

**WRKY and Na<sup>+</sup>/H<sup>+</sup> antiporter genes conferring tolerance to salinity in interspecific derivatives of peanut (*Arachis hypogaea* L.)**

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**Abstract**

Peanut is an important edible oilseed crop in the world. Salinity is one of the important abiotic stresses affecting peanut productivity by hampering germination, arresting vegetative and reproductive growth and affecting seed quality. Ten lines developed through introgressive hybridization along with 23 popular varieties were screened *in vitro* for their germinability under high salt concentration (250mM NaCl) in test tube. Interspecific derivatives NRCGCS-296 (J11 x *A. duranensis*) and NRCGCS-241 (GG 2 x *A. cardenasii*) had high germination stress tolerance index (GSTI) and promptness index (PI) and were regarded as tolerant whereas susceptible genotype (TMV-2) had low GSTI and PI. Tolerant, moderately tolerant and susceptible genotypes were evaluated further for biomass accumulation and salt uptake. Root length, shoot length, chlorophyll *a*, *b* and carotenoid contents reduced under high salt conditions. Transportation of the absorbed Na<sup>+</sup> ions from roots to leaves was more in susceptible plant (TMV-2) compared to tolerant genotypes (NRCGCS-241 and NRCGCS-296). These four genotypes were further screened with gene specific primers (Na<sup>+</sup>/H<sup>+</sup> antiporter, NAC, *WRKY* and PR10) synthesized from the sequences available in the NCBI database. *WRKY* and Na<sup>+</sup>/H<sup>+</sup> antiporter gene specific primers discriminated tolerant and susceptible genotypes and amplified about 350bp and 1000bp amplicons, respectively in NRCGCS-241 which were absent in rest of the three genotypes. Besides, Na<sup>+</sup>/H<sup>+</sup> primer amplified a separate amplicon of about 900bp in all genotypes except NRCGCS-296. Rest two primers were monomorphic among these genotypes and did not differentiate these four genotypes. *WRKY* and Na<sup>+</sup>/H<sup>+</sup> genes might be responsible for imparting tolerance to salinity stress in peanut.

**Keywords:** Peanut, interspecific derivatives, salinity, physiological parameters, *WRKY*, NAC, Na<sup>+</sup>/K<sup>+</sup>, PR10.

**Abbreviations:** C\_control (0mM NaCl); DMRT\_Duncan multiple range test; GSTI\_Germination Stress Tolerance Index; LEA\_Late Embryogenesis Abundant; PR\_Pathogenesis-related; PI\_Promptness Index; S\_Stress (250 mM NaCl); SNAC\_stress-responsive NAC.

**Introduction**

Peanut (*Arachis hypogaea* L.) is the second most important cultivated grain legume and the fourth largest edible oilseed crop grown in the world (Shilman et al., 2011). Salinity is a serious problem because it limits plant growth and productivity (Qin et al., 2010). The problem of soil salinity continues to grow further because of increasing area under irrigated crops, use of poor quality water for irrigation, poor drainage facility and ingress of sea water. Peanut cannot germinate and grow at salinity levels exceeding electrical conductivity (EC) 8. Peanut could be grown with water having EC up to 3.0 dS m<sup>-1</sup>, but studies have shown that peanut plant starts facing salinity stress above 2.0 dS m<sup>-1</sup> and EC above 4.5 dS m<sup>-1</sup> kills the plant. However as enough genotypic variation exists, salinity level between 3-4 dS m<sup>-1</sup> during most of the cropping period was ideal to screen for salinity tolerance (Singh et al., 2010). Salinity affects physiological process negatively including water relations and gas exchange attributes (Maeda et al., 2008), nutritional imbalance (Yang et al 2008) and disturb the stability of membranes (Dogan et al., 2010). Although various crop specific agronomic practices have been proposed to elevate the problem of salinity, they depend largely on availability of good quality of water for irrigation which is limiting the success in majority cases. Development of salt tolerant crop

varieties either through conventional or molecular breeding or transgenics to improve their adaptation to salt stress conditions are becoming a more viable option to deal with the salinity problem (Khan et al., 2001). Salt tolerance in plants has been reported to be controlled by several gene families. The *WRKY* gene family has been suggested to play important roles in the regulation of transcriptional reprogramming associated with plant stress responses. *WRKY* proteins also function *via* protein-protein interaction and auto regulation or cross-regulation is extensively recorded among *WRKY* genes (Chen et al., 2012). Similarly, NAC proteins, a plant specific transcription factors, have a variety of important functions in plant development and abiotic stress responses and over 100 of them have been identified in Arabidopsis and rice. Transgenic Arabidopsis and rice plants over expressing stress-responsive NAC (SNAC) genes have exhibited improved drought and salt tolerance (Nakashima et al., 2011). Earlier studies have shown that plants adapt at in salinity stress condition by restricting the uptake of environmental Na<sup>+</sup>, increasing the efflux of Na<sup>+</sup> from the cell and sequestering Na<sup>+</sup> into the large intracellular vacuole to reduce Na<sup>+</sup> accumulation. Thus, the Na<sup>+</sup>/H<sup>+</sup> antiporter play a major role in improving the salt tolerance capacity of most plant species (Shu et al., 2011). Recently, pathogenesis-related

