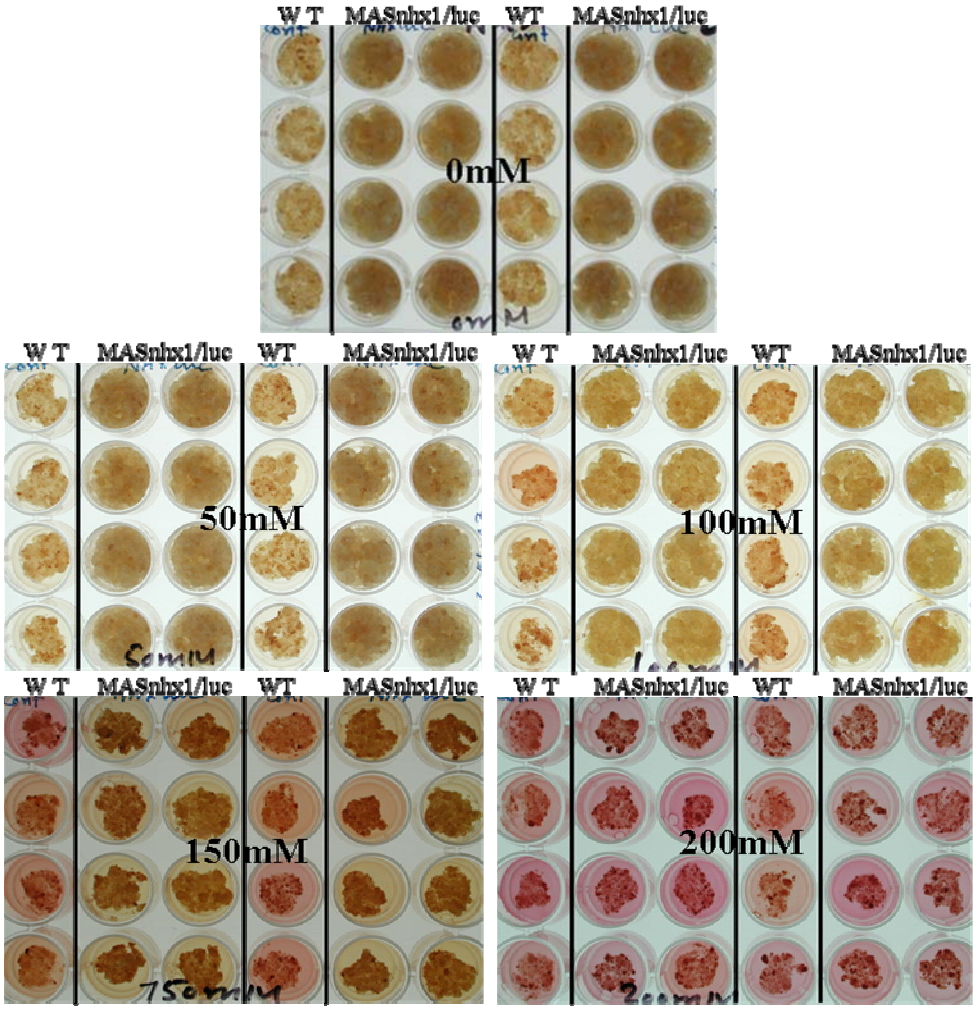

Fig. 29:.Growth of transgenic TO plants derived suspension cells (MASnhx1/luc) under different NaCl concentrations.

Equal amount of wild type and transgenic cells were spread on Gambor B5 (4x) growth medium containing CR pH indicator and different NaCl concentrations (0 to 200 mM).

Wild type and non transgenic cells showed reduced growth and luciferase expression with increasing salt concentrations. In transgenic cells only those cells showed any

luminescence under the imager which could survive under salt selection as shown in Fig. 30.

3.3.1 Luciferase expression in transgenic cells under NaCl challenge

Observation for the luciferase expression in the suspension cells under NaCl challenge were made using Fuji imager LAS300 after 4 weeks of cells growth.

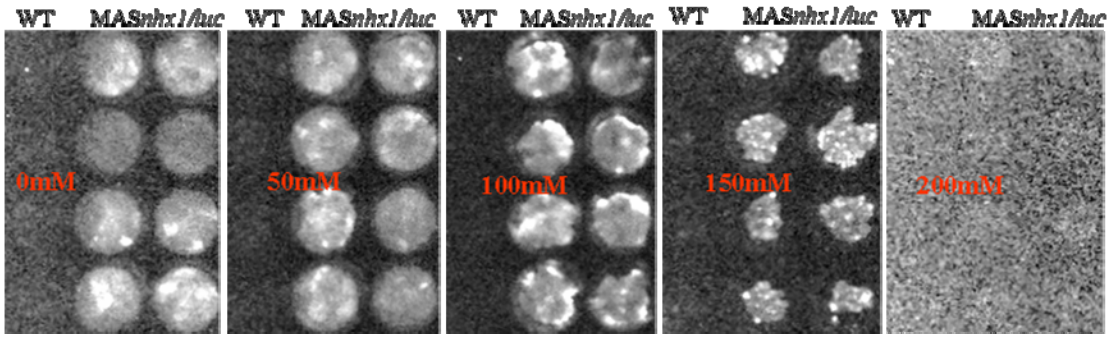

Fig. 30: Luciferase expression in MASnhx1/luc suspension cells under NaCl challenge.

With increase in NaCl concentration there is decrease in cell mass but increase in luciferase expression, maximum at 100 mM NaCl challenge and null expression at 200 mM.

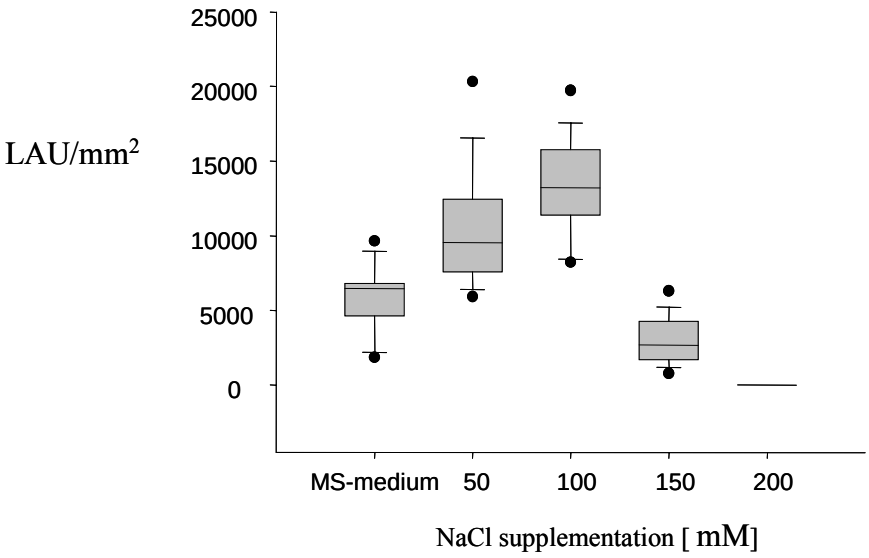

Fig. 31: Luciferase activity of AtNHX1 transgenic suspension cells on solid medium (LAU: linear arbitrary) units).

Luciferase expression was quantified in the cells growing at various NaCl selection levels by the Fuji imager LAS 3000. with the increase in NaCl concentration, gradual increase in luciferase expression (LAU/mm^2) was recorded with a maximum at 100 mM NaCl challenge and then decline in expression took place with the increase in NaCl level a as shown in Fig. 31 and 32.

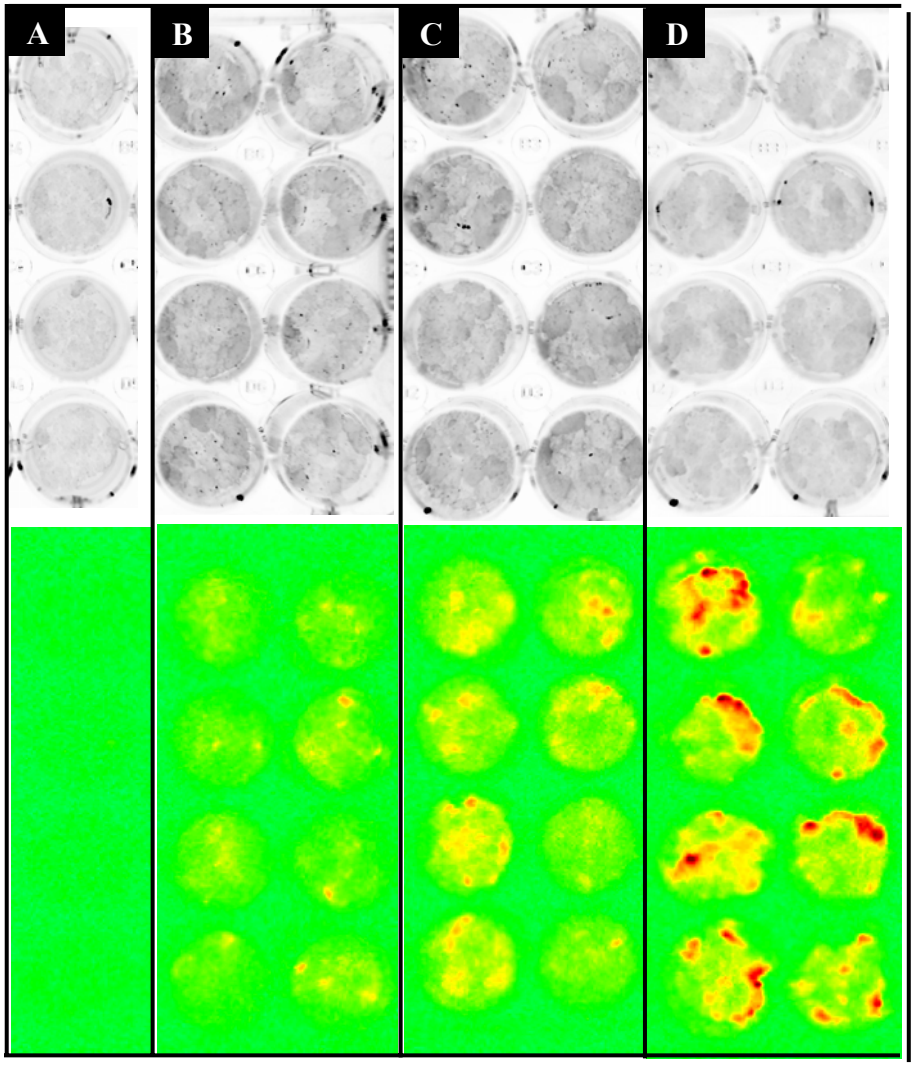

Fig. 32: Luciferase activity imaging in titer plate cultures.

