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Research Article

Dilemmas between barley cultivars under salinity stress

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ABSTRACT

Salinity is one of reasons causing the greatest yield-reducing factors leads various physiological and biochemical effects. Recently, long non-coding RNAs (lncRNAs) became prominent as crucial regulators in various biological processes, including developmental processes and stress responses such as salinity. In this study, physiological responses of four *Hordeum vulgare* L. cvs genotypes (Beyşehir 99, Hasat, Konevi 98 and Tarm 92) were assessed for 150 mM salinity treatment for 3 days germination period, maize (CNT0018772) and rice (CNT0031477) lncRNAs localizations in barley chromosomes via fluorescence in situ hybridization (FISH). Physiological responses of barley varieties, including root and shoot heights, fresh and dry weights, water content (WC) and protein content were affected differently by 150 mM salinity stress. The germination percentage of Beyşehir 99 and Tarm 92 reduced while Konevi 98 was slightly increased. However, proline content changed only in Beyşehir 99. According to FISH results, the localizations of CNT0018772 and CNT0031477 were revealed up 6 and 8 signals, respectively. This study may contribute new insight into lncRNAs functions underlying the salinity tolerance mechanisms in different barley varieties.

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INTRODUCTION

Salt stress is one of the greatest yield-reducing factors, leading various physiological and biochemical effects on plants. Salinization is a global problem that effects approximately 20% of the globally cultivated and nearly half of the total irrigated land, also reduces crop yields significantly [1-3]. High salt stress, which causes growth inhibition, acceleration of development and senescence and death during prolonged exposure, influences plants in numerous ways such as water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion [4-6]. Therefore, salt stress tolerance shows extensive diversity in plants, especially cereals that rice (*Oryza sativa*) is considered as the most sensitive, and durum wheat (*Triticum turgidum*) is regarded as sensitive and bread wheat (*Triticum aestivum*) is moderately

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tolerant. However, barley (*Hordeum vulgare* L.) is the most tolerant and is the fourth most important cereal crop in the world after wheat, maize, and rice [7-10].

In recent years, reports on the plant lncRNAs have demonstrated they act as key regulatory elements in the diverse biological mechanisms played in developmental process, biotic and abiotic stress responses. Additionally, several molecular functions and biological processes have been regulated by lncRNAs such as vernalization, fertility, photo morphogenesis, protein re-localization, phosphate homeostasis, alternative splicing, modulation of chromatin loop dynamics [11-14]. To date, 7970 among 19,908 lncRNAs have been annotated by CANTATAdb [15, 16]. Annotation and sequencing studies revealed that CNT0018772 had two exon and one intron, while sequencing data of cDNA did not contain any intron part, indicating that this lncRNA may undergo the excision of introns or alternative splicing [17]. According to our previous studies, expression analysis of CNT0018772 and CNT0031477 demonstrated that these two lncRNAs were affected by salt stress. Expression levels of CNT0018772 were down-regulated compared controls on roots and shoot. Additionally, the expression levels of CNT0031477 in 150 mM salt applied groups were also down-regulated compared controls on roots and shoot except for Tarm 92 variety which was up-regulated [18]. In another study, Huang and colleagues [19] performed RNA-Seq analysis to reveal interactions between barley and Fusarium graminearum which causes reduction in barley production. They identified 604 lncRNAs as Fusarium head blight responsive. In 2019, Unver and Tombuloglu [20] reported ~8000 barley lncRNA related to boron stress. Based on expression analysis, sensitive and tolerant varieties indicated some lncRNAs were specific for the tolerant variety contributing to boron stress response.

Barley is widely utilized for food, livestock feed and brewing beer, moreover, is an important crop research model with well-studied genetics analysis, including a sequenced genome, and physiological characteristics [8, 21]. Due to higher salt tolerance of barley is considered as a good model for understanding the mechanisms of salt tolerance in cereal crops [9, 22]. In this study, salt stress was investigated at germination level to determine the differences between four barley (Beyşehir 99, Hasat, Konevi 98 and Tarm 92) varieties (Beyşehir 99, Hasat, Konevi 98 and Tarm 92). Salt stress treatment was applied as control and 150 mM NaCl. Physiological and biochemical responses were determined under salt stress conditions during 3 days of germination period. Salt stress application effects were evaluated in terms germination percentage, root and shoot growth, dry and wet weight, water content, total protein, and proline contents. Additionally, we carried out fluorescence *in situ* hybridization (FISH) analyses in samples obtained from roots that were harvested after 72 h during germination period. We investigated maize (CNT0018772) and rice (CNT0031477) lncRNAs localizations under confocal microscope via *in situ* hybridization on barley root preparations.

MATERIAL AND METHODS

Plant Materials and Treatments

Grains of four different *Hordeum vulgare L.* cv. varieties were utilized in this study: Beyşehir 99, Hasat, Konevi 98 and Tarm 92 were obtained from different Agricultural Research Institutes in Turkey. Beysehir 99 and Konevi 98 were provided from Bahri Dagdas International Agricultural Research Institute, Tarm 92 was from Field Crops Central Research Institute and, Hasat were obtained from Directorate of Trakya Agricultural Research Institute. Grains were disposed randomly in Petri dishes containing filter papers saturated in (a) only H2O as control, (b) 150 mM NaCl application. Salt treated groups were kept for 72 hours at dark. Totally 180 barley grains were utilized for each control and salt treated groups (10 grains in each petri dish, three replicates for each treatment). The plants were grown under controlled growth chambers at 18-25°C with dark period. Relative humidity of growth chamber was kept at 60-75%. Roots and shoots were harvested separately after 72 hours treatment. Harvested samples were directly treated with liquid nitrogen. Afterwards, samples were stored at -80°C until RNA extraction and probe synthesis.

The Physiological Experiments

After 72 h of application salt stress, three replicates of the control and salt applied plants (three plants of each) were harvested. The root and shoot of each plant were collected separately for estimation of shoot fresh weight (FW) and height. The shoot FW and height were expressed as mg g-1 plant-1 and cm shoot-1, respectively. Germination percent for the control and salt treated plants was also calculated.