proteins are induced in plants in response to stress, pathogen attack or abiotic stimuli, thus playing a cardinal role in plant defense system. Several lines of evidence corroborate and implicate a role of PR10 proteins under abiotic stress such as drought (Dubos and Plomion, 2001), salinity and cold stress (Hashimoto et al., 2004; Kav et al., 2004; Srivastava et al., 2006). Salinity tolerant crop varieties could be developed successfully in three different ways. Firstly, existing cultivars could be screened for tolerance based on morpho-physiological traits as well as molecular markers and promising lines can be used in affected areas (Kingsbury and Epstein, 1984). Secondly, variations can be created by artificial crossing and selecting the most promising lines (Ashraf et al., 1986). Finally, the salinity tolerance could be improved by using genes from wild relatives by crossing techniques, or through genetic engineering. The present study was therefore conducted to i) evaluate different peanut germplasm, cultivars and interspecific derivatives to categorize them as salt tolerant and sensitive on the basis of morphological parameters and ii) In depth study of tolerant and susceptible genotypes to identify traits responsible for tolerance for use in breeding programme.

## Results

### *Screening for germination under salinity stress*

Under normal condition at 7<sup>th</sup> day on an average 93% germination was observed ranging between 73 to 100 per cent. Both germination per cent and range improved in majority of the genotypes on 14<sup>th</sup> day but not much increment was observed on 21<sup>st</sup> day. In majority of the genotypes maximum germination was observed at 14<sup>th</sup> day except NRCGCS-241 and NRCGCS-296 which showed germination up to 21<sup>st</sup> day. When salinity stress was imposed, germination was delayed in majority of the genotypes. Paired t-test for germination between control and stress for 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> day was significant. Very less mean germination was observed at 7<sup>th</sup> day (39 %), improved from 14<sup>th</sup> day (64%) to 21<sup>st</sup> day (69%). Germination per cent of NRCGCS 241 and NRCGCS 296 ranged from 87 (7<sup>th</sup> day) to 93 % (21<sup>st</sup> day). Germination stress tolerance index (GSTI) differed significantly among the genotypes studied. NRCGCS-241 and NRCGCS-296 showed maximum value of GSTI (79) followed by MH-2 (70.63) (Table-1). The lowest value of GSTI was observed in NRCG-4659 (0.61) which is an exotic germplasm. High GSTI indicates that these genotypes are tolerant to salinity stress.

### *Na<sup>+</sup> and K<sup>+</sup> uptake under salt stress*

Genotypes with high (NRCGCS-241 and NRCGCS-296), moderate (NRCGCS-237) and low (TMV-2) GSTI were evaluated further for root and shoot length; root and shoot weights as fresh and dry; Na<sup>+</sup> and K<sup>+</sup> content in leaves and roots. Root dry weight did not show much difference under control and salt stress conditions but significant differences were observed among genotypes for shoot dry weight. NRCGCS-237 recorded similar biomass under both conditions. Significant differences among genotypes were observed for Na<sup>+</sup> and K<sup>+</sup> accumulation in leaves, root and total (leaves plus roots) between control and salt stress conditions except leaf potassium content. Under controlled condition NRCGCS-237 absorbed maximum total Na<sup>+</sup> (0.70) whereas TMV-2 absorbed the lowest (0.36). In contrast, under salt stress condition TMV 2 absorbed maximum total Na<sup>+</sup> (4.39 %) and NRCGCS-241 absorbed lowest Na<sup>+</sup> (2.58

