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# Characterization of a T-DNA insertion mutant for NaCl-hypersensitivity in *Arabidopsis thaliana*

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**Abstract:** Salinity is a major abiotic stress which reduces crop productivity. Determination of the molecular components involved in salinity stress signaling and understanding the mechanisms of adaptation to salinity is essential for genetic improvement of crop plants. Forward genetics, using the model plant *Arabidopsis thaliana*, has been and will continue to be a powerful method to identify the determinants of salt stress adaptation. Several families of T-DNA insertional mutagenesis of *Arabidopsis* was generated by *Agrobacterium*-mediated transformation. Genetic characterization of a subset of the transformants indicates that they have in average 1.35 inserts each, as assayed by kanamycin resistance segregation. These lines have been screened under varying growth conditions for the monitoring of visible alterations (phenotype). Several putative mutants were observed, including a NaCl-hypersensitive mutant (mut-46). This mutant exhibited a salt-sensitive phenotype manifested as reduction in primary root growth, and exhibited reduced length as well as density of root hairs in the presence of NaCl. However, the phenotype of the non-stress mut-46 root hairs is similar as the WT under salt stress. The fresh weight of wild-type and mut-46 seedlings was reduced by exposure to NaCl. Western blot analysis indicated that salinity reduced the expression of actin proteins in both wild-type and mut-46 compared with control *Arabidopsis* seedlings.

**Key words:** T-DNA insertion mutant, *Arabidopsis thaliana*, salinity

## INTRODUCTION

Salinization is a widespread agricultural problem affecting 20% of the world's irrigated croplands, and many other regions of the earth designated as arid and desert lands. Salinization of croplands will present challenges for the future given the predictions of climate change, population growth and the greater demand for more intensive agriculture (Liu *et al*., 2007). Soil salinity is a major abiotic stress that reduces plant growth and ranks among the leading factors limiting agricultural productivity. $Na^+$ is not essential for plant growth, and under salt stress, it hinders uptake of the important mineral nutrient $K^+$ and competes for its enzyme binding sites. (Quan *et al*., 2007).

Insertional mutagenesis approaches by using *Agrobacterium* T-DNA, play important roles in plant functional genomics. The use of T-DNA as an insertional mutagen has several advantages. T-DNA integration results in stable mutations in the genome. Also, the low number of insertions per transformant significantly reduces the additional work required to remove undesired mutations. The development of a simple *in planta Agrobacterium* transformation method for *Arabidopsis thaliana* allowed the high-throughput production of T-DNA insertion mutants in this model plant (Li *et al*., 2007).

Much progress has been made in recent years in understanding the primary signaling pathway in plants responding to salt stress. A forward genetic screen for mutations that cause NaCl hypersensitivity of *Arabidopsis* seedlings led to the identification of the first major components of the Salt-Overly-*S*ensitive (SOS) signalling pathway, SOS1, SOS2, and SOS3. The signaling pathway involves a salt-elicited $Ca^{2+}$ signal, which is sensed by SOS3 and activates SOS2, a serine/threonine protein kinase. Together SOS2 and SOS3 regulate the activity and expression level of SOS1, a plasma membrane $Na^+/H^+$ antiporter. SOS2 and possibly other $Ca^{2+}$-activated protein kinases initiate a protein phosphorylation cascade channeled downstream through mitogen-activated protein (MAP) kinases (Quan *et al*., 2007).

In Arabidopsis, Rigas *et al*. (2001) generated plants homozygous for a complete loss-of-function *tiny root hair 1* (*trh1*) mutation by means of the T-DNA–tagging method. Where, trichoblasts of the *trh1* plants form

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initiation sites but fail to undergo tip growth. *TRH1* mediates $K^+$ transport in *Arabidopsis* roots and is responsible for specific $K^+$ translocation, which is essential for root hair elongation. When Yamamoto *et al.* (2005) grow wild-type and mutant *Arabidopsis*, in which the T-DNA was inserted into ascorbate oxidase gene, plants under normal conditions, both plants germinated with similar rates. However, when the growth medium contained 0.1 M NaCl, the root length of the T-DNA mutant was longer than that of the wild-type plants. Zhu *et al.* (2007) isolated and characterized a T-DNA insertional mutant called *enh1-1* that enhances the salt sensitivity of *sos3-1* (myristoylated calcium sensor) and also causes increased salt sensitivity by itself. Liu *et al.* (2007) described T-DNA insertion mutations in AtS1P and AtbZIP17 that blocked upregulation of these genes by salt stress. Salt stress induces a signaling cascade involving the cleavage of AtbZIP17 by AtS1P, the translocation of the N-terminal fragment of AtbZIP17 to the nucleus and the up-regulation expression of several salt stress response genes by AtbZIP17.