Water content (WC %) of plants as control and treated salt were evaluated after germination. After 72 h, the plants fresh weight was measured. The fresh plants were evaporated in the oven for 3 days at 70°C and oven-dry plants weight (DW) were measured. Water content was calculated by using WC (%) = [(FW−DW)/FW] × 100 formula [23].

Total soluble protein content of plants was evaluated based on Bradford [24] using bovine serum albumin (BSA) as a standard. Controls and treated plants were homogenized by liquid nitrogen. 1ml extraction buffer [50 mM PBS (pH 7.0), 0.1 mM EDTA, 4 % polyvinylpyrrolidone] was added per 0.01 mg plant material. The mixture was centrifuged at 14.000 rpm for 20 min at 4°C [25]. Supernatant was utilized for determination of soluble protein content, measured spectrophotometrically at 595 nm against a reagent blank and expressed as mg g-1 DW.

Proline content of plants was calculated according to Bates et al. [26] and Abraham et al. [27]. Approximately 100 mg FW of plants was extracted in 3% sulfosalicylic acid (5 µL/mg fresh weight). The extract was centrifuged

at maximum speed for 5 min at RT. 100 µL from the supernatant of the plant extract was mixed with 100 µL of 3% sulfosalicylic acid, 200 µL glacial acetic acid, 200 µL acidic ninhydrin solution (1.25 g 1,2,3-indantrionemonohydrate, 30 mL glacial acetic acid, 20 mL of 6 M orthophosphoric acid) for reaction in a centrifuged tube for 1 h at 96°C. The reaction was stopped on ice, and mixture was extracted with 1 ml toluene. The chromophore-containing toluene was aspirated from the aqueous phase, and its absorbance was measured at 520 nm against a reagent blank. Proline content was calculated by using a standard curve (0-100 µg ml-1) and calculated as mg g-1 DW.

Results of the physiological and molecular experiments were expressed as mean ± standard deviation (s.d.) and each data point is the arithmetic mean of triplicates (n=3). All the experiments were repeated at least three times starting from different and independent RNA extractions. The significance of data was analysed by One-Way Analysis of Variance (ANOVA). Afterwards, Tukey test was utilized to compare the means ($p<0.05$ were considered significant).

Probe Design

For RNA extraction, plant samples (roots and shoots) were completely powdered using pre-chilled mortar and pestle, in the presence of liquid nitrogen. Total RNAs were extracted separately from root and shoot samples by using TriPure® (Roche Diagnostics) reagent based on manufacturer's instructions with some modifications. Total RNAs were resuspended in RNase-free water and stored at -80°C. The quality of the extracted RNA samples was checked by agarose gel electrophoresis (2% agarose gel); three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent. The purity of RNA is roughly indicated by measuring the absorbance ratios for protein and reagent contaminations (A260/A280 and A260/A230 nm ratios, respectively) by using NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific). A 260/280 and a 260/230 ratio around 2 is usually interpreted as 'pure'. For the removal of gDNA, 4 µg of total RNA was treated with five units of RNase-free recombinant DNase I® (Roche Diagnostics) based on the manufacturer's instructions. DNase I® treatment reaction was incubated at 37°C for 15 min and terminated by adding 2 µL of 0.2 M EDTA (pH 8.0) to a final concentration of 8 mM and heating to 75°C for 20 min.

The first strand of cDNA was synthesized by using Transcriptor High Fidelity® cDNA Synthesis Kit (Roche Diagnostics) based on suggestions of the manufacturers' instructions. Total RNA (4 µg) was processed with random hexamer primers. Obtained cDNAs were stored -20°C until use.

The probe amplification reaction of CNT0018772 and CNT0031477 (approximately 180 bp) were performed by using forward and reverse primers indicated in below. The probes for CNT0018772 and CNT0031477 were designated with GC% and Tm values as around 60 and between 58°and 60°C by using IDT's PrimerQuest© Tool (2012), respectively. The sequence of target lncRNAs was provided from CANTATAdb which a database of lncRNAs is identified computationally in 39 species, 36 plant and 3 algae (Szcześniak et al. 2016). The sequence of CNT0018772 was obtained from maize and, CNT0031477 sequence was provided from rice. Probe sequences are as listed below:

CNT0018772 F: 5' – CACCCTTCTTCACAATCAC,
CNT0018772 R: 5' – GTTATGGCAGGCGTAATG,
CNT0031477 F: 5' – GATGAGGGCCAAGTGGAAG,
CNT0031477R:5'–TTGGATCTCTGCCTCATCTTTC.

cDNA probe synthesis was carried out by using CNT0018772 and CNT0031477 lncRNAs as template mentioned above. The reaction was carried out in a total volume of 50 µl including 18.25 µl nuclease-free dH2O, 25 µl of HotStart PCR Master Mix (Bio-Rad), 1.5 µl of each primer (10 µM/µl), 1.75 µl of tetramethylrhodamine-dUTP (TRITC) (1 mM), and 2 µl template cDNA (10 ng/µl). PCR conditions were as follows: 94°C for 5 min followed by 40 cycles of 94°C for 25 s, annealing 55°C for 25 s and 72°C for 30 s. The reaction was accomplished by a final extension step at 72°C for 7 min.

Fluorescence *in situ* Hybridization (FISH)

The seeds of *Hordeum vulgare* L.cv. Hasat were disposed on wet filter paper in a petri dish to germinate in an incubator at 18-25°C in the dark for 3 days. Root tips from 1-2 cm germinated roots were harvested and directly fixed in Carnoy fixative (ethanol:acetic acid solution, 3:1) without any chemical pre-treatment and stored roots at 4oC. Chromosome preparations and FISH procedure were performed according to Jenkins and Hasterok [28, 29] with modifications. The slides were checked under the light microscope (Olympus U-TVO.5XC-3) and kept in a freezer at -20 °C.