A: wild type B: *AtNHX1* transgeinc at 0 mM NaCl, C: *AtNHX1* transgeinc at 50 mM NaCl and D *AtNHX1* transgeinc at 100 mM NaCl

In the following experiment luciferase expression was also quantified (RLU) in transgenic cells growing on medium supplemented with different NaCl concentrations at different time intervals as shown in Fig. 33. With the increase in NaCl level although expression was lower at the beginning stage (after 4 hours of challenge) but it was going to increase with time interval. luciferase expression was quantified by luminometer Lumat L B 9501 by Berthold using promega *luciferase* assay kit.

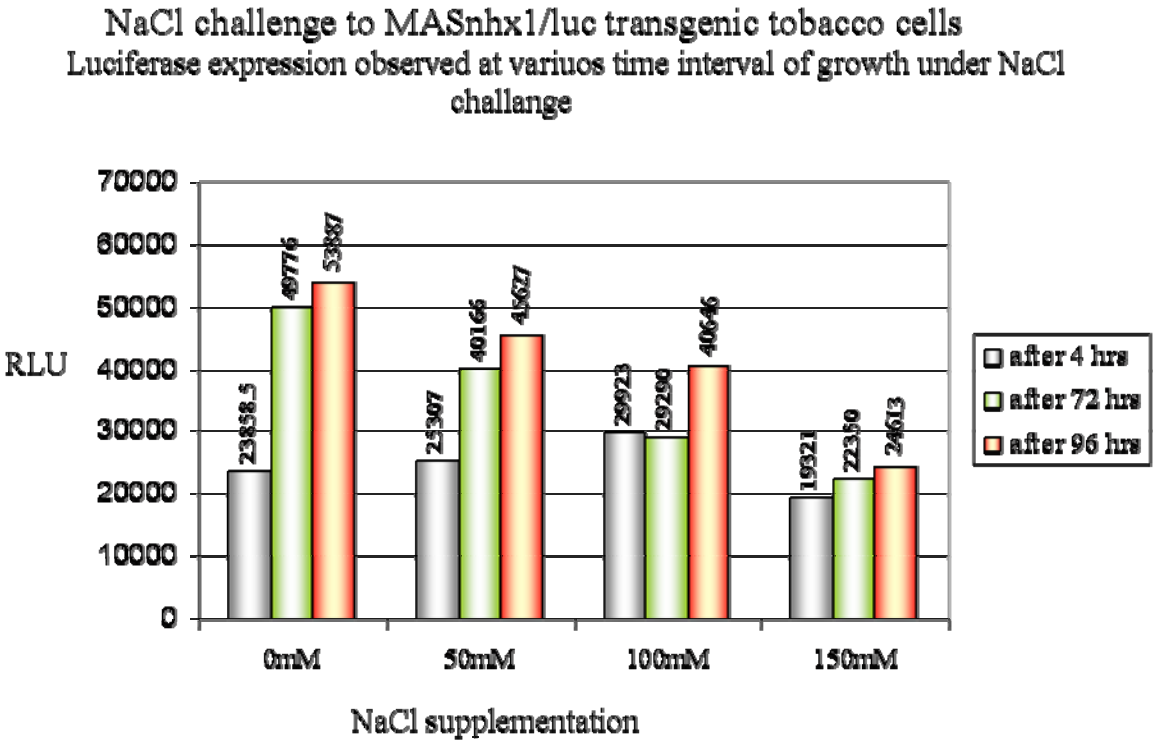

Fig. 33: Luciferase expression quantification in suspension cells at different time intervals,

Growth on Gamborg B5 medium supplemented with different NaCl concentration. Cell mass (g) measurement under NaCl challenge. RLU is relative light units.

Fresh weight (g) was measured of the transgenic and non transgenic suspension cells after 4 weeks of growth. At 0 mM NaCl the fresh weight of the transgenic cells is higher than of the wild type which is also very visible in Fig. 34, an observation we made not only in this experiment. With increasing NaCl concentration, decrease in growth was measured but increasing luciferase activity up to 100 mM, none at 200 mM as shown in Fig. 29 and 30.

In the following the transient data were cross- checked with stably transformed *N. tabacum* suspension cells by comparing the fresh weight of suspension cells after 4 week.

In Fig. 34 the different growth rates of *AtNHX1* transgenic cells in comparison with wild type cells are shown.

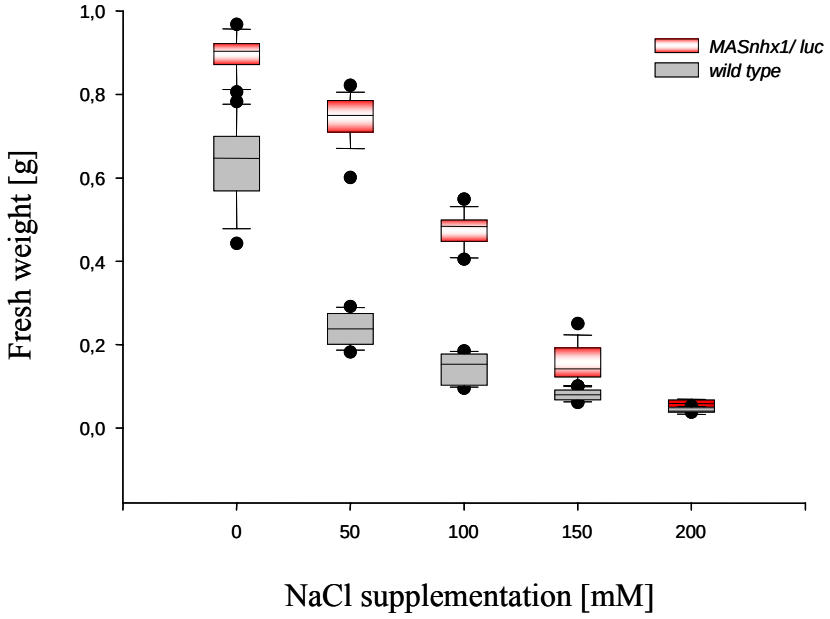

Fig. 34: MASnhx/luc transgenic vs wild type cells - fresh weight measurement after 4 weeks of growth under NaCl challenge.

Finally the integration of the *AtNHX1* and the *luciferase* gene in transgenic T1 tobacco plants and suspension cells derived from transgenic plants were analyzed by using specific primers designed against each gene as shown below in Fig. 35 and 36.

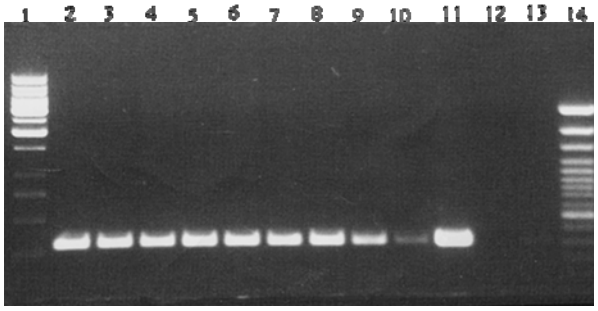

Fig. 35: Analysis of AtNHX1 integration in T1 tobacco plants and in suspension cells.

Lane 2-7 are T1 plants, 8-10 are suspension cells. Lane 11 is +ve control and 12 is wild type plant and 13 is H_2O control. Lane 1 is 1 kb DNA marker and 14 is 100 bp marker.

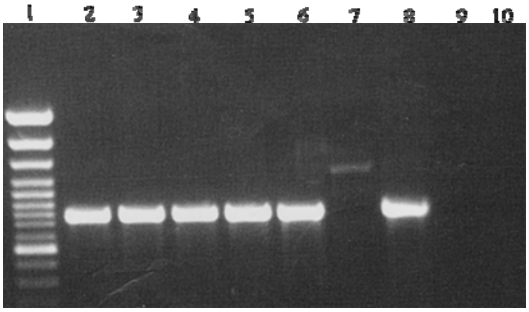

Fig. 36: Analysis of luciferase gene integration in T1 tobacco plants and in suspension cells.

Lane 2-4 are T1 plants, 5-7 are suspension cells. Lane 8 is +ve control, 9 is wild type plant and and 10 is H_2O control. Lane 1 is 100 bp marker.

3.4 Selection of suspension cells under phosphinothricin (ppt) vs sodium chloride (NaCl)

On the basis of the results of all previously described transient and stably transformation experiments, further investigations were made under phosphinotricin selection and NaCl challenge.

3.4.1 Selection of suspension cells under phosphinothricin (ppt)

After 3-4 weeks of growth under ppt selection no or very poor growth could be seen in wild type cells but performance in growth of transgenic cells was quite normal. Although transgenic cells grew very good under ppt selection, but no luciferase expression could be seen after applying luciferin under the Fuji imager as shown in Fig. 37.

3.4.2 Growth of suspension cells under (NaCl)

In the 2nd approach, growth of transgenic cells was investigated on media containing different NaCl concentration ranging from 0 mM to 200 mM. All wild type and most of transgenic cells also were dead at 150 mM NaCl stress. At 100 mM all wild type cells showed strongly reduced growth but not in transgenic cells. Although transgenic cells also showed growth reduction slightly but luminescence activity was very high at 100 mM NaCl selection as shown in Fig. 38.

3.4.3 Suspension cells under Sodium chloride (NaCl) + phosphinothricin (ppt) selection

The 3rd suspension cells selection experiments was made under NaCl salt and ppt together. In the previous experiments no luciferase activity was observed when cells were grown only under 5 mg/l ppt selection. When cells grown on medium containing both NaCl and ppt together, luciferase expression could be observed as shown in Fig. 37. Growth of the cells was significantly reduced under 100 mM NaCl +5 mg/l ppt as shown in Fig. 39.