Recent genome wide analyses of assayed gene expression showed that 5–30% of the assayed gene expression was modulated by abiotic stress. Exposure of rice and sorghum seedlings to high salinity (150 mM NaCl) modified the expression of genes encoding components of the cytoskeleton (e.g., actin); (Yan *et al.*, 2005; Buchanan *et al.*, 2005). Scherer (2006) used cytochalasin D to test the involvement of actin on carrot callus growth in the presence of 50 mM NaCl. Halotolerance was enhanced by the influence of cytochalasin D, as indicated by increasing the growth of callus.

In this study, T-DNA insertion mutagenesis was used to produce a large number of independent T-DNA insertion mutants in *Arabidopsis* plants. We also present the characterization of an insertionally induced mutant with salt-sensitive phenotype. The effects of salt stress on root phenotype, fresh weight and actin expression of this mutant were examined.

## MATERIALS AND METHODS

### *T-DNA mutagenesis and isolation of the mut-46 mutant:*

A collection of *Arabidopsis* T-DNA mutants were generated according to Weigel and Glazebrook, (2002). To generate T-DNA insertional mutagenesis, more than 20 treated *Arabidopsis thaliana* plants ecotype Columbia were transformed by the vacuum-infiltration procedure using the *Agrobacterium* strain LBA4404 carrying the pBIN19 vector. Infiltrated plants were allowed to set seed, and the seeds were collected. These seeds are referred to as the T1 generation. The harvested T1 seeds were surface sterilized by immersion in 70% (v/v) ethanol for 2 min, followed by immersion in 10% (v/v) laundry bleach (Na+ hypochlorite) solution for 5 min. Seeds were then washed four times with sterile distilled water and sown onto 1% agar containing MS medium. Selection of plants having T-DNA insertions was performed on 100 µg/ml kanamycin-containing medium. Seeds were then stratified for 2 d in the dark at 4°C. After stratification seeds were incubated at 22°C in a 16 h light, 8 h dark regime. The kanamycin-resistant T1 plants from each treatment were planted in soil to produce T2 seeds.

Seeds from T2 plants were screened for a mutant that exhibited increased sensitivity to NaCl. Sowings were performed by plating mutant and wild-type seeds on MS agar medium supplemented with 80 mM NaCl or non-supplemented. Individual seeds were sown along a level horizontal line onto the surface of the media. Plates were oriented vertically with the seed line parallel to the shelf surface in the growth chamber. Salt sensitivity was determined based on the growth of primary root.

### *Growth Measurements:*

For primary root length measurement, wild-type and NaCl-hypersensitive mutant (mut-46) seeds were germinated on MS agar plates supplemented with 0 or 80 mM NaCl. The plates were placed vertically with seedlings in the upright position. During the entire period of treatment, the Petri dishes were angled at 30° to encourage $^{root}$ growth towards the bottom of the media. Three replicates were run for each treatment. Increases in primary root length were measured with a ruler after 3, 6 and 10 days (Kandasamy *et al.*, 2005).

Roots of 10 days old seedlings were placed on a glass microscope slide under a coverslip. The number of root hairs in a 1-mm-region length at the midpoint of a root was counted under a light microscope. From the midpoint of this 1-mm region, the length of 10 root hairs from each root was measured (Rahman *et al.*, 2002).

Fresh weight of 10 days old seedlings was determined immediately after harvesting (Quesada *et al.*, 2000).

### *Western Blot Analysis:*

Western blot analysis was performed using a general plant-actin–specific antibody as described previously (Mabrouk, 2007). Quantification of actin protein bands was performed by the TopSpot evaluation program (Algorithmus, Berlin, Germany).

## RESULTS AND DISCUSSION

### *Production of Arabidopsis Insertion Mutant Collection:*

To determine the average number of functional inserts, T2 families from the treatments were tested on kanamycin-containing medium. The average number of independent insertion loci was estimated by the determination of the segregation ratio of kanamycin-resistant to kanamycin-sensitive plants in 100 randomly selected T2 families (Table 1).