The FISH procedure was adopted from Jenkins and Hasterok [28, 29] with modifications. Chromosome spreads were scanned under ×40 objective light microscopes to determine the number and quality of well-spread metaphase and anaphase plates, and then they were treated with 100 µg/ml of RNase at 37°C for 1 h. The hybridization mixture consisted of 20 µl of deionised formamide (50%), 8 µl of dextran sulphate (10%), 4 µl of 20X SSC (2X SSC), 2 µl of 10% SDS (0.5%), 10 µl of probe (75-200 ng/slide), 1 µl of blocking DNA (sonicated salmon sperm DNA) (25-100X probe) and added sterile distilled water to bring final volume 40 µl. The mixture was denatured at 85°C for 10 min and kept on ice for 10 min. A 40 µl aliquot of the hybridization mixture was applied onto each slide, covered with a coverslip, and sealed with paper bond. Both chromosomal DNA and probe DNA on the slides were denatured together in a thermal cycler at 70°C for 6 min and hybridized with each other at 37°C overnight in a humid dark box.

After hybridization the chromosome spreads were washed three times in 2X SSC: once 2X SSC to float coverslips off; once in 15% formamide/0.1X SSC, and again once in 15% formamide/0.1X SSC, each for 10 min at 42°C. After, slides were washed in 2X SSC for 3 min at 42°C. This step was repeated twice with fresh 2X SSC at 42°C. Finally, at RT, slides were washed three times in 2X SSC for 3 min. Later, slides were dehydrated in alcohol series (70, 90 and 100%), each for 1 min at RT and waited in the dark for 15-20 min. Vectashield-DAPI mounting-staining medium (7-10 µL) was dropped onto the chromosome spreads, which were then stored at 4°C until used.

FISH results were observed under confocal microscope (Leica DM5500). The wavelengths were used for fluorescence detection: 551-575 nm for probes labelled with TRITC and 420-480 nm for DAPI. The different fluorescent images were acquired separately, and then they were merged into single composite images. The signal images were analysed by Adobe Photoshop CC 2014.

RESULTS AND DISCUSSION

Salt Treatment Affected Divergently Four Different Barley Cultivars

Higher salt accumulations according to evaporation and capillary rise of water is one of the major problems in surface soils where barley germination and early seedling occur. Therefore, barley genotypes are more often exposed to higher salinity stress at germination and early stages of growth [30]. Understanding of salt effects on barley germination may contribute new insight for increasing the production of important cereals. In this study, four barley varieties were selected to investigate the response patterns on germination percentage, root and shoot growth, dry and wet weight, water content, total soluble protein and proline contents under control and salt stress conditions. According to results, 150 mM salt application significantly reduced germination percentages, root and shoot heights, fresh and dry weights, and WC while protein content increased. However, changes in proline content were only observed in Beysehir 99, indicating that barley varieties display distinct responses under salinity. In this regard, diversity of barley accessions to salinity offers a valuable tool for investigation of salt tolerance mechanisms [31]. Due to their direct connection with soil and absorption of water and nutrients from soil, roots, are the most sensitive organ to salt stress and, are important to study salinity effects on plants [32]. The effects of salinity on barley seedlings growth were investigated regarding differences in germination percentage, root and shoot fresh and dry weight content, and heights. Comparison of germination percentages demonstrated that there was no significant difference between barley varieties for control groups ($p>0.05$). However, salinity affected ($p<0.01$) germination percentage of Beyşehir 99, Konevi 98 and Tarm 92 in response to 72 h of treatment with 150 mM NaCl (Table 1) (Figure 1).

Interestingly, germination percentage of Hasat was not significantly affected by 150 mM salt treatment compared with control ($p>0.05$). Beyşehir 99 and Tarm 92 showed a reduction for germination percentage. On the contrary, germination percentage of Konevi 98 was slightly increased under salt stress conditions. Measurement of root and shoot heights of all barley varieties used in this study was performed at the time of sample harvested. Moreover, 150 mM salt treatment remarkably reduced root and shoot heights, although differences between groups were observable in all varieties ($p<0.01$) (Table 2) (Figure 1). The extreme

Table 1. Effects of salt application on shoot and root heights

	Shoot heights (cm)		Root heights (cm)	
Genotype	**Control**	**150 mM Salt Treatment**	**Control**	**150 mM Salt Treatment**
Hasat	1,61±,02	0,43±0,01**	4,42±0,09	2,77±0,05**
Beyşehir 99	1,87±0,02	0,73±0,02**	4,57±0,07	3,19±0,05**
Konevi 98	1,97±0,02	0,62±0,04**	4,09±0,03	3,08±0,05**
Tarm 92	2,18±0,08	0,87±0,02**	4,26±0,07	2,80±0,06**

Data are presented as mean (n=3)±standard error (s.e.)

*p<0.05; **p<0.01 (data are different from control)

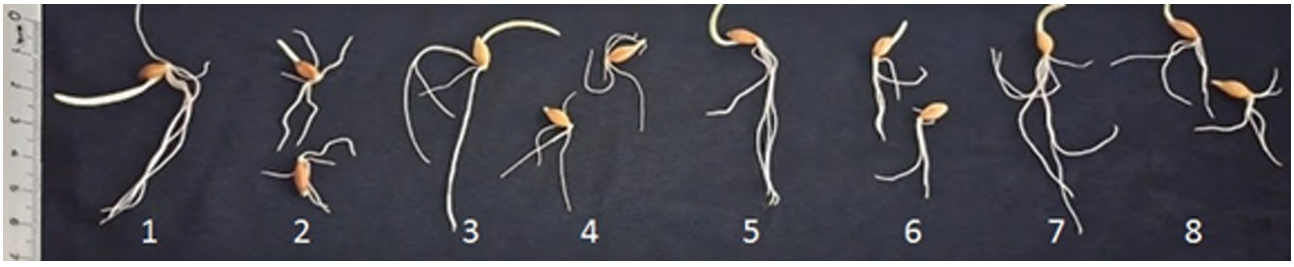

Figure 1. Morphological view of *Hordeum vulgare* L. cv. seeds germinated between filter papers at dark for 72 h. (1) Tarm 92 control; (2) 150 mM NaCl applied Tarm 92; (3) Konevi 98 control; (4) 150 mM NaCl applied Konevi 98; (5) Beyşehir 99 control; (6) 150 mM NaCl applied Beyşehir 99; (7) Hasat control; (8) 150 mM NaCl applied Hasat.