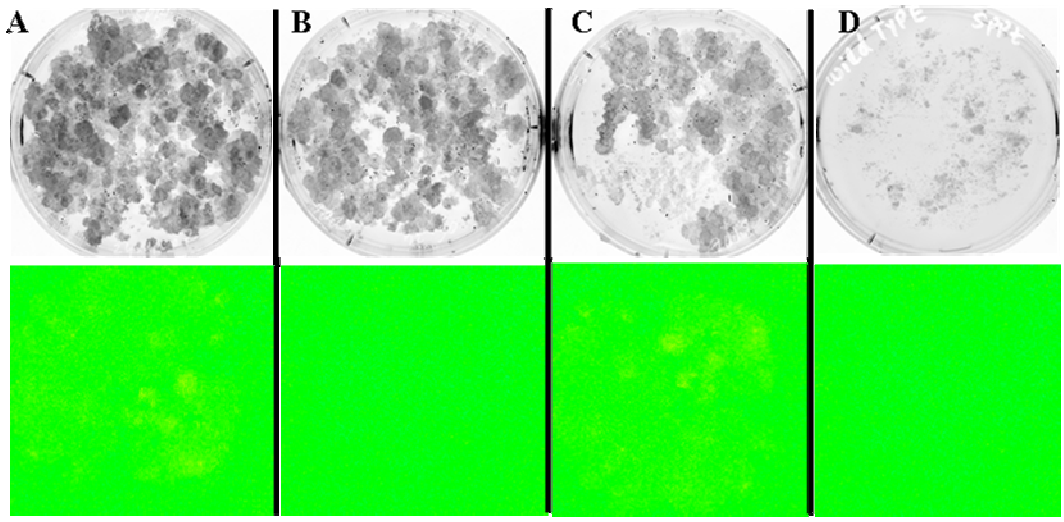

Fig. 37: MASnhx1/luc transgenic tobacco cells growing under 5 mg/l ppt in Gamborg B5 (4x) medium.

A, B, and C are MASnhx1/luc transgenic tobacco cells growing under 5 mg/l ppt in Gamborg B5 (4x) medium. Cells grew very good but luciferase expression could not be found (lower green figures) after 4 weeks of growth. .D is wild type tobacco cells.

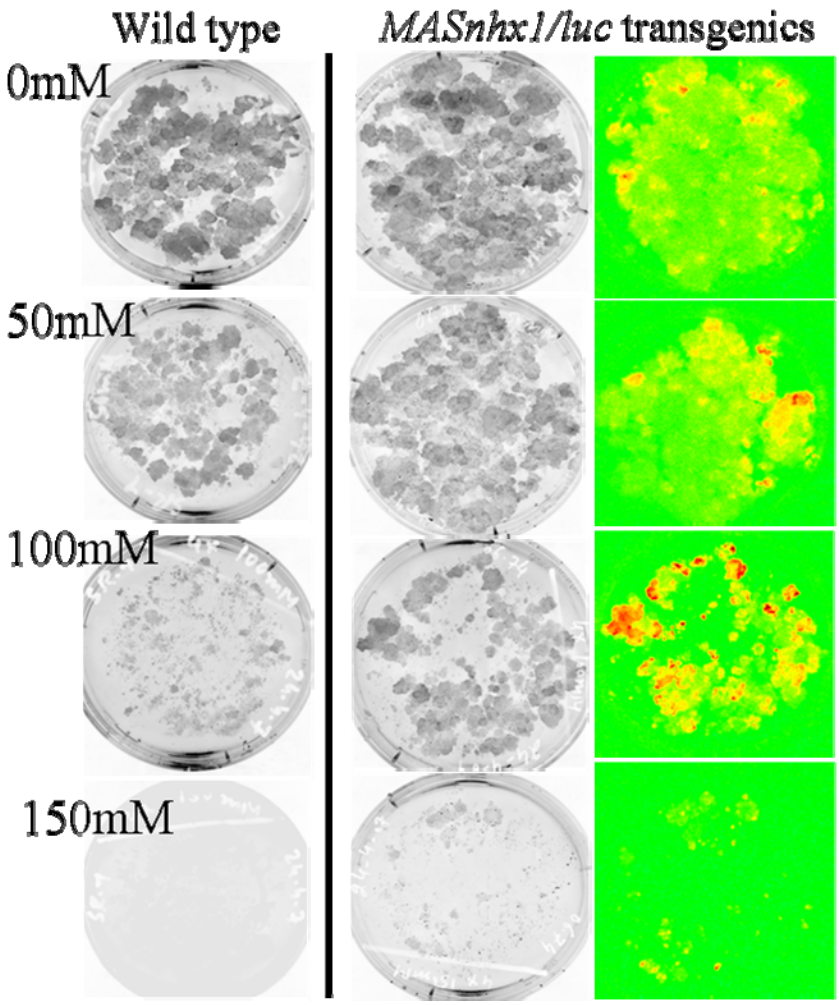

Fig. 38: Selection of wild type and MASnhx1/luc transgenic cells under different NaCl concentration.

All wild type cells were dead at 100 mM NaCl selection but transgenic survived. Left black and white pictures are without luminescence where as green pictures (right) are MASnhx1/luc transgenic cells after chemiluminescence under Fuji imager LAS 300.

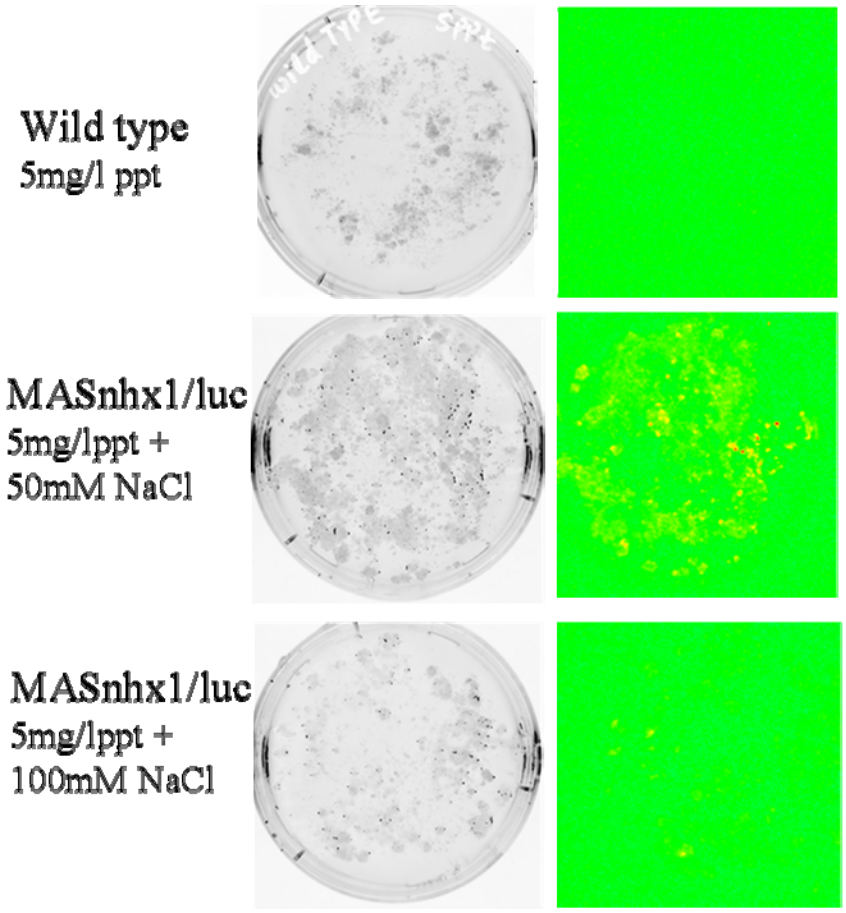

Fig. 39: MASnhx1/luc transgenic cells under NaCl + ppt selection.

Right images are under Fuji LAS 3000 after applying luciferin and left side without luciferin application.

3.5 Southern blot analysis of transgenic tobacco cells.

Integration of gene was investigated in 0229MASnhx1/luc transgenic suspension cells using Southern blot analysis with nhx1/luc probes as shown in Fig. 40. ApaI and StuI in combination excise the whole cassette consisting of, MAS-promoter, *AtNHX1* gene, IRES element and the *luciferase* gene inclusive terminator. Therefore the single band my represent several copies from different integration events.

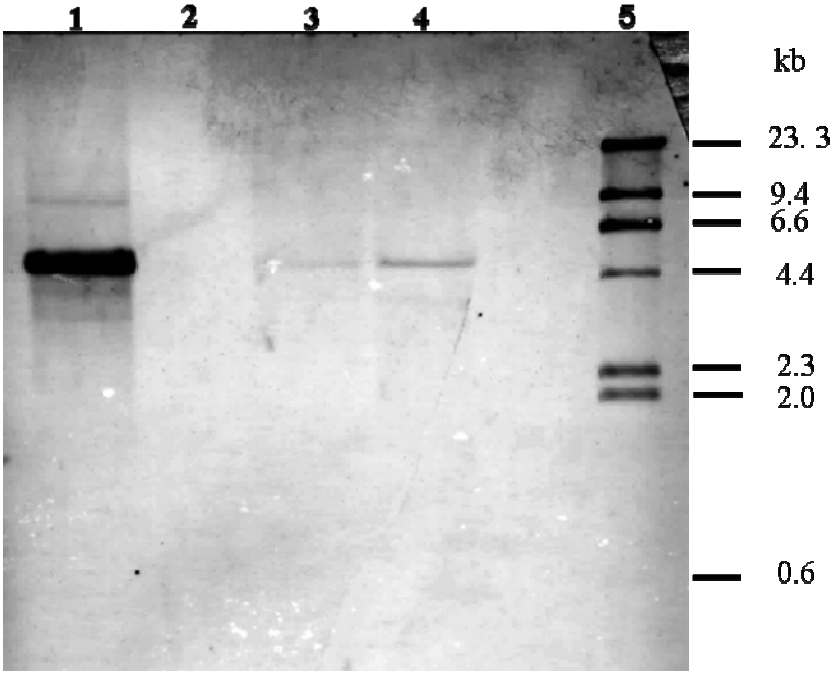

Fig. 40: Southern blot analysis of 0229MASnhx1/luc transgenic suspension cells

1 - +control plasmid DNA 0229MASnhx1/luc restricted with *Kpn*I and *Stu*I
2 – ve control
3 & 4 gDNA isolated from 0229MASnhx1/luc transgenic tobacco suspension cells digested with *Apa*I and *Stu*I enzymes.
5 Dig labelled high molecular weight marker II

3.6 Pea transformation

After confirming the functionality of the dicistronic vector system in transient and stably transformed tobacco cells, transformation was made with pea (*Pisum sativum* L.) as a legume model. The *Agrobacterium* mediated transformation system according to the modified protocol of Schroeder *et al*., 1993 and Bean *et al.,* (1997) has been used. The

explants and calli which were transformed induced the color shift of the medium containing CR pH indicator, from red to yellow whereas no color shift was observed in non transgenic and wild type embryos as shown in Fig. 41. All of the non transgenic and wild type embryos started to die after the second round of selection. Regenerating shoots from these clones were further subcultured with increased ppt concentrations (7.5 mg/l) and used for in vitro grafting on a wild type root stock.