**Table 1:** Segregation analysis of the T-DNA encoded kanamycin-resistance marker in 100 randomly chosen T2 families

| $Kan^R$ : $Kan^S$ | Number of lines |
|---|---|
| 3 : 1 | 62 |
| 15 : 1 | 23 |
| 63 : 1 | 4 |
| < 3 : 1 | 11 |

$\chi^2$ analysis for each class indicated no significant deviation from the expected ratio at a level of $P$=0.05. At least a total of 300 T2 seedlings were scored in each family.

The majority of T2 families (62%) segregated the T-DNA-encoded kanamycin-resistance marker at a ratio of 3: 1, whereas 15: 1 and 63: 1 segregation ratios were observed in 23% and 4% of examined families, respectively. Eleven per cent of T2 families segregated for the KanR marker in an exceptional manner (< 3 : 1). Excluding these later lines, the average number of inserted loci was estimated as 1.35 per plant.

### *Mutant Identification:*

All of the families segregated for the T-DNA marker gene, neomycin phosphotransferase II (NPT II), as shown by segregation for resistance to kanamycin. The segregated families were screened for visible alterations in phenotype by growing them in soil and on agar-solidified medium to identify mutations. A number of the families were also segregating for a variety of altered phenotypes including embryo and seedling lethality, size variant, reduced-fertility and pigment were observed.

A NaCl-hypersensitive mutant (mut-46) was identified during an initial screen of T2 seedlings from the T-DNA insertion lines. The mut-46 mutant was identified as conferring increased sensitivity to 80 mM NaCl. To further analyze this NaCl-hypersensitive mutant, 156 seeds of mut-46 line were examined for the NaCl-hypersensitivity. Within this population, 125 had normal root length and 31 had short root length ($\chi^2$ (3 : 1) = 2.19, P > 0.05). Thus, the trait appears to be determined by a single recessive allele. To ascertain the number of T-DNA inserts in the family, the ratio of kanamycin-resistant to kanamycin-sensitive ($kan^r$ to $kan^s$) members was calculated. In a sample of 259 plants there were 183 $kan^r$ to 76 $kan^s$ individuals ($\chi^2$ (3 : 1) = 2.48, P > 0.05), which indicates the presence of one T-DNA insert. The salt-sensitive mutant was confirmed based on genetic inheritance of the phenotype in the T3 and further generations.

### *Characterization of Salt-sensitive Mutant:*

The mut-46 seedlings were compared with wild-type (WT) plants under the inclusion or exclusion of NaCl in the medium. Root length was measured in 3-, 6- and 10d-old plants to compare plant growth in NaCl treatments. Root growth clearly showed the differences between standard media and NaCl treatment (Figs. 1A and 1B).

When the wild-type and knock-down line of *Arabidopsis* plants were grown under normal conditions, both plants germinated with similar rates. However, when the growth medium contained 80 mM NaCl, the growth of the primary root for the T-DNA mutant was reduced compared to the wild-type plants as shown in Fig. 1C. The mut-46 primary roots averaged 33.9% the length of those on the wild-type *Arabidopsis* seedlings when grown on MS medium supplemented with NaCl for 10 days (Fig. 1C). The primary root length of mut-46 was found to be considerably shorter than that of wild-type on standard media at 10 d, but slightly longer than the NaCl-treated wild-type plants. A 3- and 2-fold decrease in primary root length of mut-46 was also observed (Fig. 1C) by NaCl application at 6 and 10 d, respectively. These results collectively indicate that mut-46 plays a role in salt response. Images of a root hair treated with 80 mM NaCl for 10 d and an untreated $^{root}$ hair are shown in Fig. 2A-D.

Root hair length and density were greater in wild-type plants than mut-46 plants on standard media. The percentage of root hair length in untreated mut-46 mutant was approximately 62.7%, less compared with the wild-type (Fig. 2E). NaCl decreased root hair length and density of the wild-type plants to approximately 29 and 44.5%, respectively (Figs. 2E and F). In contrast, NaCl treatment failed to induce any change in mut-46 root hair phenotype. The root hairs of wild-type plants treated with NaCl resembled the untreated mut-46 root