%). Root Na<sup>+</sup> content under control condition was lowest in TMV-2 (0.26 %) while NRCGCS-237 absorb the maximum (0.59 %). Under salt stress condition, NRCGCS-241 absorbed lowest Na<sup>+</sup> in root, while NRCGCS-296 (2.54 %) absorbed the maximum depicting NRCGCS-241 as a shy Na<sup>+</sup> absorber under excess salinity stress condition by activating root specific antipoter mechanism. The genotype NRCGCS-296 showed minimum Na<sup>+</sup> content (0.09 %) and NRCGCS-241 showed maximum (0.23 %) under controlled condition. Under salt stress condition the genotype NRCGCS-296 transported minimum Na<sup>+</sup> and TMV-2 transported the maximum to the leaf. Under controlled condition leaf K<sup>+</sup> content was higher in NRCGCS-237 (1.24 %) and it was less in TMV-2 (0.82 %). In roots higher K<sup>+</sup> content was observed in NRCGCS-296 (0.48 %) and less in TMV-2 (0.35 %). Under stress condition, higher K<sup>+</sup> content in leaves was observed in NRCGCS- 296 (1.04 %) and it was less in TMV-2 (0.82 %) whereas root K<sup>+</sup> content was higher in NRCGCS-237 (0.39 %) and it was less in TMV-2 (0.20 %). Root as well as leaf Na<sup>+</sup> content among genotypes increased significantly from control to stress condition due to higher Na<sup>+</sup> ion in soil solution. Similarly root K<sup>+</sup> content decreased significantly among genotypes differed significantly between control and stress condition which decreased under stress condition due to increase in Na<sup>+</sup> ion surrounding the root surface. However, leaf K<sup>+</sup> content in all genotypes did not differ between control and salt stress condition indicating that plants maintained K<sup>+</sup> pump unhurt in all the genotypes under stress condition in spite of significantly higher Na<sup>+</sup> concentration in root zone condition. Na<sup>+</sup>/K<sup>+</sup> ratio, which is an indicator of salinity stress, ranged from 0.09 to 0.23 in leaves and 0.75 to 1.35 in roots under controlled condition whereas under salinity stress condition it ranged from 0.43 to 2.41 and 4.88 to 11.76 in leaves and roots, respectively (Fig 3). Na<sup>+</sup>/K<sup>+</sup> ratio in root though varied under controlled condition but under salt stress condition by and large it was uniform. The ratio of Na<sup>+</sup>/K<sup>+</sup> in leaf varied both in controlled and stress condition. It was minimum in NRCGCS-296 under controlled condition; however under salt stress it was minimum in NRCGCS-241. Root and shoot length decreased significantly under salinity stress conditions. Reduction in root length was maximum in NRCGCS-237 and minimal in NRCGCS-241. Similarly maximum reduction in shoot length was observed in NRCGCS-241 and minimal in NRCGCS-296 (Fig 1). Salinity level also had influence on photosynthetic pigment of leaf like chlorophyll *a* (chl *a*), chlorophyll *b* (Chl *b*) and carotenoids which were high in NRCGCS-241 and NRCGCS-237 under control and stress conditions, respectively (Fig 2).

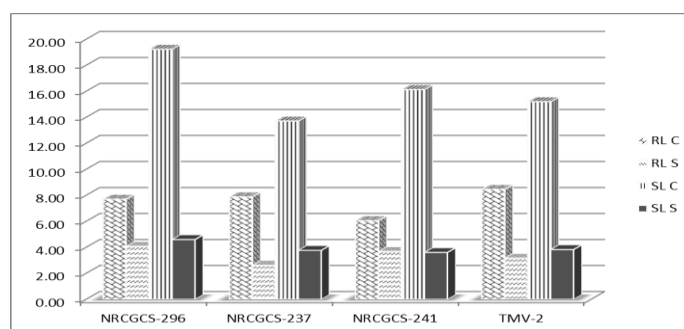
### *Expression of gene specific primers under salt stress*

These four genotypes were further screened for previously reported genes (Na<sup>+</sup>/H<sup>+</sup> antipoter, NAC, *WRKY* and PR10) conferring tolerance to salinity in various crops. Full length cDNA sequence reported for Na<sup>+</sup>/H<sup>+</sup> antipoter, NAC, *WRKY* genes were selected upon blasting NCBI data base (Table- 3) and primers were designed (Table-4) from the sequence selected. In addition, Primer of PR10 gene was synthesized directly using the sequence reported by Jain et al. (2012). Amplification of multiple bands was observed in case of Na<sup>+</sup>/K<sup>+</sup>, *WRKY* and NAC genes while single band was observed in case of PR10 primer. Na<sup>+</sup>/H<sup>+</sup> antipoter and *WRKY* gene specific primers showed polymorphism among genotypes used in molecular analysis and amplified about 350bp and 1000bp amplicons, respectively in NRCGCS-241 which were absent in rest three genotypes (Fig. 4 and 5).

