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Hydroponic Method of Halophobic Response Elicitation in Flax (*Linumusitatissimum*) for Precise Down-Stream Gene Expression Studies

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ABSTRACT: *A nutraceutical crop like flax,* Linumusitatissimum*, faces multiple abiotic stresses viz. drought and salt that are detrimental for optimum plant growth and reduce seed yield as well as fibre production. Thus, yield stagnation at 1.4t/ha among major flax growing countries has been a major concern. Though, this genomically orphan crop recently received attention of the scientific communityand structural genome was sequenced; molecular search for identifying novel genes imparting abiotic stress tolerance is in its infancy. In a pursuit to standardize and isolate salinity responsive genes in flax, we adopted a hydroponic system for precise stress induction in flax. The halophobic stress imparted by 250mM NaCl for 12hours is the break-even point for salinity tolerance in flax variety T-397. Though, the salinity stress had impact on total cellular RNA homeostasis; it had no effect on the quality of isolated RNA. Our results suggest, the stress induction method was precise, repeatable and isolated RNA was amenable to down-stream molecular experiments such as cDNA synthesis and semi-qRT-PCR.*

Key words*: Flax/linseed, Salt-stress induction method, Hydroponics, RNA isolation, Gene expression.*

INTRODUCTION

Flax, known as linseed, (*Linumusitatissimum* L.) is a multipurpose cash-crop that provides dietary supplements of omega-3 fatty acids in human consumption [1]as well as industrial requirement offiber and solvents. Both, its fiberand seeds have been used since early human civilization [2][3]. Although, the crop is speculated to have originated in India/ Middle-Eastregions of Asian continent [4], currently, it is being cultivated both in temperate and tropical regions of world comprising Canada, China, Russia including Europe. Globally, average yield of linseed stagnates at less than 1 ton per hectare (t/ha) [5] although a maximum yield of 2.5 t/ha is reported in field conditions [6]. However, the yield average of meager 1.4 t/ha among major flax producing countries is a cause of concern.

Genetically, flax is a self-pollinated annual crop having thirty chromosomes (2n = 2x = 30) and belongs to the family "Linaceae"[7]. Despite being a diploid crop, the perceived major bottlenecks in flax yield enhancement are (i) absence of inherent hybrid system with flax breeders[3], (ii) lack of genomic resources for improvement of the cultivar (iii) lack of suitable varieties tolerant to abiotic stresses prevalent in marginal lands of India, and (iv) failure to compete in acerage against other priority crops like rice, wheat and sugarcane.Additionally, like many other field crops, flax cultivation too is threatened by salt stress, especially in central arid zones of India.

Under field conditions, flax faces two types of salt stresses (i) mild salt stress that primarily has negative impact on house-keeping metabolic activities affecting plant development, agronomic traits and agricultural productivity; while (ii) chronic salt stress negatively impacts nutrient acquisition from soil, impairs lipid metabolism there by disrupting integrity of cellular membranes and photosynthesis followed by destructive processes of hydropenia, hyperosmotic stress including ionic toxicity, secondary oxidative stress [8-9] and eventually death of plant. Thus, salt stress across crop species has been considered an increasingly serious problem [10-13] and highlights the importance of developing salt-tolerant plants, through the use of genetic engineering.The salt tolerance mechanism of flax (if any) at the molecular level is not known as it has been a "Genomic orphan crop" [14-19] till the structural genome was deciphered few years ago [20].

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Since root is the first organ exposed to salt stress; we adopted a hydroponic system to precisely impart halophobic stress in flax. The objective of the investigation was to assess the phenotypic responses of flax to *in vitro* salt stress to get a realistic impression of how consistent plants responded to stresses and identify the correct stage and concentration of salt that flax perceives as a break-even-point before eliciting the phenotypic responses.We applied varied concentration of sodium chloride (NaCl) ranging 100 mM – 300 mM in a time scale manner at 0-, 6-, and 12-hours to precisely induce the salt toxicity symptoms and specifically address the question, is there a significant change in RNA homeostasis in flax in response to high salt stress? To our knowledge, this is the first report of standardization of salt stress induction in Indian flax variety T-397 and elicitation of thehalophobicsymptoms. In a pursuit to explore the transcriptome profiles and to identify salt-responsive genes in flax, our results show that the salt induction method through hydroponics is precise, reproducible and yielded good quality of RNA that can be used for down-stream gene expression study to identify putative candidate genes involved in salt tolerance in flax.

MATERIAL AND METHODS

Plant material

In-house multiplied seeds (obtained from Linseed project coordinating unit, Kanpur) of flax variety T-397 were used for all the salt–stress studies. Plants were grown under controlled condition of temperature (22°C) and relative humidity (75%) in tissue culture facility. 10cm X 10 cm pots were tightly filled with a 1:1 mixture of coco peat and vermiculite and were completely saturated with water. Thirty seeds per pot were germinated and plants were grown for 30 days with proper irrigation. At the end of 30 days, plants devoid of soil were carefully transferred to 1X Hoagland media (HimediaPvt ltd, India). Plants were allowed to acclimatize in the Hoagland media for 2 days and damaged plants if any were removed. Further, salt stress treatments were performed in hydroponics.