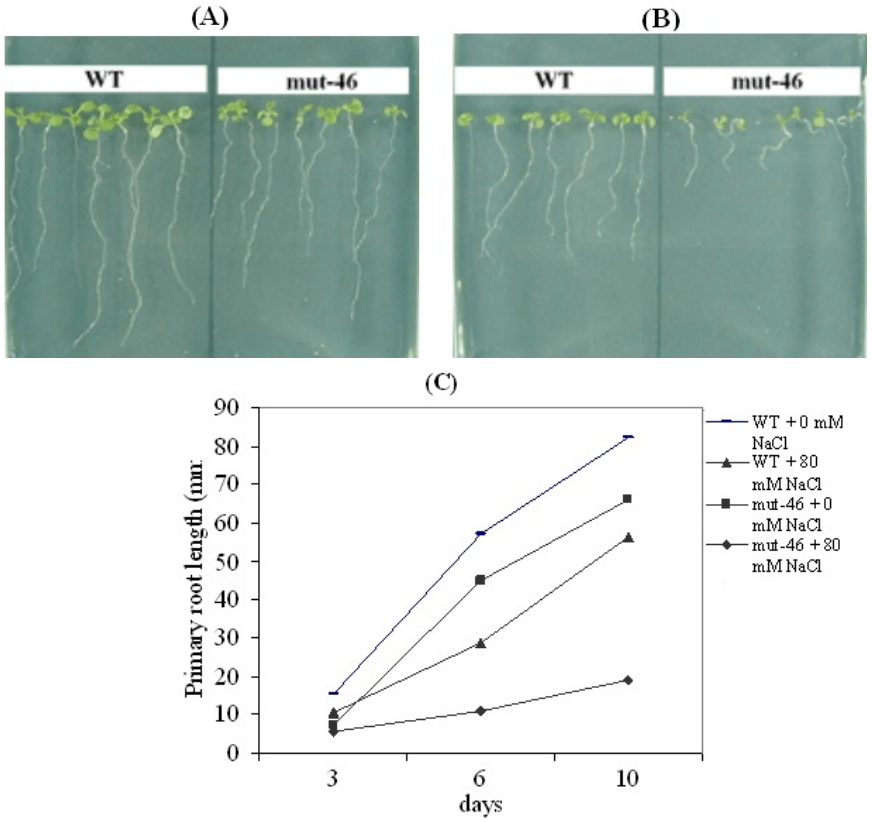


**Fig. 1:** Primary root length of 10-d-old wild-type (WT) and mut-46 plants grown (A) without or (B) with 80 mM NaCl. (C) graph representing the average of primary root growth data of wild-type and mut-46 *Arabidopsis* seedlings grown for 3 d, 6 d or 10 d in media containing 0 or 80 mM NaCl ($n$ =18).

hairs in length and number (Figs. 2E and F). These results collectively suggest that mut-46 copied the NaCl-treated wild-type root hair phenotype by decreasing the length and density of hair roots. The length and number of the mut-46 root hairs is not altered under either salt stress or non-stress conditions (Figs. 2E and F). Application of 80 mM NaCl dramatically changed the root hair phenotype of wild-type without altering that of mut-46. Application of NaCl, which has been shown to have almost no effect on root hair growth of mut-46 (Fig. 2), dramatically changed primary root elongation (Fig. 1).

To confirm that mut-46 plays a critical role in the process of salt response in Arabidopsis, we analyzed the fresh weight of mut-46 mutant in response to salinity. Figure (3) shows the fresh weight of wild-type and mut-46 knockout plants grown on NaCl-containing Murashige and Skoog (MS) agar plates without (control) and with 80 mM NaCl for 10 days. The mut-46 mutant gained much less fresh weight during NaCl treatment when compared to wild-type plants. The primary root growth was limited, as further deduced from the reduction in fresh weight. Both fresh weight and primary root length in NaCl-containing media were more severely reduced in mut-46 knockout plants than in wild-type plants; the fresh weight of mut-46 was reduced to 61% of wild type, and primary root length was decreased to 34% of wild type (Figs. 1C and 3). However, in the control medium without the addition of NaCl, wild-type plants had 1.4-fold higher fresh weight and 1.2-fold higher primary root length than mut-46 plants (Figs. 1C and 3). In contrast, mut-46 plants on standard media did not display any differences in root hair length when compared with NaCl-containing media (Fig. 2E). On 80 mM NaCl medium, the overall fresh weight of mut-46 was reduced relative to the wild type, but the number of root hair was reduced only 13% (Figs. 2F and 3).