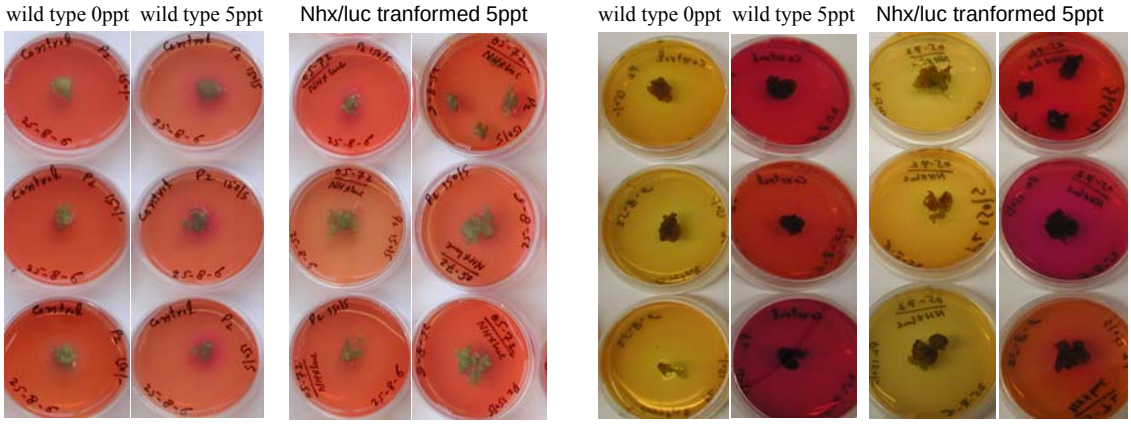

Fig. 41: WT and transformed pea embryos on P2 media containing chlorophenol red indicator and ppt 5mg/l.

W.T also without ppt selection (left side). Wild type without ppt selection and some transgenic with ppt selection induced color shift. All non transgenic under ppt selection were dead. Photo made with Nikon digital camera.

3.6.1 Luciferase expression in T0, T1 and T2 generation of MASnhx/luc transgenic Pea plants

luciferase expression was observed from the shoot proliferating transgenic pea calli as shown in Fig. 42.

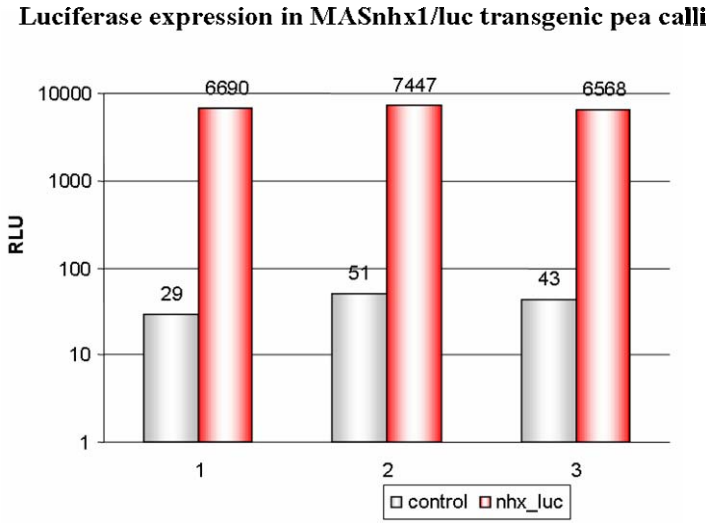

Fig. 42: Luciferase expression(RLU) in shoot regenerating transgenic pea calli.

Expression was observed by luminometer using the Promega luciferase asssy kit. (RLU-relative light units).

From these calli shoots were further subcultured under ppt selection.To recover transgenic shoots without delay; grafting was done on non transgenic root stocks as shown in Fig. 43.

Fig. 43: MASnhx/luc transgenic shoot grafted on non transgenic root stock

From the transgenic T0 and wild type plants growing in greenhouse leaves were taken and life luciferase assay was made using the Fuji imager. No difference between transgenic and wild type pea leaves could be seen as shown in Fig. 44.

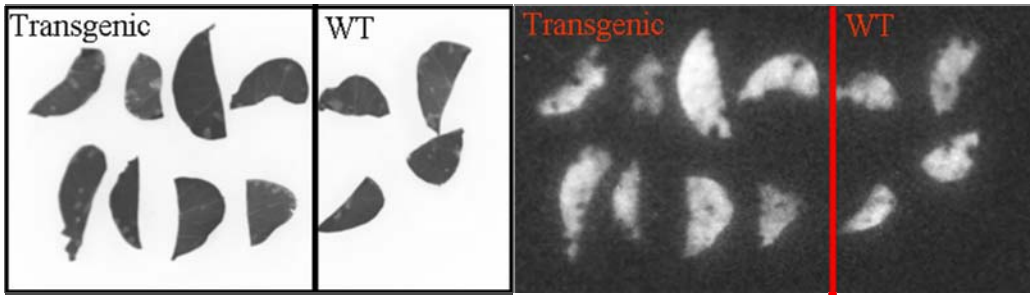

Fig. 44: Luciferase expression inMASnhx1/luc transgenicT0 and WT pea leaves by spraying luciferin over the leaves

Observations were made under Fuji LAS 3000 imager.

From the T0 freshly picked seeds (T1) were used for life luciferase assays. In the preliminary approach for investigation of transgenic seeds, small pieces of transgenic and wild type cotyledons were cut and luciferin was applied. Observation of luciferase expression was made under the Fuji imager. luciferase expression in transgenic cotyledons is shown in Fig. 45.

On the basis of these observations luciferin salt was directly applied to the freshly picked pea pods containing seeds. Interestingly luciferase expression could be observed even in freshly picked pea seeds (Fig. 46).

3.6.2 Expression of luciferase in transgenic pea cotyledons

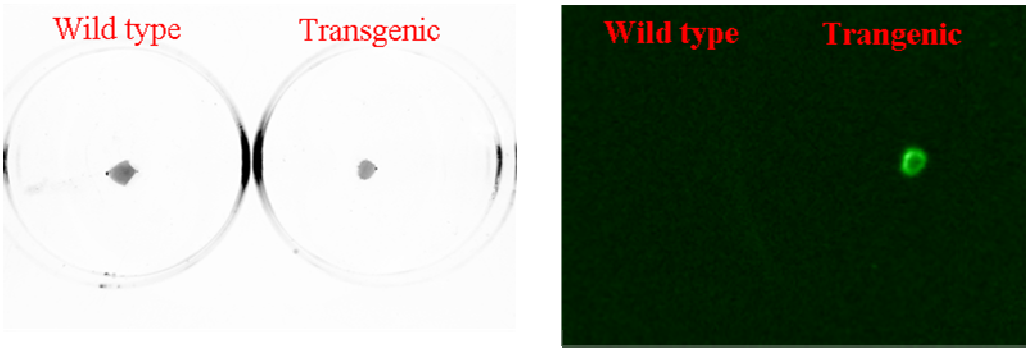

Fig. 45: MASnhx/luc transgenic T1 pea cotyledon expressing luciferase under Fuji imager LAS 3000.

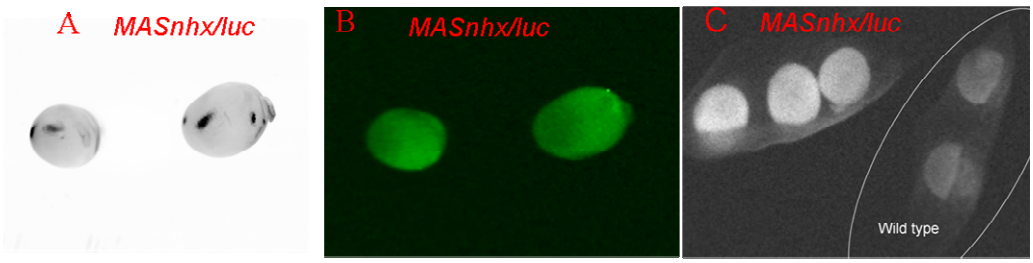

Fig .46: Luciferase expression in MASnhx/luc transgenic T1 pea seeds.

A shows the points of injection of luciferin with the help of syringe into seeds whereas B, shows luciferase expression in seeds under imager. In C luciferase expression in MAS nhx/luc transgenic seeds bt applying lucefrin on seeds attached in side pods. In C lower right side are wild type seeds and there is only background expression.

From the T0, T1 and T2 pea plants growing in the greenhouse leaf material was taken and quantitative *luciferase* assays were performed using promega *luciferase* kit by observing in the Luminometer as shown in Fig. 47.