Salt-stress treatment

For studying the effect of salinity on the growth and development of flax variety T-397, thirty two days old experimental plants were treated with varying concentrations of sodium chloride (NaCl) ranging from 100 mM, 200 mM, 250 mM, and 300 mM for 24 hours. Control plants were not treated with NaCl (0mM). The variations in symptoms as a measure of salinity stress were observed and carefully noted at 0-, 6-, 12-, and 24-hours. 200 mg fresh (100mg root +100mg shoot) tissue was used as starting material for isolation of RNA and down-stream experiment.

RNA isolation

200 mg of tissue from stress induced as well as control flax plant were finely powdered by grinding in liquid nitrogen. A modified borax decahydrate extraction buffer [21] was used for the extraction of RNA from the tissues. Total RNA isolated was re-suspended in 100ìL of RNase free water. Quality and integrity of isolated RNA was verified both by spectro-photometerand gel electrophoresis oftotal RNA.

Quantitative and qualitative estimation of RNA

To estimate the quality of isolated RNA,1 % denaturing agarose gel electrophoresis was carried out at 80 volt current (115cm^2 gel size) until the bromophenol blue migrated at least 2/3rd of the length of the gel. The bands were visualised and photographed using gel documentation unit. Precise estimation of isolated RNA was carried out using Nanodrop 1000 (Thermo Scientific, USA) that spectro-photometrically measured RNA quality and quantity at 230, 260 and 280 nm wave length.

cDNA synthesis and semi-quantitative RT-PCR

Good quality total RNA was used for first-strand cDNA synthesis using oligo-dT primer and Superscript III system according to the manufacture's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, 1000 ng of total RNA was used for cDNA synthesis using RNase out. The quality of cDNA was assessed by semi-quantitative PCR technique using gene specific primers for actin. First, cDNA was diluted tentimes and equal volume of cDNA was subjected to 35 PCR amplification cycles. Each reaction tube contained 1 µl cDNA, 1.25 units of Taq DNA polymerase (Bangalore genei), and 0.2mM each of dNTPs (Takara Inc. Japan), and 10pmolactin primers. Amplified product was electrophoresed on 1% agarose gel and photographed in Alpha imager gel documentation system.The primer pairs that were used in qRT-PCR reactions wereActin Fd: 5'-TTGCTGACCGTATGAGCAAG-3'; Actin Rev: 5'-ACCCTCCAATCCAGACACTG-3'.

RESULT AND DISCUSSION

Halophobiacausesrapid and excessive damage to the plant cells by production of reactive oxygen species

(ROS) that leads to impairment of biochemical processes by oxidative damages. To scavenge the over-production of reactive oxygen species and to mitigate the cellular damage, plants trigger a complex antioxidant defense system by combinatorial action of antioxidant enzymes like SOD, CAT, and POD [22]. The massive recruitment of these enzymes to neutralize the damaging effect of salt–stress is modulated by transcriptional and translational level changes [23]. However, to date, transcriptional level changes involved in salt tolerance in flax is largely unknown. Identification of specific genes underpinning halophobicresponse in flax requires seizing of candidate genes in a time scale manner. To capture the candidate genes, it is important to carry out molecular analysis at correct stage of stress which is a pre-requisite for precise downstream discovery.

In our experiment, salt stress was imparted on 32d old flax plants by treating them with different concentrations of NaClranging from 100 mM-300 mM salt over a period of 24 hours. For this, a hydroponic system (Fig. 1) of treatment in 1X Hoagland media was standardized by a pre-experiment in which 30d old flax plants were conditioned for additional two days to adapt to the hydroponics. Additionally, hydroponic system ensured uniform salt stress at root level and eliminated diffusion related experimental error. The stress symptoms on flax shoots were monitored over 0-, 6-, 12- and 24 hours. It was observed that plant elicited no visible difference in symptoms between 12- and 24 hours. However, the control plants were not treated with NaCl and were maintained in Hoagland media till the end of the experiment.

It was found that 100 mMNaClhad no effect on the plants, as no visible symptoms were observed after 24h of treatment (Fig. 1-1 A, B, C). Contrastingly, 300 mM NaCl was toxic to plants (Fig. 1-4A, B, C), as the plants started showing visible symptoms of stresswithin 2 hours and were unable to recover from the stress injuries after 12hours of treatment due to high salt concentration. Plants treated with 200 mM and 250 mM salt showed progressive symptoms (Fig. 1-2A, B, C; 1-3A, B, C) and recovery from stress after six hours. However, the rate of symptom appearance was slow in 200mM as compared to 250 mMNaCl.