***Western Blot Analysis of Actin Level:***

T-DNA insertion mutagenesis strategy was tested for its applicability to analyze actin expression and to gain knowledge on the interconnections of actin and NaCl in Arabidopsis. To characterize actin expression in wild-type and mut-46 mutant, experiments were performed in which Arabidopsis was grown for 10 days under

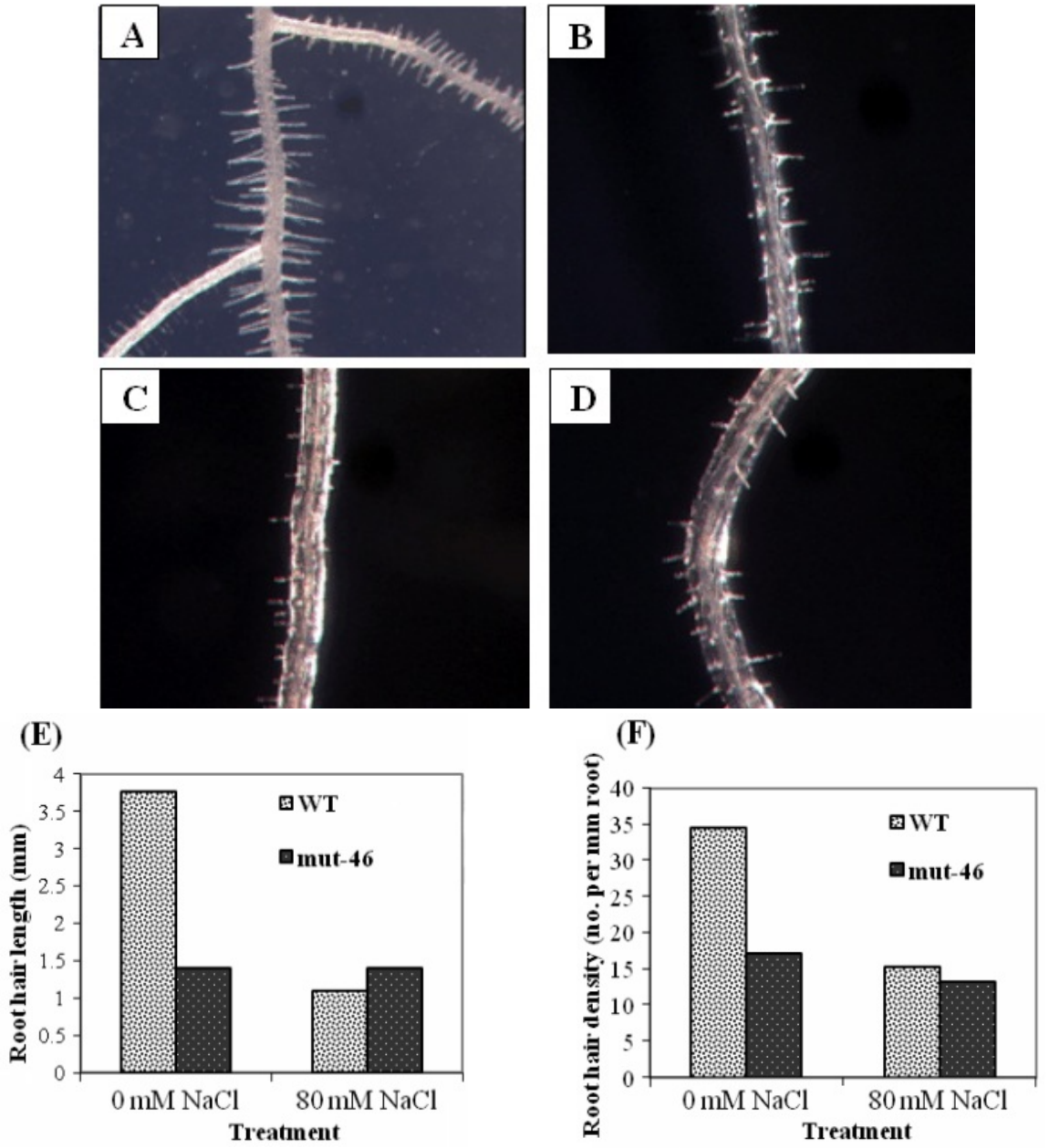


**Fig. 2:** Root hair phenotypes of 10-d-old Arabidopsis seedlings. The wild-type seedlings (A) displayed elevated root hair length and density than those of mut-46 (B), when grown on MS medium without NaCl. When the same medium is supplemented with 80 mM NaCl, the wild-type seedlings (C) display short root hair length and density, resembled the phenotype of mut-46 (D) root hairs. Root hair length (E) and density (F) of WT and mut-46 Arabidopsis plants grown on 0 or 80 mM NaCl for 10 d. Values shown are means of ten observations.

salinity stress. The corresponding proteins were quantified using Western blot, after supplying MS medium with 80 mM NaCl. Figure 4 shows the results obtained when Arabidopsis was exposed to 10 days to NaCl at 80 mM NaCl for 10 days. The amount of actin protein was quantified by TopSpot and the results are shown graphically in Fig. 4B.