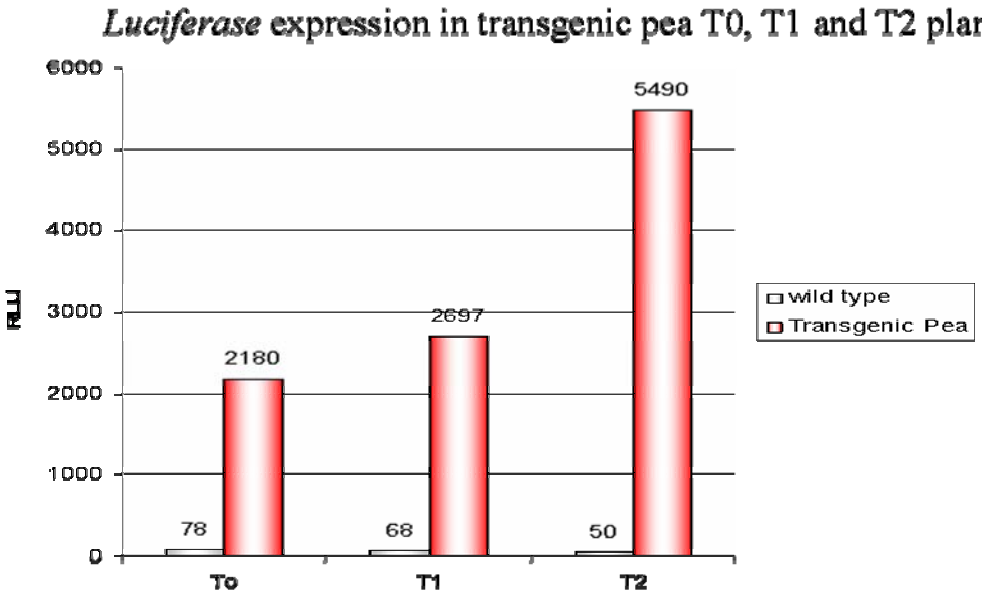

Fig. 47: Luciferase expression in T0, T1 and T2 MASnhx/luctransgenic pea plants.

Assays were made by using promega luciferase kit.

On the basis of the previous results it can be estimated that the expression of *luciferase* as a second cistron can indicates the expression of *AtNHX1* (first cistron).

3.6.3 Analysis of transgenic (MASnhx/luc) pea plants by PCR

Confirmation of the integration of *AtNHX1*, *luciferase* and bar gene were made by PCR amplification using specific primers against each gene of interest as shown in Figs. 48-53.

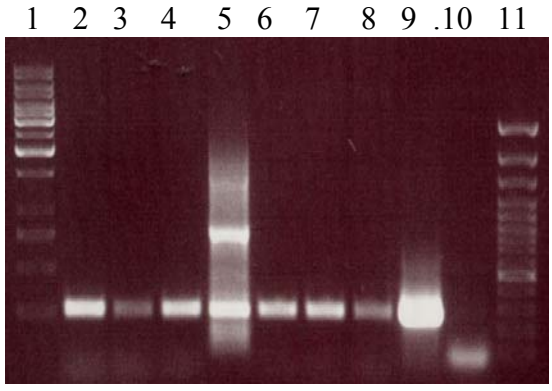

Fig. 48: AtNHX1 integration in T0 MASnhx/lucPea plants.

From left to right 2-8 *AtNHX1* integration in T0 MASnhx/lucPea plants No.9 is + ve control & No.10 is –ve control. Lane 1&11 are 100 bp markers.

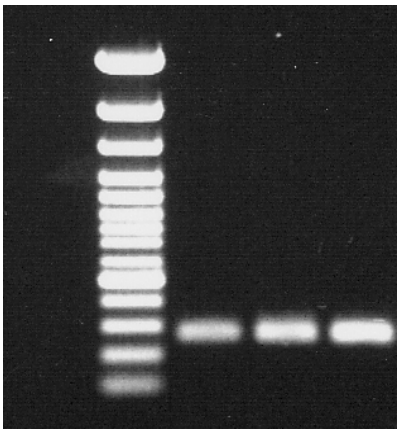

Fig. 49: T1 pea plants expressing bar gene

From left to right 1 is – ve control, 2 is 100 bp marker,3 & 4 are transgenic plant and 4 is + ve control.

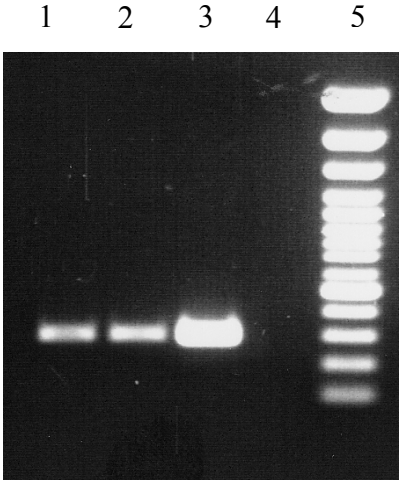

Fig. 50: AtNHX1 integration (300 bp) in T1 pea plants.

Confirmations were made using specific primers. From right to left No 5 is 100 bp marker, 4 is –ve control, 3 is + ve control and No 1 & 2 are transgenic pea plants.

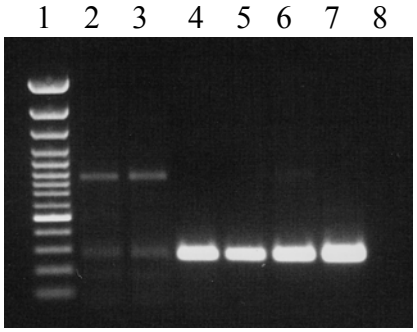

Fig. 51: T2 pea plants - Integration of bar gene.

No 1 is 100 bp marker, 2&3 wild type pea plant, 4&5 T2 transgenic plants 6 is T1 transgenic pea plant No 7 is + ve control and No 8 is –ve control.

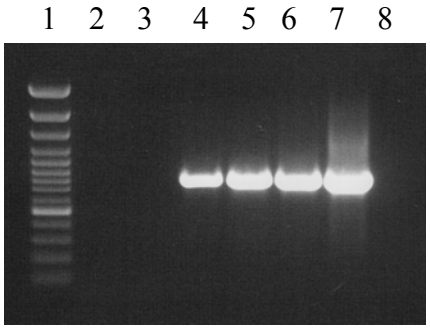

Fig. 52: T2 pea plant - Integration of luciferase gene.

No 1 is 100 bp marker, 2&3 wild type pea plant, 4&5 T2 transgenic plants 6 is T1 transgenic pea plant No 7 is + ve control and No 8 is –ve control.

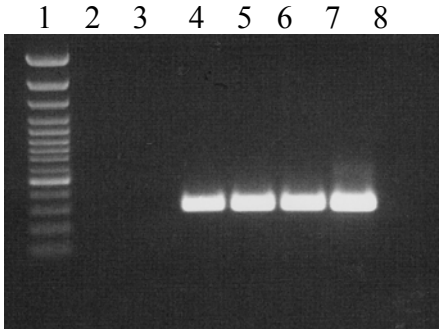

Fig. 53: T2 pea plants - Integration of AtNHX1 gene.

No 1 is 100 bp marker, 2&3 wild type pea plant, 4&5 T2 transgenic plants 6 is T1 transgenic pea plant No 7 is + ve control and No 8 is –ve control.

4 DISCUSSION

Coordinated expression of multiple proteins under control of the same promoter may be helpful to achieve transformed plants with improved traits and is still a big challenge in plants. To achieve this task, it seems to be reasonable to involve IRES elements as intercistronic spacers although IRES mediated expression is lower. Genetic engineering of crop plants with stress tolerance genes has the potential to improve among others also drought and salt tolerance (Cixin *et al*., 2005; Shi *et al*., 2003; Zhang *et al.,* 2001; Apse *et al*., 1999). Various stress tolerance genes have been identified and their functional properties were reported (Chinnusamy *et al*., 2004; Wang *et al*., 2003). In this study a sodium/proton antiporter gene (*AtNHX1*) has been used as a proof of principle for a type of new vectors, since the functionality of this gene is already proven by transformation of different crop plants (Sottosanto *et al.,* 2007*;* Cixin *et al.,* 2005; shi *et al.,* 2003; Zhang *et al*., 2001).

In the first approach the functionality of the *AtNHX1* gene (see 3.1.1.5), under the control of a CaMV35S promoter in monocistronic system (see Fig. 13) was shown by transforming *N. tabacum*. Transgenic T1 tobacco plants were grown on MS salt medium containing different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM and 200 mM respectively). In these T1 tobacco plants enhanced salt tolerance could not be observed because of the already very high tolerance of wild type plants. There might be already a high level of Na^+ antiporters in tobacco plants.

The objective of this study was not to develop salt tolerant plants but to establish a model system for testing the functionality of *AtNHX1*. For testing its functionality in plants like pea (*Pisum sativum* L.) it would take much more time. For the identification of appropriate candidate genes in transgenic approaches it is essential to test the constructs in a model system and than in the target plant species.

Salt challenge to *AtNHX1* non-transgenic tobacco calli showed significantly improved growth over the wild type (see Fig. 14b). Even at low salt concentrations (0 mM NaCl) transgenic calli performed better than the wild type. This might be due to Gamborg´s B5 salt (4x) medium which was used for callus induction and growth, because it contained comparably high amount of NaCl i.e. 2 g l^{-1} (*Duchefa*). As long as the NaCl concentration was increased, severe reduction in growth up to 100 mM NaCl was

observed in wild type but not in transgenic calli. At higher concentration of NaCl, growth was also reduced in transgenic and at 150 mM most transgenic and all of wild type calli were dead after 4 weeks of growth (Figs. 14 and 15).

The accumulation of high amounts of Na^+ in the cytosol and vacuole is lethal and created osmotic imbalances for the cells. Thus there was no further storage capacity available for cells to accumulate more Na^+ and Cl^- ions and all cells died.

The experimental data obtained for calli in this study are consistent with those obtained from other species such as *Arabidopsis*, rapeseed, tomato cotton (Apse *et al*., 1999; Blumwald, 2000; Zhang *et al*., 2001. Cixin *et al*., 2005), thus confirming the present results. The expression of *AtNHX1* can indeed improve the cellular tolerance against salt stress, although previous studies have been made with entire plants expressing this gene. Another interesting and major finding which differs from other previous studies but is consistent with Cixin *et al.,* (2005) is that even under relatively low salt conditions the *AtNHX1* -expressing plant cells performed better than wild-type cells. Cixin *et al.,* (2005) reported that under low salt conditions in the field, the *AtNHX1* expressing cotton plants performed better than wild-type plants, exhibiting higher yields and better fiber quality.