accumulation of salts, which alter the metabolic activities and restrict the cell wall elasticity, may cause the reduction of the shoot heights. Furthermore, the cell wall becomes rigid and the turgor pressure efficiency in cell enlarges the damages [33, 34]. The reduction of plant heights may also be attributed to the decrease of physiological activities as a resultof water and nutrients stress which consequently might be related to reduction of plant growth and productivity [35-37]. However, our previous studies demonstrated CNT0018772 and CNT0031477 lncRNAs expression profile under salt stress were found to be down- regulated. In addition, comparison of CNT0018772 expression levels showed there was no significant difference between 150 mM salt treated barley varieties and controls ($p>0.05$). On the contrary, the alterations of expression levels of CNT0031477 demonstrated there was statistically significant difference between 150 mM salt applied and control groups ($p<0.05$). To reveal significance between groups, Tukey test was performed following to One-Way Analysis of Variance. The expression level of Konevi 98 shoot control was statistically higher than Tarm 92 shoot control [18]. Although CNT0018772 and CNT0031477 lncRNAs were found to be related to salt stress, these lncRNAs effect on salt stress may not be associated with barley cultivar.

Total FW for all varieties, was dramatically ($p<0,05$) affected by salinity andreduced significantly in response to 150 mM NaCl salt application for 72 h. Control groups of Hasat, Konevi 98 and Tarm 92 demonstrated the highest total FW (94-100 g plant-1) (Table 3) and root FW (55-60 g plant-1). However, Beyşehir 99 had lowest root FW, in

Table 2. Effects of salt application on total FW, DW and WC

Genotype	Control			150 mM Salt treatment		
	FW (mg g^{-1})	DW (mg g^{-1})	WC%	FW (mg g^{-1})	DW (mg g^{-1})	WC%
Hasat	99,47±5,82	7,51±0,44	92,23±0,78	29,75±1,80**	4,42±0,38**	91,02±0,80
Beyşehir 99	87,06±7,84	8,48±3,80	91,05±0,50	34,66±3,27**	3,80±0,35**	88,63±1,68
Konevi 98	100,83±3,33	7,81±0,47	90,85±0,21	41,64±1,61**	4,79±0,08**	87,72±0,23**
Tarm 92	94,00±0,00	8,16±0,17	90,20±0,29	41,83±2,75**	4,86±0,48**	87,69±0,69**

Data are presented as mean (n=3)±standard error (s.e.)

*p<0.05; **p<0.01 (data are different from control)

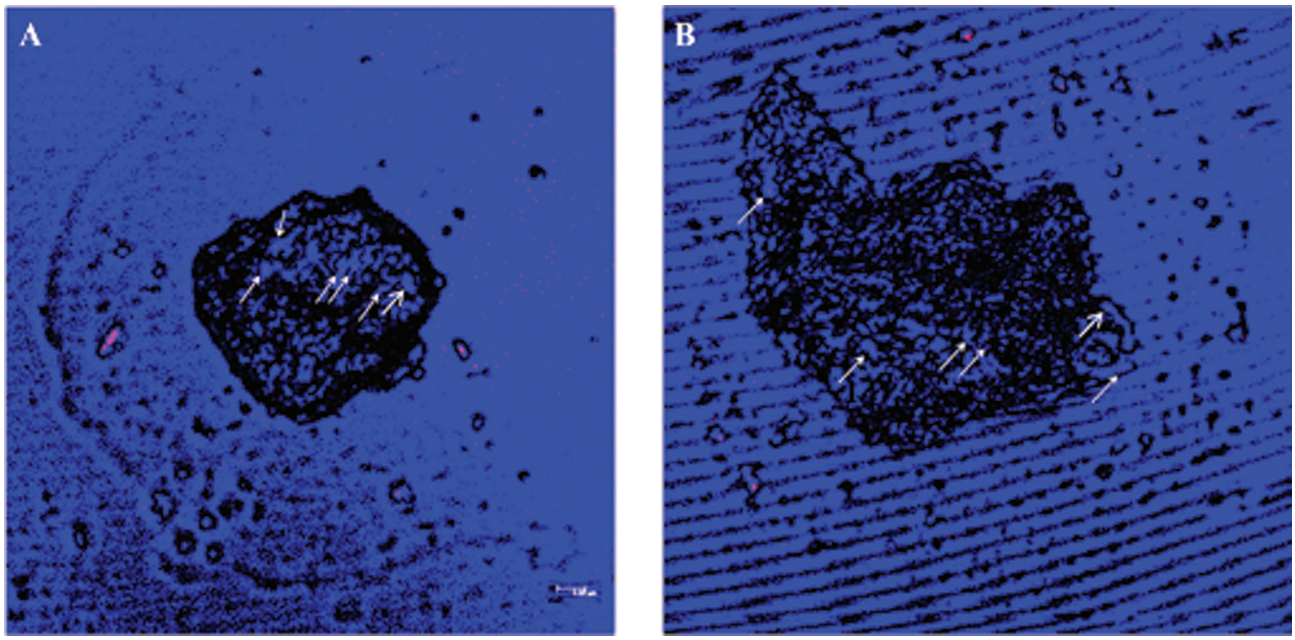

Figure 2. FISH analysis showing *CNT0018772* (maize) in nucleus and cytoplasm. DAPI-stained chromatin in the nucleoplasm was shown as blue fluorescence. Regions hybridized with TRITC-labelled probes were indicated as red fluorescence. Display of *CNT0018772* with 6 signals in the nucleoplasm. FISH analysis is also showing localization patterns of lncRNAs in the cytoplasm. Scale bar=5 µm.

contrast, shoot FW of Beyşehir 99 was the highest for control (see in Table 4 and 5). Hasat showed significant reduction in total and shoot FW compared to control and other three barley varieties. The root and shoot FW and DW also affected under salinity stress. Specifically, shoot FW of Hasat (Table 4) and root FW of Konevi 98 (Table 5) reduced in response to 150 mM salinity stress. However, root FW of Beyşehir 99 did not show significant differences between other control groups and salt applied Beyşehir 99 ($p>0.05$) (Table 5). Also, total shoot and root of DW dramatically reduced by 150 mM salt application ($p<0.01$), except Hasat showed no alteration compared as control group ($p>0.05$). FW and DW of shoots and roots for all four barley cultivars significantly reduced with 150 mM salt application for 72 h (Table 4 and 5). The reduction fresh weight of shoots may depend on decrease of cell contents, development and differentiation of tissues, unbalanced nutrition, damage of membranes and disturbed avoidance mechanisms [38].