**Table 1.** Effect of NaCl induced salt stress on germination percent of thirty selected peanut genotypes.

Sl. No	Genotype	7 <sup>th</sup> day		14 <sup>th</sup> day		21 <sup>st</sup> day		PI		GSTI
		C	S	C	S	C	S	C	S	
1	NRCGCS-241	86.67	86.67	93.33	93.33	100.00	93.33	38.50	30.50	79.22a
2	NRCGCS-296	86.67	86.67	93.33	93.33	100.00	93.33	38.50	30.50	79.22a
3	MH-2	92.86	60.00	92.86	86.67	92.86	86.67	35.75	25.25	70.63ab
4	DH-3-30	80.00	46.67	80.00	73.33	80.00	73.33	33.00	20.75	62.88bc
5	NRCG-2588	100.00	75.00	100.00	75.00	100.00	75.00	38.50	20.25	52.60cd
6	NRCGCS-242	86.67	46.67	100.00	66.67	100.00	73.33	39.25	20.00	50.96cde
7	TMV-12	93.33	46.67	93.33	66.67	93.33	66.67	38.50	19.50	50.65cdef
8	NRCG-7206	92.31	77.78	100.00	88.89	100.00	88.89	34.75	17.00	48.92cdefg
9	TAG-24	100.00	53.33	100.00	53.33	100.00	80.00	41.25	20.00	48.48cdefg
10	Panjab-1	73.33	33.33	80.00	46.67	80.00	66.67	32.00	15.25	47.66defgh
11	S-206	93.33	26.67	93.33	73.33	93.33	80.00	38.50	18.25	47.40defgh
12	NRCGCS-256	100.00	35.71	100.00	57.14	100.00	64.29	33.00	15.50	46.97defgh
13	M-522	92.86	46.15	92.86	61.54	92.86	69.23	35.75	16.50	46.15defghi
14	NRCGCS-317	80.00	41.67	93.33	75.00	93.33	75.00	36.50	16.25	44.52defghi
15	ICGS-76	92.86	15.38	92.86	84.62	92.86	84.62	35.75	15.75	44.06defghi
16	MA-16	100.00	36.36	100.00	90.91	100.00	90.91	38.50	16.50	42.86defghi
17	GG-2	92.86	33.33	92.86	75.00	92.86	75.00	35.75	15.25	42.66defghi
18	NRCGS-237	100.00	7.69	100.00	100.00	100.00	100.00	41.25	17.25	41.82defghi
19	Tirupati-3	86.67	30.77	93.33	69.23	93.33	69.23	37.50	15.25	40.67defghi
20	NRCG-6450	100.00	54.55	100.00	63.64	100.00	63.64	41.25	14.75	35.76efghij
21	NRCGCS-238	92.86	26.67	100.00	46.67	100.00	53.33	37.50	13.25	35.33fghij
22	TMV2(NML)	100.00	35.71	100.00	35.71	100.00	35.71	33.00	11.25	34.09ghij
23	NRCG-6820	93.33	38.46	93.33	46.15	93.33	46.15	38.50	12.50	32.47hijk
24	TG-17	100.00	16.67	100.00	66.67	100.00	66.67	38.50	12.00	31.17ijk
25	GG-20	92.31	10.00	92.31	50.00	92.31	70.00	33.00	8.25	25.00jkl
26	TMV-2	100.00	25.00	100.00	33.33	100.00	58.33	41.25	9.50	23.03jkl
27	NRCG-6131	100.00	20.00	100.00	50.00	100.00	50.00	38.50	8.25	21.43jkl
28	JL-24	92.86	8.33	100.00	25.00	100.00	58.33	37.50	6.75	18.00kl
29	NRCG-4659	100.00	12.50	100.00	25.00	100.00	25.00	33.00	3.50	10.61L
	Average	93.09	38.77	95.60	64.09	96.04	69.42			
	Paired t-test	<0.0001		<0.0001		<0.0001				

Values followed by same letter do not differ at 5% level of probability.



**Fig 1.** Effect of NaCl induced salt stress on shoot and root length of four peanut genotypes. Each value is the mean of ten replicates.

Besides, Na<sup>+</sup>/H<sup>+</sup> primer amplified an additional amplicon of about 900bp in all the genotypes except NRCGCS-296. In contrast, NAC and PR10 primers were monomorphic among these four genotypes and failed to discriminate these genotypes. Thus expression of WRKY and Na<sup>+</sup>/H<sup>+</sup> genes probably resulted in highest GSTI (Table-1), less total Na<sup>+</sup> % (Table-2), higher root length under NaCl induced salinity stress in both NRCGCS-241 and NRCGCS-296 (Fig-1). Lowest root Na<sup>+</sup>/K<sup>+</sup> ratio in NRCGCS-241 and shoot Na<sup>+</sup>/K<sup>+</sup> ratio in NRCGCS-296 could be due to activation of these two transcription factor responsive genes under NaCl induced salt stress.