The resulting actin band corresponding to the treated plants was less intense than the band corresponding to the control plant. NaCl decreased actin expression in wild-type and mut-46 by 52.07% and 39.20%,

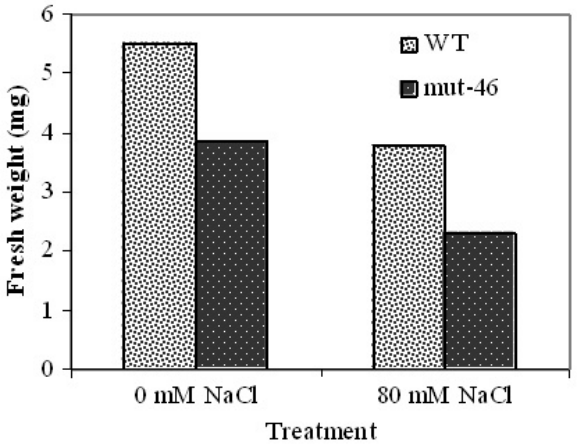


**Fig. 3:** Fresh weight of wild-type and mut-46 knockout plants grow in media without and with 80 mM NaCl for 10 d. Values are means; $n$ = 8.

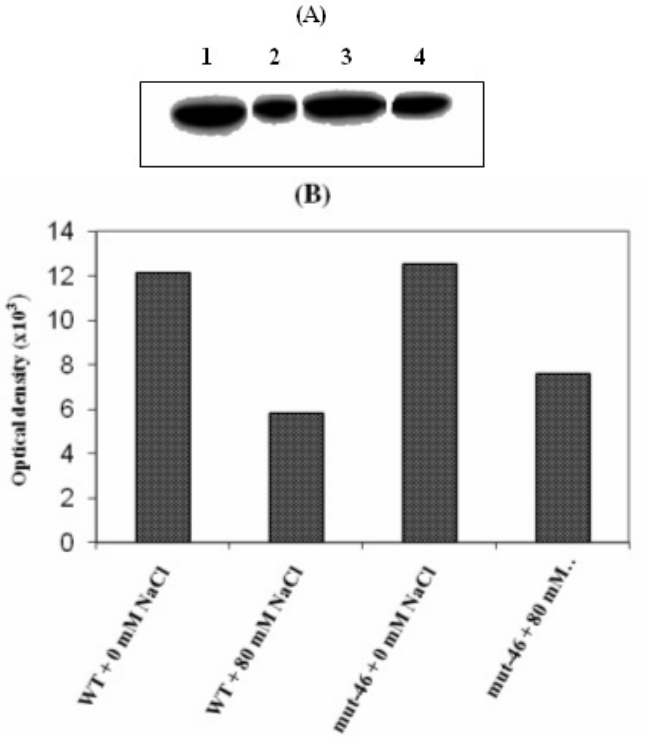


**Fig. 4:** (A) Western blot analysis of actin protein expression in 10-d-old Arabidopsis seedlings of wild-type and mut-46 plants mediated by 0 or 80 mM NaCl. (B) Quantification of actin expression using the TopSpot software on 1- WT + 0 mM NaCl, 2- WT + 80 mM NaCL, 3- mut-46 + 0 mM NaCl and 4- mut-46 + 80 mM NaCl.

respectively, relative to the untreated control. The down-regulation effect of actin expression was detected when we quantified the protein after 10 days under NaCl treatments. However, this effect was seen with Western blot for both wild-type and mutant Arabidopsis plants.