Significant reduction of total WC (%) was observed for Konevi 98 and Tarm 92 under 150 mM salt stress conditions ($p<0,01$) (Table 3). On the contrary, salinity did not significantly affect total WC (%) of Hasat and Beyşehir 99 ($p>0.05$). The root and shoot WC (%) were also determined that Hasat, Konevi 98 and Tarm 92 showed a significant reduced ($p<0.05$), however Beyşehir 99 did not affected by salinity and maintained the highest root and shoot WC (%), 90 % and 89 %, respectively. Furthermore, DW/FW is a measure of water intake associated with the WC [39]. The reduction of WC was demonstrated for Konevi 98 and Tarm 92. On the other hand, it was observed Hasat and Beyşehir 99 showed no alteration in WC. The reduction in WC due to salinity induce to utilize inorganic ions such as Na^+ and K^+ and, synthesize organic compatible solutes [40]. Maintenance of high WC might be important to prevent salt stress effects [41].

The soluble protein content of Konevi 98 ($p<0.05$), Hasat and Beyşehir 99 ($p<0.01$) were increased in response to 150 mM salt stress compared with controls. However, Tarm 92 did not show significant difference between treatment and control ($p>0.05$) (Table 5). Differences of proline content between salt treatment and control were not statistically significant in Hasat, Konevi 98 and Tarm 92 ($p>0.05$). On the other hand, proline content of Beyşehir 99 was significantly increased in response to salt stress ($p<0.01$) (Table 5). Summary of the comparison of physiological effects on barley cultivars were demonstrated in Table 6. Alterations in gene expression and protein accumulation, which are important to investigate differences between the varieties underlying the salt stress sensitivity and tolerance, are the first plant defensive response to salinity. Some reports showed that the protein content in salt stressed plants is generally reduced [42, 43]. On the other hand, our results demonstrated protein content was increased in three varieties (Hasat, Beyşehir 99, Konevi 98) among four barley genotypes. However, protein content of Tarm 92 did not demonstrate significant difference

Table 3. Effects of salt application on shoot FW, DW and WC

Genotype	Control			150 mM Salt treatment		
	FW (mg g^{-1})	DW (mg g^{-1})	WC%	FW (mg g^{-1})	DW (mg g^{-1})	WC%
Hasat	30,80±0,18	3,17±0,29	90,23±0,28	4,61±0,09**	1,40±0,09**	74,07±1,60**
Beyşehir 99	42,53±9,08	3,00±0,21	90,74±1,60	9,08±0,80**	1,44±0,10**	86,25±3,50
Konevi 98	39,75±3,46	3,38±0,04	90,73±0,06	9,86±0,41**	1,55±0,09**	87,27±0,77**
Tarm 92	36,80±1,68	3,94±0,34	89,42±0,85	11,55±0,09**	1,53±0,21**	85,51±0,26**

Data are presented as mean (n=3)±standard error (s.e.)

*p<0.05; **p<0.01 (data are different from control)

Table 4. Effects of salt application on root FW, DW and WC

Genotype	Control			150 mM Salt treatment		
	FW (mg g^{-1})	DW (mg g^{-1})	WC%	FW (mg g^{-1})	DW (mg g^{-1})	WC%
Hasat	53,33±2,65	3,70±0,09	92,66±0,18	27,33±1,15**	3,58±0,08	91,02±0,80*
Beyşehir 99	43,84±7,43	4,43±0,38	90,10±1,39	34,44±2,22	3,45±0,23*	90,06±0,95
Konevi 98	53,83±6,42	4,58±0,52	92,75±0,01	23,28±1,80**	2,85±0,13**	90,33±0,40**
Tarm 92	55,19±2,13	4,20±0,18	92,76±0,28	29,61±2,33**	3,42±0,38*	88,45±0,58**

Data are presented as mean (n=3)±standard error (s.e.)

*p<0.05; **p<0.01 (data are different from control)

between control and treated plants (Table 6). Accumulation of protein content may depend on genotype of barley or saline conditions. Proline accumulation is considered to occur under water deficit, salinity, low temperature, heavy metal exposure plays a significant role in reducing the damage of salinity and boosting of the repairing process following stresses [44, 45]. It was observed that proline accumulation in salt applied plants was a primary defense response maintaining the osmatic pressure in a cell [46, 47]. Moreover, Ashraf and Foolad [48] reported proline contributed to stabilizing sub-cellular structure, dealing with free radicals and also, enhancing redox potential by buffering under saline conditions. Increase of proline content in plants was demonstrated under salt stress and other stress conditions [49, 50]. Similar result was observed for Beyşehir 99 which accumulated proline approximately two-fold than other barley cultivars in this study. Some reports showed that tolerant genotypes to salt stress have more ability to adapt salinity and accumulate proline acting as a compatible osmolyte much higher than sensitive genotypes [51, 52]. However, Hasat, Konevi 98 and Tarm 92 showed no alteration in proline content in this study. These barley genotypes may possibly survive by using other defense mechanism such as antioxidation, ion homeostasis and hormonal systems under saline conditions [53-55].