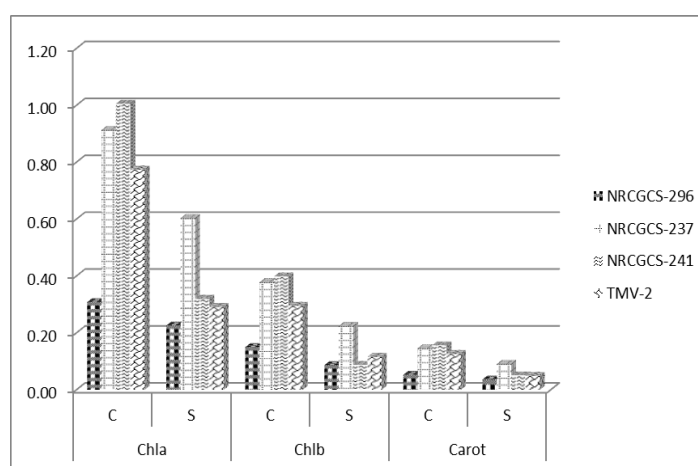
## Discussion

Germination was reported to be less susceptible to salinity stress (Khatun and Flowers, 1995). This may be due to production of late embryogenesis abundant (LEA) proteins

which protect embryo from various environmental stresses like salinity and dehydration (Wang et al., 2003). Paired t-test in the present investigation indicated significant reduction in germination due to salinity during initial stage. Several reports have indicated that seed germination, seedling emergence and early survival are susceptible to salinity (Kausar et al., 2012). Higher salinity retard germination and root emergence due to osmotic effects and prevent plant from maintaining proper nutritional requirement (Krishnamurthy et al., 2007). Some efforts have been made to study the performance of peanut cultivars by recording germination and studying plant till vegetative phase in pots (Vadez et al., 2005) and in field (Janila et al., 1999) and very few till maturity in field (Mensah et al., 2006). Also recently attempts were made for developing screening protocol using rate of survival under NaCl treatment in glass house (Vadez et al., 2005) as well as *in vitro* regenerated shoots grown on

**Table 2.** Effect of NaCl induced salt stress on Na<sup>+</sup> and K<sup>+</sup> content of four peanut genotypes.

Genotypes	Leaf Na <sup>+</sup> (%)		Root Na <sup>+</sup> (%)		Total Na <sup>+</sup> (%)	
	C	S	C	S	C	S
NRCGCS-296	0.09	0.45	0.37	2.54	0.45	2.99
NRCGCS-237	0.11	0.85	0.59	2.29	0.70	3.14
NRCGCS-241	0.23	0.80	0.38	1.78	0.63	2.58
TMV-2	0.10	1.98	0.26	2.41	0.36	4.39
Paired t-test	P = 0.007		P = 0.001		P = 0.009	
Genotypes	Leaf K <sup>+</sup> (%)		Root K <sup>+</sup> (%)		Total K <sup>+</sup> (%)	
	C	S	C	S	C	S
NRCGCS-296	0.97	1.04	0.48	0.38	1.65	1.42
NRCGCS-237	1.24	0.98	0.44	0.39	1.63	1.37
NRCGCS-241	0.99	0.93	0.42	0.36	1.45	1.29
TMV-2	0.86	0.82	0.35	0.20	1.21	1.02
Paired t-test	P=0.45		P = 0.028		P = 0.002	

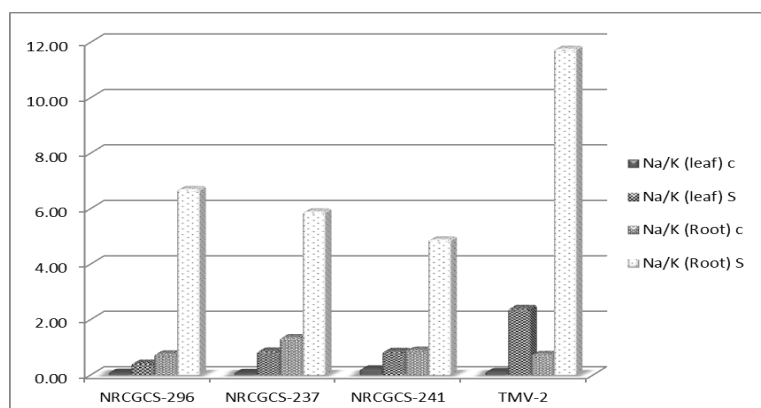
**Fig 2.** Effect of NaCl induced salt stress on chlorophyll *a*, *b* and carotenoids of four peanut genotypes. Each value is the mean of ten replicates.