***Discussion:***

We established a collection of T-DNA insertion mutants defective in genes with essential functions during plant growth and development. Such knockouts will be of great importance to determine the biological function of *Arabidopsis* genes. The average number of T-DNA inserts per transformed line in this study was 1.35. This is similar to the values observed for other *Arabidopsis* T-DNA insertion mutant collections (1.29; Rios *et al*., 2002). The analysis presented by Feldmann and Marks (1987) showed that 56, 26, 5, and

1 %, of the tested families, have 1, 2, 3, and 4 inserts, respectively. Ten per cent of the lines segregated for the KanR marker in an exceptional manner (<3:1). The average number of inserts among the tested transformants was 1.4.

Lee *et al*. (2004) presented evidence for the role of *AtMRP5* (ABC transporter) in the salt stress response of *Arabidopsis*. The function of *AtMRP5* was analyzed by isolating a plant with a disruption in this gene caused by T-DNA insertion. Root growth of atmrp5-2 was substantially inhibited with 100 mM NaCl in contrast to the almost normal growth of wild-type seedlings. Mitsuya *et al*. (2005) investigated whether the lack of *RCI2A* gene causes a salt sensitive phenotype in *Arabidopsis*. Two T-DNA insertional mutants of RCI2A were identified. Although the growth of *RCI2A* mutants was comparable with that of wild type under normal conditions, high NaCl treatment caused more reduction of the growth of roots and shoots of *RCI2A* mutants than that of wild type. Furthermore, when wild-type and rci2a plants were treated with NaCl, NaNO3, $Na_2SO_4$, KCl, $KNO_3$, $K_2SO_4$ or LiCl, the *RCI2A* mutants showed more reduction of shoot growth than wild type. These results suggested that *RCI2A* contributes to salt tolerance. Manabe *et al*. (2008) found that loss-of-function mutations rag1-1 (root attenuated growth1-1) and rag1-2, in the locus encoding KAPP, cause NaCl hypersensitivity in *Arabidopsis thaliana*. The NaCl hypersensitive phenotype exhibited by rag1 seedlings includes reduced shoot and primary root growth. The phenotype exhibited by rag1-1 seedlings is associated with a specific response to Na(+) toxicity. This observation is consistent with the findings described herein, where under the conditions of salinity; primary root elongation of mut-46 was inhibited. The genetic evidence presented here is consistent with the hypothesis that a T-DNA insert has interrupted a gene required for salt tolerance.

In the present study, mut-46 mutant exhibited a similar root hair phenotype to the NaCl-treated wild-type, i.e. the root hair length and density of mut-46 were reduced. Shi and Zhu (2002) reported that plants deficient in *sos4* gene, which codes for pyridoxal kinase, are hypersensitive to $Na^+$ ions. Interestingly, this mutant also blocks the initiation of root hairs and impairs the tip growth of existing $^{hair}$s. Halperin *et al*. (2003) assessed the effects of salinity (NaCl) stress on growth of root hairs of *Arabidopsis thaliana*. Exposure to increasing NaCl concentrations, up to 90 mM, for 2 days or 6 days reduced hair extension. Root hair development is controlled by environmental signals. Wang *et al*. (2008) showed that root epidermal cell types and root hair development are highly regulated by salt stress. Root hair length and density decreased significantly in a dose-dependent manner on both primary roots and junction sites between roots and shoots. The root hair growth and development were sensitive to inhibition by ions but not to osmotic stress.

Length and fresh weight of the soybean hypocotyl and root were reduced under salt stress (Aghaei *et al*., 2008).

To examine the effects of salinity more thoroughly on *Arabidopsis* plants, actin expression was assessed by Western blot. Compared with the control, the actin protein level was decreased in wild-type and mut-46 plants in the presence of NaCl. These observations are consistent with previously reported results. Jiang *et al*. (2007) found in Arabidopsis that one actin protein (ACT8) and one tubulin β-chain decreased, while tubulin α-6 chain was induced, in response to 150 mM NaCl treatment. In addition, Nicot *et al*. (2005) reported that the level of actin expression in potato during salt stress (100 mM NaCl) was low after 14 days compared to the control. Our results showed that exposure of Arabidopsis plants to 80 mM NaCl cause rapid inhibition of primary root growth and reduction of fresh weight. Therefore it is not surprising that exposure of Arabidopsis seedlings to salinity reduced the expression of the cytoskeleton genes encoding actin.

The availability of DNA from the region interrupted by the insert will permit the eventual cloning and sequencing of this interesting gene. Understanding the molecular mechanism of the alterations in this mutant may provide valuable information on the kinds of mechanisms involved in the control of plant salt stress response.

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