FISH or RISH (RNA *in situ* hybridization) provide great potential to study of lncRNAs enable quantification and spatial resolution of single RNA molecules within cells via hybridization of multiple, labelled nucleic acid probes to a target DNA or RNA [56]. In this study, we also performed FISH analyses to observe the localization two barley lncRNAs and sequencing analyses to identify and evaluate the putative barley lncRNAs. Selected two barley lncRNAs localizations, are homologous with maize (CNT0018772) and rice (CNT0031477) lncRNAs, were investigated in barley chromosomes that samples obtained from roots were harvested after 72 h during germination period under confocal microscope. According to FISH results, CNT0018772 and CNT0031477 probes were exhibited 6 and 8 signals (Figure 2 A-B and 3 C-D), respectively. However, cellular localizations were also exhibited under confocal microscope via in situ hybridization on barley root preparations for CNT0018772 and CNT0031477. TRITC labelled probes for both CNT0018772 and CNT0031477 lncRNAs were obtained from cDNA. DAPI-stained chromatin structure in the nucleoplasm observed via confocal microscope that we observed 6 and 8 signals for CNT0018772 and CNT0031477 lncRNAs in barley nucleus, respectively which is also consistent with sequence analysis. Sequence analysis also showed CNT0018772 have six copies with some sequence differences in barley genome. However, copy number of CNT0031477 in reference barley genome is two according to sequence analysis. These results are not consistent with FISH signals which were 8 for CNT0031477, suggesting that copy numbers of these lncRNAs may be different between barley cultivars. Moreover, we were able to demonstrate intracellular locations of two lncRNAs (CNT0018772

Table 5. Effects of salt application on protein and proline contents

	Protein content (mg g^{-1})		Proline content (mg g^{-1})	
Genotype	**Control**	**150 mM Salt Treatment**	**Control**	**150 mM Salt Treatment**
Hasat	6,99±0,90	12,37±1,34**	1,60±0,16	2,03±0,33
Beyşehir 99	10,29±0,56	14,99±1,20**	2,41±0,40	4,33±0,38**
Konevi 98	8,81±0,38	12,69±2,09*	1,60±0,10	1,97±0,57
Tarm 92	8,44±0,36	8,42±0,39	1,86±0,23	3,30±1,16

Data are presented as mean (n=3)±standard error (s.e.)

*p<0.05; **p<0.01 (data are different from control)

Table 6. Effects of salinity stress on barley genotypes during germination [18]

Physiological features		Most effected variety > least effected variety
Germination percentage		Tarm 92 > Beysehir 99 > Hasat > Konevi 98
Shoot heights		Hasat > Konevi 98 > Beysehir 99 = Tarm 92
Root heights		Hasat > Tarm 92 > Beysehir 99 > Konevi 98
Total	**FW**	Hasat > Beysehir 99 > Konevi 98 > Tarm 92
	DW	Beysehir 99 > Tarm 92 > Hasat > Konevi 98
	WC	Konevi 98 > Tarm 92 > Beysehir 99 > Hasat
Shoot	**FW**	Hasat > Beysehir 99 > Konevi 98 > Tarm 92
	DW	Tarm 92 > Hasat > Konevi 98 > Beysehir 99
	WC	Hasat > Beysehir 99 > Tarm 92 > Konevi 98
Root	**FW**	Konevi 98 > Hasat > Tarm 92 > Beysehir 99
	DW	Konevi 98 > Beysehir 99 > Tarm 92 > Hasat
	WC	Tarm 92 > Konevi 98 > Hasat > Beysehir 99
Protein content		Hasat > Beysehir 99 > Konevi 98 > Tarm 92
Proline content		Beysehir 99 > Hasat = Konevi 98 = Tarm 92

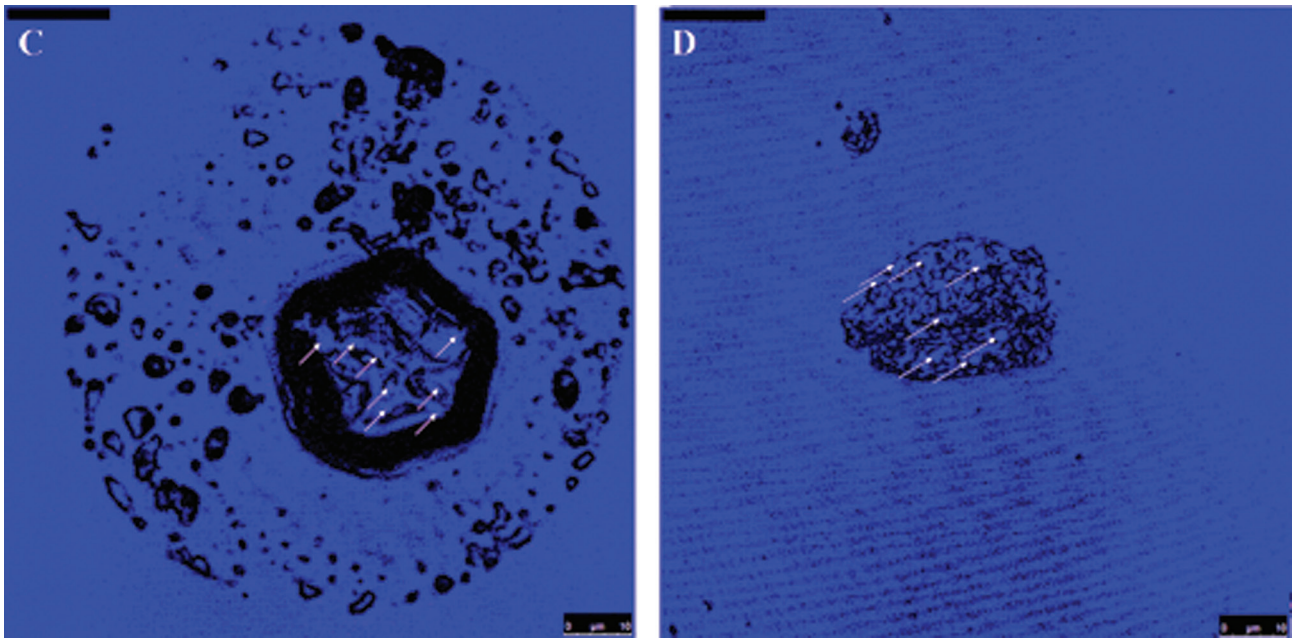

Figure 3. FISH analysis showing *CNT0031477* (rice) lncRNAs in nucleus and cytoplasm. DAPI-stained chromatin in the nucleoplasm was shown as blue fluorescence. Regions hybridized with TRITC-labelled probes were indicated as red fluorescence. Display of *CNT0031477* with 8 signals in the nucleoplasm. FISH analysis also showing localization patterns of lncRNAs in the cytoplasm. Scale bar= 10 μm.

and CNT0031477) by FISH probes. However, plant fluorescence microscopy is observed to be complicated by endogenous auto fluorescence, which is minimal in root cells, thus they are especially used to image quantification of RNAs and determination of cell-to-cell variations [57]. By establishing FISH protocols, the relationship between sense and antisense transcription at the single cell and single locus level are able to be demonstrated. Furthermore, the ability to visualize lncRNAs at sub-cellular resolution can uncover nuclear or cytoplasmic localization, providing information for gene expression [58-60].