media (Mungala et al., 2008) as a measure of their tolerance to salinity. Thus salt tolerant lines have been identified based on germination stress tolerance index (GSTI) by previous authors (Kausar et al., 2012) to identify salt tolerant genotypes. On the basis of GSTI genotypes NRCGCS-241, NRCGCS-296 and MH-2 were found to be tolerant whereas genotypes GG-20, TMV-2, NRCG 6131, JL-24 and NRCG-4659 were susceptible to salinity stress in the present study. Salinity tolerance is a relative term depending mainly upon its intensity and relative performance of cultivars. Peanut cultivar with high field emergence followed by high plant stand and low mortality under saline conditions could be considered as tolerant to salinity stress. However, data on yield ability is more vital as increasing salinity decreases pod yield (Hunshal et al., 1991). In a recent field screening trials peanut genotypes were ranked (Singh et al., 2010) based upon mortality and better yield for their tolerance to salinity stress. Two tolerant (NRCGCS-296 and NRCGCS-241), moderately susceptible (NRCGCS-237) and susceptible (TMV-2) genotypes were selected and further subjected to physiological and molecular studies. K<sup>+</sup> concentration between control and stress conditions did not change much in leaves and roots but significant increase in Na<sup>+</sup> content was observed under salt stress compared to controlled conditions. Under controlled conditions Na<sup>+</sup> accumulation in leaves was very low. Under salinity stress genotypes tend to absorb more Na<sup>+</sup> salts in both leaves and roots irrespective of tolerance level (Pandey and Srivastava, 1991), but in the present study the level of accumulation was comparatively more in susceptible genotype (TMV-2) than in tolerant genotypes (NRCGCS-296 and NRCGCS-241). Salinity

caused accumulation of Na<sup>+</sup> in leaves and to compensate that and maintain proper ratio of various nutrients there was accumulation of Ca<sup>+</sup> and K<sup>+</sup> content. However salinity tolerant cultivars tend to accumulate comparatively less Na<sup>+</sup> and K<sup>+</sup> in their leaves than that of sensitive cultivars (Singh et al., 2010). These absorbed salts are transported from roots to leaves by transporters. The amount of Na<sup>+</sup> salts transported from root to leaf is very less in tolerant genotype than in susceptible genotype. Though tolerant genotypes were similar with respect to GSTI, NRCGCS-296 transported very low quantity of Na<sup>+</sup> salt to leaves compared to NRCGCS-241. This indicates difference in capability of genotypes to transport salts to cytosol under saline conditions. Selective transporters in NRCGCS-296 may confer resistance to salt stress. The Na<sup>+</sup> uptake in the cytosol was much higher in the salt sensitive cultivar than salt tolerant cultivar and tolerance to salt depends on preventing Na<sup>+</sup> influx into the roots and sequestering of Na<sup>+</sup> from cytosol into the vacuole of the shoots (Shu et al., 2011). This is supported by the fact that these transporters are very active in susceptible genotypes resulting greater accumulation of salts in roots and transported to leaves. Roots are the first developing organs of the plant and are sensitive to increased levels of salinity (Akram et al., 2007). In fact, lower availability of O<sub>2</sub> under saline conditions deprives the plants from energy source and accumulation of high level of ethylene inhibits root growth (Akram et al., 2007). In the present investigation salinity stress had reduced root growth. Reduction in root length was highest in NRCGCS-237 and lowest in NRCGCS-241. Chlorophyll content had reduced under salinity stress condition among genotypes studied which is in agreement

**Table 3.** Details of salinity tolerance genes along with accession number used in designing gene specific primer.

Reported salinity tolerant gene	Accession No.	Plant in which sequence reported	Length of sequence (bp)
NAC gene	NM-1041675.1	<i>Arabidopsis thaliana</i>	1280
N+/H+ antiporter gene	AY685183.1	<i>Arabidopsis thaliana</i>	1617
WRKY gene	CS137502.1	<i>Arabidopsis thaliana</i>	1458
PR10	DQ813661.1	<i>Arachis hypogaea</i>	474



**Fig 3.** Effect of NaCl induced salt stress on Na/K ratio in root and shoot of four peanut genotypes. Each value is the mean of ten replicates