In order to analyze the functionality of *AtNHX1* regarding the direction of integration attempts were made to establish dicistronic gene expression mediated by IRES elements. The functionality of the system was analyzed using the marker genes *β-glucuronidase* (first cistron) and firefly *luciferase* (second cistron). Testing the three different internal ribosomal entry sites (IRES) of TMVcp148 IRES, polio IRES and *Zea mays* IRES elements as an intercistronic sequences clearly showed that TMVcp148 IRES elements showed better performance and higher expression of *luciferase* (being second cistron) in plants. The activity of IRES elements is not kingdom specific thus IRES elements working in animal system can work in plants system as well (Urwin *et al*., 2000) but according to Hennecke *et al*., (2001) the composition and arrangement of genes also has an influence on the IRES mediated translation. The objective was to find which IRES elements are initiating internal translation best in plant cells.

For functional investigations transient expression studies were conducted among various gene expression systems. *Agrobacterium* based transient expression systems take advantages by efficiently transferring the specific DNA construct located within the T-DNA regions of the bacterial Ti plasmid (Cazzonelli *et al.,* 2006) as compared to direct

gene transfer e.g. biolistic and induced DNA uptake of protoplasts (Taylor and Fauquet, 2002). Additionally *Agrobacterium* infusion into plant provides results in almost homogeneous expression pattern in contrast to the direct DNA transfer technology in tissues where only a limited number of the cells actually express the recombinant gene (Kapila *et al.,* 1997; Johensen *et al.,* 2001, Cazzonelli, CI. *et al.,* 2006). The functionality of all the tested constructs could be proven in transient expression and the results of this transient studies were consistent with the previous reports (Kapila *et al.,* 1997; Dorokhov *et al.,* 2001; Johensen *et al.,* 2001, Cazzonelli *et al.,* 2006).

Furthermore the activity of TMVcp148 IRES was compared with polio and maize IRES elements, in transient expression in tobacco leaves. No activity of polio IRES elements in tobacco leaves could be seen, although they are working well in animal cells (Dirks *et al*., 1993). The putative maize IRES elements also showed significant lower expression of the second cistron (data not shown). The performance of TMVcp148IRES in the present study is consistent with Dorokhov *et al.,* (2002). Dorokhov also compared the activity of TMVcp148 IRES with EMCV IRES in Hela, tobacco and yeast cells and reported that the activity of EMCV IRES was higher in Hela cells than in tobacco and yeast cells whereas the relative activity of TMVcp148 IRES was higher in variability in all cells tested. Hennecke *et.al*., 2001 reported in his study that synthetic mRNA assembly can exert strong negative effects on IRES mediated translation. He further explained that certain coding sequences can exert a negative effect on IRES mediated translation. Which factors are involved influencing the activity of IRES still needs further detailed studies.

The cap independent expression of *luciferase* due to TMVcp148 IRES elements, it could be confirmed by cloning these IRES elements in antisense orientation in the same vector pG0229MASguscp148luc. In this comparative study no expression or only slight expression of the *luciferase* gene could be seen when TMVcp148 IRES were in antisense orientation. (see Fig. 18) The slight *luciferase* activity might be due to the formation of a fusion protein as *glucuronidase* and *luciferase* genes were in frame (see Fig. 5).

Cap dependent translation of the *β-glucuronidase* was examined in both sense and antisense IRES elements constructs. There were no significant differences in glucuronidase expression among constructs which indicate the steady state level production of mRNA from dicistronic expression plasmids. The approximate equal glucuronidase expression in 0229MASgus/luc infiltrated and 0229MASgus/luc(antisense)

infiltrated leaves (see Fig. 19) proved that *luciferase* expression in 0229MASgus/luc is due to tobamovirus IRES elements as almost no *luciferase* activity could be seen when IRES elements were in antisense orientation although glucuronidase expression in IRES antisense was slightly even higher.

Different methods have been used for confirming the functionality of IRES driven translation. Martin *et al.,* (2006), showed that the insertion of a *Hin*dIII site, in place of the initiating AUG codon of the wild type EMCV IRES, is responsible for the dramatic loss of expression from the second cistron, whereas expression from the first cistron remains unaffected.

By confirming the functional performance of TMVcp148 IRES, *AtNHX1* cDNA was subcloned as a first cistron in the vector of 0229MASgus/luc by replacing the *gus* gene with the *AtNHX1* gene, resulting in the construct 0229MASnhx1/luc.

In transient assays higher *luciferase* expression was observed with *Agrobacterium* infiltrated leaves harbouring the 0229MASnhx/luc construct in comparison to 0229MASgus/luc constructs (see Fig. 22). This observation could again be due to the possibility that the leaf infiltration medium (1 x MS-salt) is causing salt stress when injected into the intercellular lumen, which then is smoothed by transient sodium antiporter (*AtNHX1*) expression. The higher *luciferase* expression level is possibly due to the higher viability of the cells which are co-expressing the *AtNHX1* gene. These results also strengthened the previous results which were obtained with the mono cistronic *AtNHX1* transgenic calli (see Figs 14 & 15).

Further comparative transient expression studies under NaCl stress (100 mM NaCl provided in irrigation water after leaf infiltration) also support the previous results that luciferase expression is increased when co-expressed with *AtNHX1* gene over luciferase co-expression with glucuronidase (see Fig. 23) at NaCl concentration of 0 mM and 100 mM. There was no significant difference in luciferase expression at 0 mM and 100 mM with the 0229MASnhx1/luc infiltrated leaves but *luciferase* expression was already low with the 0229MASgus/luc construct at 0 mM and further dropped down at 100 mM salt stress. This observation has to be seen in context with the physiological status of the leaf cells.

The expression of the *luciferase* gene can confirm that *AtNHX1* is also being expressed. As according to Martin *et.al*., (2006) the expression of the product encoded by the second

cistron (IRES elements) is the assurance that the first cistron is also being expressed since both genes are linked by an IRES element under the control of same promoter.

Nevertheless the high variance in transient assays did not allow proper statistics of the discussed observations, therefore the analysis of the functional performance of dicistronic vectors was made in stably transformed tobacco cells. From the stably transformed tobacco plants with MASgus/luc and MASnhx1/luc, calli were induced and positive luciferase and glucuronidase activity were observed which confirmed the functional performance of di-cistronic vector system in stable transformed plants also (see Figs. 24 and 25). Subsequent all investigations were made MASnhx1/luc transgenic tobacco plant derived calli and suspension cells. From the transgenic T0 tobacco plants (MASnhx1/luc) calli were derived and studies were made under salt challenge according to the same procedure as explained in (3.1.1.6 Figs. 14 and 15). Dry weights of calli were measured after 4 week of growth. The MASnhx1/luc transgenic calli gained significantly cell mass over wild type (see graph 28) with the similar pattern while *AtNHX1* mono transgenic showed enhanced growth over wild type (see Figs. 14 and 15).

Cells in calli always form clusters and only the bottom layer cells has direct contact with the medium, whereas the others have contact only through the bottom layer which may detoxify the medium components, respectively accumulate Na^+ in the vacuole. To overcome this problem, suspension cells, were generated from 0229MASnhx1/luc transgenic calli and were spread in equal quantities on Gamborg B5 medium containing different concentrations of NaCl (see Fig. 29). Observations were made on the basis of luciferase expression. No reduction in growth in MASnhx1/luc suspension cells was observed up to 100 mM NaCl, and then growth declined. All of the transgenic and wild type cells were dead at 200 mM NaCl and also no *luciferase* activity could be observed. With the increase in the NaCl concentration, although growth of transgenic cells was reduced, the *luciferase* activity was higher under salt concentrations of 50 mM and even higher at 100 mM (see Figs. 32) and 31) as compared to 0 mM. A possible explanation could be that when cells are challenged with salt non expressing cells stop growth and *AtNHX1* expressing cells are selected. So that finally a transgenic cells grown under salt challenge for some time may consists of a higher percentage of *AtNHX1* expressing cells. A callus may also consist of cells expressing higher levels of *AtNHX1*.

In the present work it is shown, that the growth of nhx1 transgenic calli is promoted. With increasing salinity of the medium the cell mass was reduced but the luciferase activity is negatively correlated to the cell mass. Highest luciferase activity was observed at 100 mM NaCl concentration (Fig. 31). Later on observations were made on the basis of fresh weight of transgenic and wild type cells and the same growth tendency was seen under salt selection (Fig 34) as in previous experiments (see Figs. 27 and 28).

The quantitative luciferase expression was also measured in transgenic cells growing under different NaCl concentrations with different time interval. As with increased salinity the growth was reduced the *luciferase* expression again increased with time interval. After 4 hours on salt supplemented medium the *luciferase* expression was decreasing with increasing NaCl concentration then an increase in expression took place when measured after 72 and even higher after 96 hours (Fig. 33). This was due to the fact that with time interval transgenic cell clusters might have started to tolerate the salt stress because of the transformed *AtNHX1* gene.

In all of the vectors, which were developed either monocistronic or dicistronic the *bar* gene was used as a selectable marker gene and all of transgenic selections were made under phosphinothricin selection. The findings of this study either transient or stable transformation experiments, indicated that as non transgenic cells were dead when using NaCl in the growth medium therefore selection could be made by using NaCl in the culture medium as a selective agent.

In the first selection approach, transgenic vs wild type cells were selected under 5 mg/l ppt. Upon ppt selection after 4 weeks of growth all of the wild type cells were dead whereas MASnhx1/luc transgenic cells grew very normal. The luciferase expression was observed in transgenic cells but no expression of *luciferase* could be seen in the cells growing on medium containg 5 mg l^{-1} ppt (see Fig. 37). In the second approach when NaCl was supplemented to the medium the cell growth decreased gradually from 50 to 100 mM and most of the cells were dead at 150 mM. Those cell clusters which were growing showed high luciferase activity (see Fig. 37). When selection was made with 5 mg l-1 ppt + NaCl in combination, the luciferase expression was detectable at 50, 100 and even at 150 mM NaCl concentration (see Fig. 39) but at 150 mM it was strongly reduced. These observations show that the expression stability of two genes under different promoters is highly variable. In this case the MAS promoter driven nhx1/luc

cassette is obviously silenced, when at the same time the bar gene is active. The dicistronic approach for functional genomics provides translational control which allows physiological investigations in vivo. In this experiment the correlation of growth and luciferase activity is clearly documented. Where as the growth under ppt is correlated to the transgeneity which is not the case with the *nhx1* gene, respectively to the luciferase activity.