As a conclusion, understanding the salt stress response in plants is one of the key challenges for improving crop breeding. Evaluation of crops, wide crossed of crops and their wilt types for their response in salt stress will contribute to improve crop yield and quality. Combination of traits will enhance the screening for salinity stress tolerance at germination.

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AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

REFERENCES

[1] Yamaguchi T, Blumwald E. Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci 2005;10:615–620. [CrossRef]

[2] FAO. FAO land and plant nutrition management service. Available at: http://www.fao.org/ag/agl/agll/spush. Accessed on Dec 21, 2018.

[3] Qadir M, Quillérou E, Nangia V, Murtaza G, Singh M, Thomas RJ, et al. Economics of salt-induced land

degradation and restoration. Nat Resour Forum 2014;38:282–295. [CrossRef]
[4] Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ. Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 2000;51:463–499. [CrossRef]
[5] Munns R. Comparative physiology of salt and water stress. Plant Cell Environ 2002;25:239–250. [CrossRef]
[6] Zhu J-K. Plant Salt Stress. In: Zhu J-K, editor. Encyclopedia of Life Sciences. 1st ed. New Jersey: John Wiley & Sons Inc; 2007.
[7] Bengtsson BO. Barley genetics — not only here for the beer. Trends Genet 1992;8:3–5. [CrossRef]
[8] Forster B, Ellis RP, Thomas WTB, Newton AC, Tuberosa R, This D, et al. The development and application of molecular markers for abiotic stress tolerance in barley. J Exp Bot 2000;51:19–27. [CrossRef]
[9] Munns R, Tester M. Mechanisms of salinity tolerance. Annu Rev Plant Biol 2008;59:651–681. [CrossRef]
[10] Shelden MC, Dias DA, Jayasinghe NS, Bacic A, Roessner U. Root spatial metabolite profiling of two genotypes of barley (Hordeum vulgare L.) reveals differences in response to short-term salt stress. J Exp Bot 2016;67:3731–3745. [CrossRef]
[11] Kim E-D, Sung S. Long noncoding RNA: unveiling hidden layer of gene regulatory networks. Trends Plant Sci 2012;17:16–21. [CrossRef]
[12] Zhang J, Mujahid H, Hou Y, Nallamilli BR, Peng Z. Plant long ncRNAs: A new frontier for gene regulatory control. Am J Plant Sci 2013;4:1038–1045. [CrossRef]
[13] Liu J, Wang H, Chua N-H. Long noncoding RNA transcriptome of plants. Plant Biotechnol J 2015;13:319–328. [CrossRef]
[14] Liu X, Hao L, Li D, Zhu L, Hu S. Long non-coding RNAs and their biological roles in plants. Genom Proteom Bioinform 2015;13:137–147. [CrossRef]
[15] Szcześniak MW, Rosikiewicz W, Makałowska I. CANTATAdb: A collection of plant long non-coding RNAs. Plant Cell Physiol 2016;57:e8. [CrossRef]
[16] Mascher M, Gundlach H, Himmelbach A, Beier S, Twardziok SO, Wicker T, et al. A chromosome conformation capture ordered sequence of the barley genome. Nature 2017;544:427–433. [CrossRef]
[17] Karlik E. Why IncRNAs were not conserved? Is it for adaptation. Front Life Sci RT 2021;2:103–110. [CrossRef]
[18] Karlik E, Gozukirmizi N. Evaluation of Barley lncRNAs expression analysis in salinity stress. Russ J Genet 2018;54:198–204. [CrossRef]
[19] Huang Y, Li L, Smith KP, Muehlbauer GJ. Differential transcriptomic responses to Fusarium graminearum infection in two barley quantitative trait loci associated with Fusarium head blight resistance. BMC Genom 2016;17:387. [CrossRef]
[20] Unver T, Tombuloglu H. Barley long non-coding RNAs (lncRNA) responsive to excess boron. Genomics 2019;112:1947–1955. [CrossRef]
[21] Mayer KFX, Waugh R, Brown JWS, Schulman A, Langridge P, Platzer M, et al; International Barley Genome Sequencing Consortium. A physical, genetic and functional sequence assembly of the barley genome. Nature 2012;491:711–716. [CrossRef]
[22] Ashraf M, Harris PJC. Potential biochemical indicators of salinity tolerance in plants. Plant Sci 2004;166:3–16. [CrossRef]
[23] Reeb JE, Milota MR. Moisture content by the oven-dry method for industrial testing. W estern Dry Kiln Association; 1999 May; Corvallis Or : Western Dry Kiln Association; 1999. p. 66–74.
[24] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254. [CrossRef]
[25] Pan Y, Wu LJ, Yu ZL. Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (Glycyrrhiza uralensis Fisch). Plant Growth Regul 2006;49:157–165. [CrossRef]
[26] Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil 1973;39:205–207. [CrossRef]
[27] Abrahám E, Hourton-Cabassa C, Erdei L, Szabados L. Methods for determination of proline in plants. Methods Mol Biol 2010;639:317–331. [CrossRef]
[28] Jenkins G, Hasterok R. Painting whole chromosome sets in hybrids using GISH, Jenkins G, Maluszynska J, Schweizer D, editors. Advanced Molecular Cytogenetics – a practical course manual. Katowice: Wydawnictwo Uniwersytetu Śląskiego; 2001. p. 35–48.
[29] Jenkins G, Hasterok R. BAC 'landing' on chromosomes of Brachypodium distachyon for comparative genome alignment. Nat Protoc 2007;2:88–98. [CrossRef]
[30] Almansouri M, Kinet JM, Lutts S. Effect of salt and osmotic stresses on germination in durum wheat (Triticum durum Desf.) Plant Soil 2001;231:243–254. [CrossRef]
[31] Bchini H, Naceur MB, Sayar R, Khemira H, Kaab-Bettaeïb LB. Genotypic differences in root and shoot growth of barley (Hordeum vulgare L.) grown under different salinity levels. Hereditas 2010;147:114–122. [CrossRef]
[32] Hussain S, Khaliq A, Matloob A, Wahid MA, Afzal I. Germination and growth response of three wheat cultivars to NaCl salinity. Plant Soil Environ 2013;32:36–43.
[33] Naseer S, Nisar A, Ashraf M. Effect of Salt Stress on Germination and Seedling Growth of Barley (Hordeum vulgare L.). Pak J Biol Sci 2001;4:359–360.[CrossRef]