with previous reports (Azooz et al., 2004). Reduction in chlorophyll content can be attributed to inhibition of chlorophyll synthesis or an acceleration of its degradation (Reddy and Vora, 1986). Reduction in chlorophyll content decreases photosynthetic activity leading to reduced growth and productivity (Netonda et al., 2004). This is further supported by the fact that in the genotype NRCGCS-296 reduction in chlorophyll content was less in comparison to other genotypes, as a result lesser reduction in shoot length was observed. In the present study *WRKY* and  $\text{Na}^+/\text{H}^+$  antiporter genes expressed in the two tolerant genotypes (NRCGCS-241 and NRCGCS-296) and was absent in other genotypes. The *WRKY* gene probably regulates transcription factor and contribute to signaling pathways and regulatory networks in these two tolerant genotypes. The *WRKY* gene family has been suggested to play important roles in the regulation of transcriptional reprogramming associated with plant stress responses. Furthermore, a single *WRKY* gene often responds to several stress factors, and then their proteins may participate in the regulation of several seemingly disparate processes as negative or positive regulators (Chen et al., 2012). Similarly, the  $\text{Na}^+/\text{H}^+$  antiporter genes play a major role in improving the salt tolerance capacity of most plant species by restricting the uptake of environmental  $\text{Na}^+$ , increasing the efflux of Na from the cell and sequestering  $\text{Na}^+$  into the large intracellular vacuole to reduce Na accumulation (Shu et al., 2011). In the present study though NRCGCS-241 and NRCGCS-296 were found tolerant, but tolerance mechanism in these two genotypes is probably different because NRCGCS-241 absorbed very low amount of  $\text{Na}^+$  under salt stress condition than NRCGCS-296 depicting that  $\text{Na}^+/\text{H}^+$  antiporter gene is active in root and preventing  $\text{Na}^+$  absorption from soil. This is further supported by the fact that primer specific to  $\text{Na}^+/\text{H}^+$  antiporter (Fig 4) and *WRKY* (Fig 5) were amplified only in NRCGCS-241. On the other hand NRCGCS-296 though absorbed more  $\text{Na}^+$  salts through roots but limited amount was transported to leaves to maintain of high cytosolic  $\text{K}^+/\text{Na}^+$  ratio which is critical for the function of cells (Zhu et al., 1998). Here signaling pathway controlled by  $\text{Na}^+/\text{H}^+$

antiporter and *WRKY* gene is probably weak resulting the expression of *WRKY* gene specific primer in TMV-2, NRCGCS-237 and NRCGCS-241 but was missing in NRCGCS-296. Hence salt tolerance mechanism acting in NRCGCS-296 is different from that of NRCGCS-241 which needs to be elucidated. Thus peanut genotypes with shy  $\text{Na}^+$  absorbing capacity from the soil as well as weak  $\text{Na}^+$  transport signaling from root to leaves should be ideal for tolerance to salinity stress. In this endeavor, interspecific derivatives NRCGCS-241 and NRCGCS-296 would be potential donors for improving tolerance to salinity stress in peanut. Further  $\text{Na}^+/\text{H}^+$  absorption by roots and its movement to leaves would be ideal trait for improving tolerance to salinity in peanut.

## Material and methods

### Germination test and screening of material

In this study selected twenty-nine peanut genotypes consisting of fifteen released cultivars, seven germplasm accessions and seven interspecific derivatives were used. The experiment was laid out with two factors (salinity and genotypes) in Completely Randomize Design (CRD). Germination test was done *in vitro* (38mm OD x 200mm length test tube, Borosil, India) with 250mM of NaCl for salinity stress (S) and tap water as control (C). One seed was placed per test tube containing filter paper wick and were considered as germinated when the radicle reached around 5 mm in length. Germination count of seed was made on 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> day after sowing. Germination test was done in 10 replications with 15 tubes per replication. The results were expressed in terms of a promptness index (PI) (George, 1967):

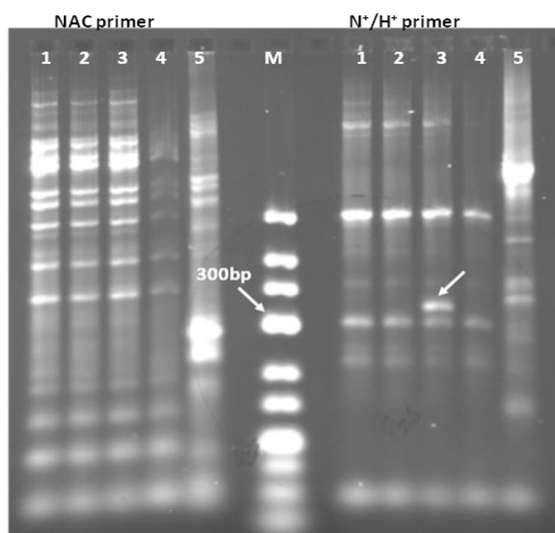
$$PI = nd7^{th} (1.00) + nd14^{th} (0.75) + nd21^{st} (0.50)$$

Where;  $nd7^{th}$ ,  $nd14^{th}$  and  $nd21^{st}$  = number of seeds germinated on the 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> day after sowing, respectively.

A germination stress tolerance index (GSTI) was expressed in percentage and was calculated as:  $GSTI = (PI \text{ of stressed seeds} / PI \text{ of control seeds}) \times 100$ .