The Southern blot analysis of the suspension culture failed several times when enzmes were used which are cutting only once in the T-DNA but were successful when the whole cassette was excised (see Fig 40). Since the suspension was derived from a T0 plant which may represent already a mixture of independent transformation events, the suspension will also be heterogeneous with respect to the transgenic character of the cells. The heterogenousity of the cell population would then result in a multiple copies like integration pattern in which the separate bands of the entire population might be under the detection level of the Southern blot analysis.

By confirming and proving the functionality of the dicistronic vector system in transient and stable transformed tobacco cells, further transformations were made in *Pisum sativum* L. using it as a leguminous modal as a relevant crop plant was the final target of this study. Since transformation efficiency of pea is very poor (Richter *et al.,* 2006; Polowick *et al.,* 2004; Schroeder *et al.,* 1993), there is a strong demand for a reliable and functional expression system on the basis of translational control in vivo. The presented dicistronic vectors are serving as a versatile tool for overcoming this problem. Further more, according to Halpin, (2005), the coordinated expression of multiple genes is also the key challenge in agro biotechnology. In this study a novel method has been established for the selection of transgenic plant tissues or entire plants starting from the initial phase of transformation. For the ultimate goal of this work, transgenic peas, transformed explants were screened for luciferase expression in the shoot proliferating calli (see Fig. 42). Those shoot proliferating calli showing positive *luciferase* expression were further subcultured and transgenic shoots were recovered from these and grafted on non transgenic rootstocks as shown in Fig. 43. From the transgenic T0 plants growing in greenhouse leaves were taken and luciferase expression was observed by spraying luciferin salt on the upper surface of leaves under a Fuji LAS 3000 imager but no difference could be seen between transgenic and wild type pea leaves because of high

back ground activity (Fig. 44). But when crud protein was extracted from the transgenic pea leaves and RLU values were recorded by Luminometer by using promega luciferase assay reagent kit, significant differences could be seen between transgenic and wild type pea leaves as shown in Fig 47. In parallel, luciferase expressing plants were also confirmed by PCR (Fig. 44).

Not all progenies of these T0 plants expressed luciferase in the subsequent T1 generation and these results were consistent with negative results in PCR. Negative plants were probably due to the chimeric character of the T0 plants, in which the germ line cells probably were not transformed or simply due to the segregation of the transgenes. However from T1 seeds a part of cotyledon was taken as well as from wild type seed and assayed under the luminescence imager. Luciferase expression could be seen in transgenic cotyledon where as no luminescence could be seen in non transgenic cotyledons as shown in (Fig. 45). Further more luciferin was directly injected with the help of syrnige into freshly picked seeds attached inside pods and expression of *luciferase* could be observed in this way also in seeds as shown in Fig. 46. T1 seeds were grown, leaf material was taken and positively tested for *luciferase* activity (Fig. 47) In parallel, confirmation of T-DNA integration was also made by PCR (Figs. 48,49 and50).

In each generation confirmations were made on the basis of luciferase expression. Finally expression of luciferase in the T2 transgenic pea plants was confirmed. In the T2 generation even higher luciferase expression level could be measured. These plants were under severe attack of powdery mildew which might have caused water deficit in the leaves and this stress might be over controlled by *AtNHX1* thus enhanced the expression of luciferasae Molecular analysis of T2 plants were made by PCR. PCR amplified products of the all target genes are shown in Figs. 51, 52 and 53.

CONCLUSIONS

The analysis made in this study in all transient and stable transformed cells and even in entire plants revealed that the sequences within the TMV cp148 IRES can mediate translation of the second ORF of a dicistronic vector construct. Urwin *et al.,* (2000) reported about the EMCV IRES elements that these elements can mediate translation of a second cistron in stable transgenic plants also. Furthermore the author explained that the EMCV IRES elements were active both in animal and plant cells, but in plant cells the activity was at moderate level. Dorokhov *et al.,* (2001) made comparative studies of tobamo IRESs (IRES cp148 and IRES MP 75) and EMCV IRESs and confirmed the results of Urwin *et al.,* (2000) as in one hand the relative efficiency of EMCV IRESs was higher in Hela cells than tobacco cells but also it was reported that the relative activity of TMVcp148 IRES was high in both animal and plant cells.

The basic novelty of this study is that for the first IRES elements were used to transform plants and plants cells with a gene transferring a functional trait linked to a reporter. Thereby it was possible to correlate the functional trait, in this case salt tolerance, in terms of cell growth with the activity of the reporter gene. This basic principle enabled us to discriminate between expressing and none expressing transgenic cells. Previously it was only possible to identify transgenic cells or transgenic cells expressing a reporter gene but not transgenic cells expressing a gene with physiological function. The *luciferase* gene has been chosen as marker gene because of its high sensitivity. Another reason was the ATP dependence of the luminescence reaction which acts as a viability proof at the same time.

Since it was possible to correlate the *AtNHX1* gene expression and the salt tolerance it provides with the activity of the reporter gene in isolated cell clusters. It can be estimated that the vector systems can be used also for any other investigation in functional genomics.

OUTLOOK

In plant cell lines the new vector system may be, either used for the screening and selection of high expressing cells on the basis of marker gene expression or for monitoring expression stability of the first cistron by measuring marker gene expression.

Especially for basic research in field performance of transformation recalcitrant plants, like legumes, the vector system would allow statistics of gene expression data based on relatively small sample sizes, because expression instabilities can be monitored.

The findings of this study show high expression of protein in transgenic cells at 100 mM NaCl selection, which is pointing our attention to the possible use of the *AtNHX1* gene as a selectable marker.

Further more by making modification in the secondary structure of IRES elements may increase the expression ratio of the second cistron in plant expression system.

SUMMARY

A direct translational control for recombinant gene products in homologous or heterologous plant expression system is the major constraint for physiological investigations. Especially in the large seed grain legume family the transformation recalcitrance is drastically limiting the number of independent lines which do not meet the basic requirements for relative expression stability, not to mention the utilization of T0 plant material. In general the coordinated expression of two genes in plants is still a problem but seems to be possible by the IRES mediated approach.

Di-cistronic binary vector constructs based on pGreenII vectors were made which allow a direct expression control on cellular- and plant level. The functionality of the constructs was proven by marker gene constructs containing a *β-glucuronidase* – and firefly *luciferase* gene. In the model dicistronic vector *β-glucuronidase* gene was cloned directly under the control of MAS promoter as a first cistron down stream IRES elements in front of firefly *luciferase* gene as second cistron. The advantage of this approach is, that the cap-dependent expression of physically independent *β -glucuronidase* can be monitored by the cap-independently co-expressed luciferase, which is located on the same mRNA thus expression of both cistrons is linked. This approach also limit the usage of multiple promoter. For the comparative luciferase expression, *luciferase* gene was also cloned behind the promoter (monocistronic).

In the first confirmation approach, the functionality of the vector constructs was proven on the basis of luciferase expression in transient leaf infiltration assay in *N. banthamiana* plants. Among three different IRES elements (polio IRES, tobamo IRES cp148 and putative Zea mays IRES) used as intercistronic spacers in dicistronic vector system, significantly higher luciferese expression could be seen only when there were tobamo IRES elements TMVcp148IRES. To prove that the expression of luciferase being a second cistron was due to TMV cp148 IRES elements, the IRES elements were cloned in opposite orientation without disturbing the rest of the architecture of dicstronic vector system. Significantly reduced luciferase expression could be seen in that case which confirmed the functionality of TMV cp148 IRES elements. The relative efficiency of *gus* gene expression was also examined in all dicistronic constructs containing TMVcp148 IRES elements in sense and anti sense orientation in transient studies.

A sodium antiporter (*AtNHX1*) from *A. thaliana* for the improvement of salinity tolerance in plants has been used as a proof of principle for the usage of dicistronic vectors for the monitoring the translation. The performance of IRES elements, in which absolute transcriptional linkage of two genes on one mRNA is combined with the translational independence of the genes, resulting in two separate proteins but driven by the same promoter. In contrast to fusion proteins, in which fused marker genes might have effects on the functionality of the genes of interest, the luciferase will not have a conformational effect on the *AtNHX1* protein.

By confirming the functionality of *AtNHX1* in transgenic tobacco cells *AtNHX1* cDNA was synthesized and subcloned into dicistronic vector construct 0229MASguscp148luc. Comparative tobacco leaf infiltration experiments were performed with 0229MASgus/luc (model system) and 0229MASnhx1/luc to proof the functionality of vectors. Observations were made on basis of luciferase expression. Higher luciferase expression was observed when the luciferase gene was in combination with the *AtNHX1* gene. The functionality of dicistronic vectors along with *AtNHX1* gene was confirmed in stably transformed tobacco plants as a model system before moving to legumes and other crop plants, as it takes much more time to transform in target plant (pea). Methodologically the mode of action of recombinant nhx1 gene can only be investigated when positive effects can be expected. Hence the major interest is the development of a reliable monitoring system for expression studies. From the transgenic T1 tobacco plants calli were derived and expression of glucuronidase in MASgus/luc was quantified Mug assay where as luciferase expression was observed in MASgus/luc and MASnhx1/luc by using Promega luciferase assay kit in Lumat luminometer.

Further studies were made with MASnhx1/luc transgenic calli on the basis of luciferase expression Investigations were made under salt challenge ranging from 0 to 150 mM. Comparative studies with salt challenged, transgenic in vitro cultured tobacco cells showed improved salt tolerance, based on the effect of an over expressed *AtNHXI* gene. Luciferase expression (LAU) was quantified in the cells growing at various NaCl selection levels by the Fuji imager LAS 3000. With the increase in NaCl concentration, gradual increase in luciferase expression (LAU/mm²) was recorded and maximum at 100 mM NaCl challenge and then decline in expression took place with the increase in NaCl level. By confirming the functionality of the dicistronic vector system in transient and

stably transformed tobacco cells, functionality of IRES elements was also confirmed in entire plant. *Agrobacterium* mediated transformation was made with pea *(Pisum sativum* L.) as a legume model. The *Agrobacterium* mediated transformation system according to the modified protocol of Schroeder *et al*., 1993 and Bean *et al*., 1997 has been used. All of the non transgenic and wild type embryos started to die after the second round of selection. Luciferase expression was observed from the shoot proliferating transgenic pea calli. Regenerating shoots from the luciferase exprssiong callis were further subcultured with increased ppt concentrations (7.5 mg/l) and used for in vitro grafting on a wild type root stock. From the T0 plants, freshly picked seeds (T1) were used for life luciferase assays. In the preliminary approach for investigation of transgenic seeds, small pieces of transgenic and wild type cotyledons were cut and luciferin was applied. Observation of luciferase expression was made under the Fuji imager. Luciferase expression in transgenic cotyledons could be seen. On the basis of these observations luciferin was directly applied to the freshly picked pea pods containing seeds. Pods were opened and luciferin was injected in the seeds. Interestingly luciferase expression could be observed in freshly picked pea seeds. Transgenic T0, T1 and T2 pea plants confirmed by PCR showed luciferase activity, as a first indicator for the *AtNHX I* expression in pea.