[34] Taghipour F, Salehi M. The study of salt tolerance of Iranian barley Hordeum vulgare L. genotypes in seedling growth stages. Biodivers Conserv 2008;4:53–58.
[35] Greenway H, Munns R, Wolfe J. Interactions between growth, Cl– and Na+ uptake, and water relations of plants in saline environments. I. Slightly vacuolated cells. Plant Cell Environ 1983;6:575–589. [CrossRef]
[36] Sharma PK, Hall DO. Interaction of salt stress and photoinhibition on photosynthesis in barley and sorghum. J Plant Physiol 1991;138:614–619. [CrossRef]
[37] Shafi M, Bakht J, Hassan MJ, Raziuddin M, Zhang G. Effect of cadmium and salinity stresses on growth and antioxidant enzyme activities of wheat (Triticum aestivum L.). Bull Environ Contam Toxicol 2009;82:772–776. [CrossRef]
[38] Kent LM, Läuchli A. Germination and seedling growth of cotton: salinity-calcium interactions. Plant Cell Environ 1985;8:115–159. [CrossRef]
[39] Naidoo G. Effects of waterlogging and salinity on plant-water relations and on the accumulation of solutes in three mangrove species. Aquat Bot 1985;22:133–143. [CrossRef]
[40] Chinnusamy V, Jagendorf A, Zhu J-K. Understanding and improving salt tolerance in plants. Crop Sci 2005;45:437–448. [CrossRef]
[41] Sairam RK, Srivastava GC. Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. Plant Sci 2002;162:897–904. [CrossRef]
[42] Muthukumarasamy M, Gupta SD, Panneerselvam R. Enhancement of peroxidase, polyphenol oxidase and superoxide dismutase activities by triadimefon in NaCl stressed Raphanus Sativus L. Biol Plant 2000;43:317–320. [CrossRef]
[43] Parida A, Das AB, Das P. NaCl stress causes changes in photosynthetic pigments, proteins, and other metabolic components in the leaves of a true mangrove,Bruguiera parviflora, in hydroponic cultures. J Plant Biol 2002;45:28–36. [CrossRef]
[44] Hare PD, Cress WA, Van Staden J. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ 1998;21:535–553. [CrossRef]
[45] Rhodes D, Nadolska-Orczyk A, Rich PJ. Salinity, Osmolytes and Compatible Solutes. In: Läuchli A, Luttge U, editors. Salinity: Environment - plants – molecules. 1st ed. Dordrecht: Springer; 2002. p. 181–204. [CrossRef]
[46] Mansour MMF, Salama KHA, Ali FZM, Abou Hadid AF. Cell and plant responses to NaCl stress in Zea mays L. cultivars differing in salt tolerance. Gen Appl Plant Physiol 2005;31:29–41. [CrossRef]
[47] Veeranagamallaiah G, Chandraobulreddy P, Jyothsnakumari G, Sudhakar C. Glutamine synthetase expression and pyrroline-5-carboxylate reductase activity influence proline accumulation in two cultivars of foxtail millet (Setaria italica L.) with differential salt sensitivity. Environ Exp Bot 2007;60:239–244. [CrossRef]
[48] Ashraf M, Foolad MR. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 2007;59:206–216. [CrossRef]
[49] El-Tayeb MA. Response of barley grains to the interactive efect of salinity and salicylic acid. Plant Growth Regul 2005;42:215–224. [CrossRef]
[50] Migdadi HM, El-Harty EH, Salamh A, Khan MA. Yield and proline content of faba bean genotypes under water stress treatments. J Anim Plant Sci 2016;26:1772–1779.
[51] Anosheh HP, Sadeghi H, Emam Y. Chemical priming with urea and KNO3 enhances maize hybrids (Zea mays L.) seed viability under abiotic stress. J Crop Sci Biotechnol 2011;14:289–295. [CrossRef]
[52] Anwar S, Shafi M, Bakht J, Jan MT, Hayat Y. Response of barley genotypes to salinity stress as alleviated by seed priming. Pak J Bot 2011;43:2687–2691.
[53] Zörb C, Noll A, Karl S, Leib K, Yan F, Schubert S. Molecular characterization of Na+/H+ antiporters (ZmNHX) of maize (Zea mays L.) and their expression under salt stress. J Plant Physiol 2005;162:55–66. [CrossRef]
[54] De Azevedo Neto AD, Prisco JT, Enéas-Filho J, Abreu CEBD, Gomes-Filho E. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ Exp Bot 2006;56:87–94. [CrossRef]
[55] Zhang J, Jia W, Yang J, Ismail AM. Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res 2006;97:111–119. [CrossRef]
[56] Dunagin M, Cabili MN, Rinn J, Raj A. Visualization of lncRNA by single-molecule fluorescence in situ hybridization. Methods Mol Biol 2015;1262:3–19. [CrossRef]
[57] Duncan S, Olsson TSG, Hartley M, Dean C, Rosa S. A method for detecting single mRNA molecules in Arabidopsis thaliana. Plant Methods 2016;12:13. [CrossRef]
[58] Battich N, Stoeger T, Pelkmans L. Control of transcript variability in single mammalian cells. Cell 2015;163:1596–1610. [CrossRef]
[59] Bahar Halpern K, Caspi I, Lemze D, Levy M, Landen S, Elinav E, et al. Nuclear retention of mRNA in mammalian tissues Cell Rep 2015;13:2653–2662. [CrossRef]
[60] Sotta N, Duncan S, Tanaka M, Sato T, Grieneisen VA, Fujiwara T, et al. Rapid transporter regulation prevents substrate flow traffic jams in boron transport. ELife 2017;6:e27038. [CrossRef]