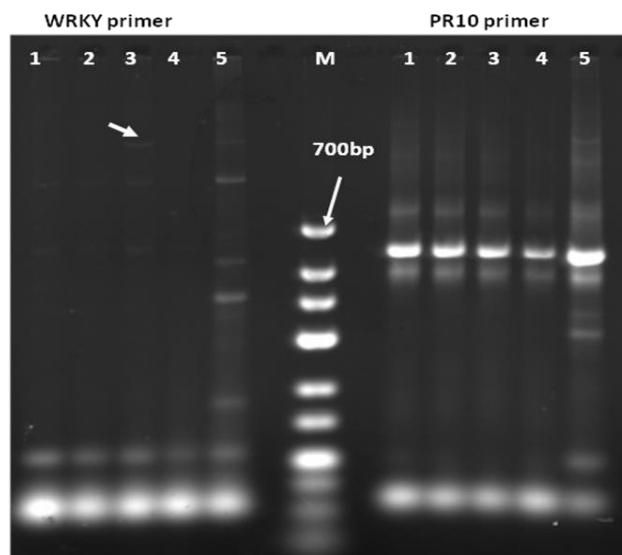
**Table 4.** Salinity tolerant gene specific primers used for screening selected tolerant and susceptible genotypes.

Salinity tolerant gene	Primer sequence (5'-3')	Length (bp)	Tm °C
NAC gene	F-TGCCGGTAATGTTGAGCATA	20	54.30
	R-ACATAAACCCAAACCCACCA	20	54.50
N <sup>+</sup> /H <sup>+</sup> antipoter gene	F-TGGACTCATGAGAGGTGCTG	20	56.50
	R-TTTGGGGTGTTGTCATCAGA	20	54.10
WRKY gene	F-CTGTCGTTGCAATCTCTCCA	20	59.98
	R-GGTCTTCGTCTTCGTCTTCG	20	59.99
PR10	F-ATGTCCATGGATGGGCGTCTTCACTTTTCGAG	31	64.50
	R-GATGACTAGTTAATATTGAGTAGGGTTGG	29	54.80



1= TMV-2, 2= NRCGCS-237, 3=NRCGCS-241, 4=NRCGCS-296 and 5= *Arachis glabrata* (wild species), not included in the study; M= DNA marker

**Fig 4.** Expression of NAC and N+/H+ genes in four interspecific derivatives and a wild species.



1= TMV-2, 2= NRCGCS-237, 3=NRCGCS-241, 4=NRCGCS-296 and 5= *Arachis glabrata* (wild species), not included in the study; M= DNA marker

**Fig 5.** Expression of WRKY and PR 10genes in four interspecific derivatives and a wild species.

### Recording of data on morpho-physiological traits

Root and shoot length, fresh root and shoot weight were measured on 21<sup>st</sup> day after germination based on 10 plants per replication. Shoot and root dry weight was measured by oven drying at 80°C till weight reached to constant. Na<sup>+</sup> and K<sup>+</sup> content in shoot and root were estimated by triacid (Nitric acid, Perchloric acid and Sulphuric acid in 10:4:1 ratio) digestion followed by estimation on a flame photometer using NaCl and KCl as standard (Jain et al., 2010). Photosynthetic pigments (chlorophyll a, b and carotenoids) of the leaf were estimated in laboratory on 21<sup>st</sup> day after germination following the methods of (Arnon et al., 1949).

#### Molecular assay

Genomic DNA was extracted from tender leaf samples collected from in vitro grown plants following CTAB methods (Saghai-Marouf et al., 1984). The quality of DNA was checked in Nanodrop spectrophotometer (Model ND 1000) at A<sub>260</sub>/A<sub>280</sub> ratio and also in 0.8% agarose gel. Gene specific primers were designed using Primer 3 software using reported sequences from NCBI database (Table 3). Primers were synthesized from Integrated DNA Technologies (IDT) Inc, USA (Table 4). PCR amplification was performed in a Thermal cycler (C1000, BIO RAD) programmed for three steps: Holding the samples at 94°C for 4 minutes for complete denaturation of the template DNA. 35 cycles of following temperatures: 1 minute at 94°C for denaturation of template, 3 minutes at 37°C for primer annealing followed by 1 minute at 72°C for primer extension. 10 minutes at 72°C for complete polymerization followed by holding at 4°C. Amplified products after completion of PCR were stored at 4°C till further use. The PCR amplified products were separated in 2% agarose (Genei, Bangalore, India) gel. The gel was scanned in gel scanner (FUJIFILM FLA 5100, Japan) and size of amplicon was determined by comparing them with DNA marker.

#### Statistical analysis

To test the effect of salinity stress we compared the response of genotypes under salt stress with the control using paired t-test to determine if salinity had any different response for the controlled conditions. Duncan multiple range test (DMRT) was performed for GSTI values using software DSAASAT (Onofri, 2007) to test the responses of genotypes under salt stress condition.

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