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APPENDIX –I

L.B Broth for *E. coli* (High salt)

Tryptone	10 g/l
NaCl	5 g/l
Yeast extract	5 g/l

pH = 7.5

APPENDIX-II

L.B Broth for *Agrobacterium* (Low salt)

Tryptone	10 g/l
NaCl	5 g/l
Yeast extract	5 g/l

pH = 7.5

APPENDIX-III

MMA medium for leaf infiltration

MS salt	4.6 g /l
Sucrose	20 g
NAA	100 mM
MES	1.95 g/l (dissolved in 7 ml H_2O add drop wise KOH to increase pH to 6.3)
pH	5.6
Acetosyrinigone	100 µM (post autoclave)

APPENDIX-IV

Callus induction medium (4X)

B5 basal micro- and macro salts + vitamins	3.16 g/l
Sucrose	20 g/l
Nz-Amin (Caseinhydrolysat)	2 g/l
2,4 D	2 mg/l
NAA	0.5 mg/l
IAA	0.5 mg/l
Kinetin	0.2 mg/l
pH	5.6
Plant Agar (for solid medium)	0.8 %

Media for Tobacco Transformation

APPENDIX-V

MS liquid

MS-salt + Vitamin (Duchefa)	4.4 g/l
MES	0.25 g/l
pH	5.6 – 5.8

APPENDIX VI

MS-1

MS-salt + Vitamin (Duchefa)	4.4 g/l
MES	0.25 g/l
Sucrose	20 g/l
NAA	0.5 mg/l
BAP	1.0 mg/l
pH	5.6 – 5.8 with 1N KOH
Plant Agar	8.5 g/l
Tic	as required (post autoclave)

APPENDIX – VII

MS-2

MS-salt + Vitamin (Duchefa)	4.4 g/l
MES	0.25 g/l
Sucrose	20 g/l
BAP	0.2 mg/l
pH	5.6 – 5.8 with 1N KOH
Plant Agar	8.5 g/l
Tic	as required (post autoclave)
ppt	as required (post autoclave)

APPNDIX – VIII

MS 0 Medium

MS-salt + Vitamin (Duchefa)	4.4 g/l
MES	0.25 g/l
Sucrose	20 g/l
pH	5.6 – 5.8 with 1N KOH
Plant Agar	8.5 g/l
Tic	as required (post autoclave)
ppt	as required (post autoclave)

Media for Pea Transformation

APPENDIX – IX

B5-i re-suspension medium

B5 basal micro and macro salts	
(Gamborg *et al*., 1968)	3.16 g/l
Glucose	10 g/l
Sucrose	10 g/l
MES	2 g/l
pH	5.6 with 1N KOH/1N HCl

APPENDIX – X

B5hT Co-cultivation medium

B5 basal micro- and macro salts +	
B5 vitamin mixture	3.16 g/l
Sucrose	30 g/l
$CaCl_2$, $2H_2O$	0.88 g/l
KNO_3	0.5 g/l
$MgSO_4$, $7H_2O$	0.5 g/l
Glutamine	0.8 g/l
Glutathione	10 mg/l
Adenine	1 mg/l
Kinetin (1 µM)	0.2 mg/l
MES	2.0 g/l
TDZ (5 µM)	1.1 mg/l
pH	5.6
GelRite	4.5 g/l

APENDIX – XI

MST Regeneration medium

MS macro- and micro salt's (Murashige and Skoog, 1962) + B5 vitamin mixture	3.16 g/l
Sucrose	30 g/l
MES	1 g/l
TDZ (5 µM)	1.1 mg/l
NAA (0.01 µM)	0.002 mg/l
pH	5.8
Plant Agar	7.5 g/l (Post autoclaving)
Ticarcillin	100 mg/l (Post autoclaving)
Combactam	100 mg/l (Post autoclaving)

APPENDIX – XII

P2 selection medium

MS basic micro- and macro salts +	
B5 vitamin mixture	3.16 g/l
Sucrose	30 g/l
MES	1 g/l
BAP (14.58 µM)	4.5 mg/l
NAA (0.1 µM)	0.02 mg/l
pH	5.8
Plant Agar	7.5 g/l
Ticarcillin	100 mg/l (post autoclaving)
Combactam	100 mg/l(post autoclaving)
ppt	2.5 mg/l (post autoclaving)

STATEMENT OF SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

_____ _____

Signature *Date*

CURRICULUM VITAE

Zahid Ali
(Email zahi06@gmail.com)

KEY SKILLS

- Extensive scientific and technical knowledge of genetics/genomics, plant biology, biotechnology, agriculture, and molecular biology
- Outstanding communication skills: verbal, written and presentation
- Excellent negotiation and rhetorical skills with demonstrated ability to influence others and work towards consensus.
- A good potential and cooperative behaviuor with the working team memebers
- Fluent in Urdu, English and also Deutsch
- Well conversant with computer applications like Microsoft Word, WordPerfect, Slide Write and PowerPoint, Internet Skills, Blast search & analysis.

Training and technical expertise

Extensive experience of working on Molecular, Microbiological & Biochemical techniques including,

- Gene Cloning: Vector Construction, Gel Elution, Ligation & Transformation
- Vector NTI
- Plant Transformation *Agrbacterium*/ biolistic (chickpea/pea/potato/ Tobacco)
- Chickpea/pea/potato/ Tobacco tissue culture.
- Establishment and maintenance of plant suspension cell cultures
- Cell suspension transformation.
- Bacterial chromosomal DNA & cccDNA Isolation & Purification.
- Plant DNA/RNA Isolation & Purification
- Genomic DNA isolation from blood & tissues
- Polymerase Chain Reaction, RT PCR
- cDNA construction
- Agarose and Acrylamide gel electrophoresis for DNA and protein
- DNA and protein quantification
- Gene sequencing and BLAST analysis
- Isolation, identification and maintenance of microbial cultures.
- HPLC / G.C
- Fuji LAS 3000 imager.
- Molecular analysis of transgenic plants.(Southern and Western blot)
- Isolation, Identification and characterization of PGPRs/Rhizobia

- Making investigation studies for plant microbe interactions

EDUCATIONAL RECORD

2003-2007, Ph.D. Natural Sciences (Biotechnology) Leibniz Universität Hannover **Germany.**

Dissertation Title: Developing Strategies for Homologous/Heterologous Plant Expression System for Physiological Investigations of Respective Target Proteins.

2001-2002, M.Sc (Hons) Agronomy obtained **CGPA, 3.76/4.00**.from University of Agriculture, Faisalabad, Pakistan.

Dissertation Title: Characterization of Plant Growth Promoting Rhizobacteria (PGPR) and their affect on seed germination, seedling vigor and growth of cotton.

1996-2000, B.Sc (Hons) Agriculture obtained **CGPA, 3.53/4.00** from University of Agriculture, Faisalabad, Pakistan.

1996, F.Sc. (Phys., Chem., Bio.) obtained 796/1100 marks, **1st div**. from Govt. College Faisalabad, Pakistan.

1993, Matric (Phys., Chem., Bio, Maths.) obtained 710/850 marks, **1st div**. from Govt. H/S/S Dijkot Faisalabad, Pakistan.

Personal details

Date of Birth	10-12 -1976
Domicile	Faisalabad (Punjab) Pakistan
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Permanent Address: H/No P-79/A Satellite town Samanabad Faisalabad, Pakistan.

►Awards

DAAD PhD fellowship

Merit scholarship in matriculation

List of Publications /Presentations

►Dicistronic binary vectors system - a versatile tool for plant gene expression studies.

Zahid ALI, Heinz Martin SHUMACHER, Hans-Jörg JACOBSEN, Heiko KIESECKER. (Invited paper, Provisionally accepted in Plant physiology)

Online ISSN: 1532-2548 Print ISSN: 0032-0889

►Novel Transformation Vectors for Plant Cell Cultures

Zahid Ali, Elke-Dobbernack Heinz Martin Schumacher, Heiko Kiesecker

ICCC 11 Conference 07. 10.-11. 10. 2007 Goslar, Germany Poster presentation.

►Developing strategies for homologous/heterologous plant expression system for physiological investigations of respective target proteins

Zahid Ali (key note speaker)

International Summer School on Plant Molecular Biology. Gottfried Wilhel Leibniz University Hannover Germany (01-07-2007 to 13-07-2007).

►Di-cistronic Binary Vectors as a Versatile Tool for Gene Expression Studies.

Z. Ali, H.-M. Schumacher, H.- J. Jacobsen, F.-Y. Hafeez, H. Kiesecker.

International conference, Plant Transfromation Technnologies Vienna Austria 4-7 February 2007 Poster presentation

►Process optimization for the Production of of Recombinant Proteins in Suspension Cultures

Heidinger B., Ali,Z., Singh M., Schumacher M., Jacobsen H.J., Kiesecker H

International conference, Plant Transfromation Technnologies Vienna Austria 4-7 February 2007 Poster presentation

►Di-cistronic Binary Vectors as a Versatile Tool for Gene Expression Studies
-potential of *AtNHX1* in pulse crops –
Zahid Ali, Fauzia Y.Hafeez, Yusuf Zafar and Heiko Kiesecker.
Microbial Technologies for Sustainable Agriculture An International Symposium held in NIBGE Pakistan March 12-16 2007 Oral presentation.

►Characterization of PGPRs and their affect on seedling vigor and growth of cotton.

F.Y.Hafeez. Z.Ali. M.E Safdar, and K.A. Malik

9th International congress for Agriculture and Biology March 2002 NIAB Pakistan. Poster presentation

References

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