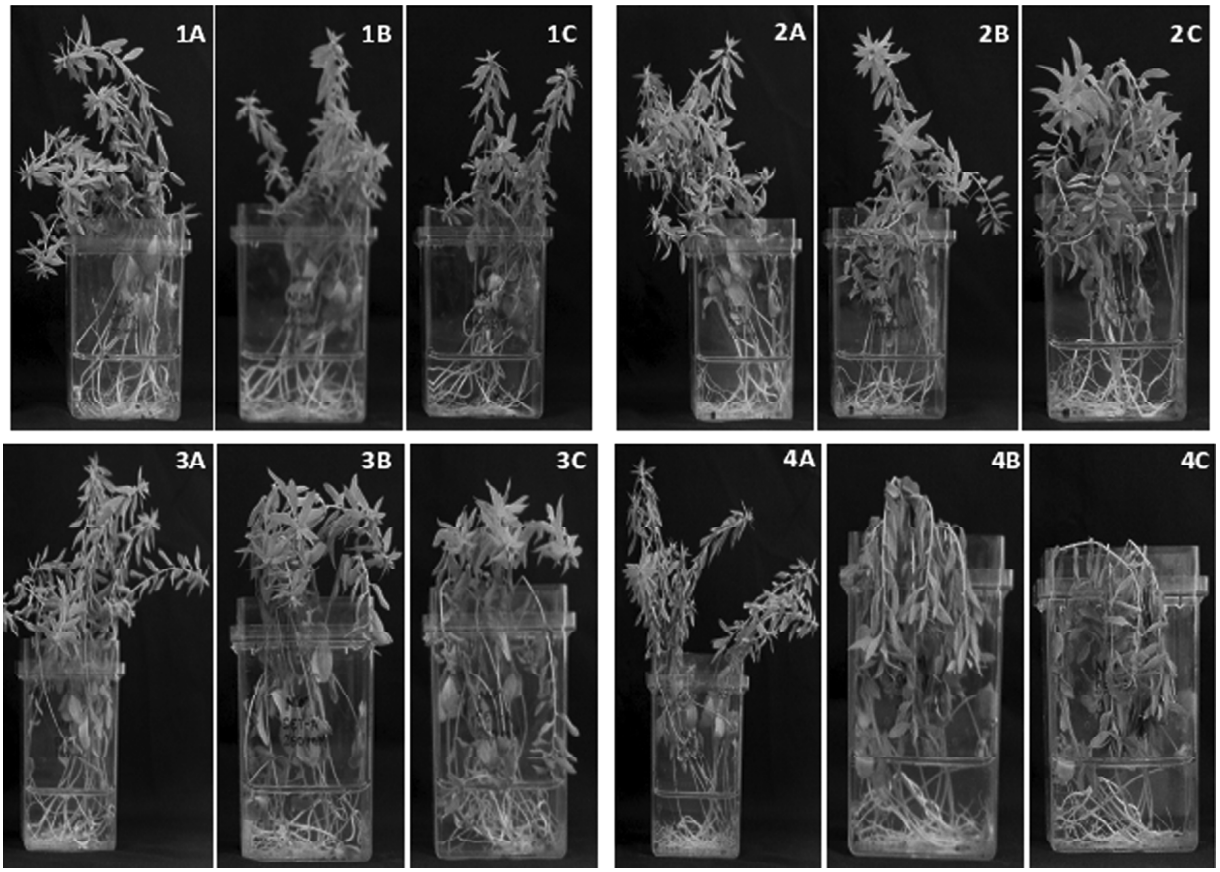

Figure 1. Hydroponic system of saline stress induction in flax. 1: 100mM NaCl concentration (A) 0 hour, (B) 6 hours, (C) 12 hours stress; 2: 200mM NaCl concentration (A) 0 hour, (B) 6 hours, (C) 12 hours stress; 3: 250mM NaCl concentration (A) 0 hour, (B) 6 hours, (C) 12 hours stress; and 4: 300mM NaCl concentration (A) 0 hour, (B) 6 hours, (C) 12 hours stress.

Many plantshave intrinsic capacity to tolerate saline-shock by buffering cellular salt concentration through a phenomenon known as "Salt exclusion" or by compartmentalizing salt in vacuoles [24]. We speculate, at 100mM salt concentration flax plants are able to completely exclude or compartmentalize the salt into vacuoles and, thus, did not display any stress symptom. At 200mM and 250mM the plants showed visible symptoms of drooping of shoots within 6 hours of stress. However, these plants slowly acquainted to the salinity stress and drooping shoots partly recovered to turgid shoot (normal state)after 12 hours of stress. Since, the degree of damage and time-span for recovery from damage depends on intrinsic property of individual plants there were minor differences between the symptoms elicited by 200mM and 250mM NaCl [25-26]. Contrastingly, 300mM salt concentration had irrecoverable damage leading to death of plants. Thus, 250mM NaCl was identified as cardinal salt concentration for imparting salt-stress in flax.

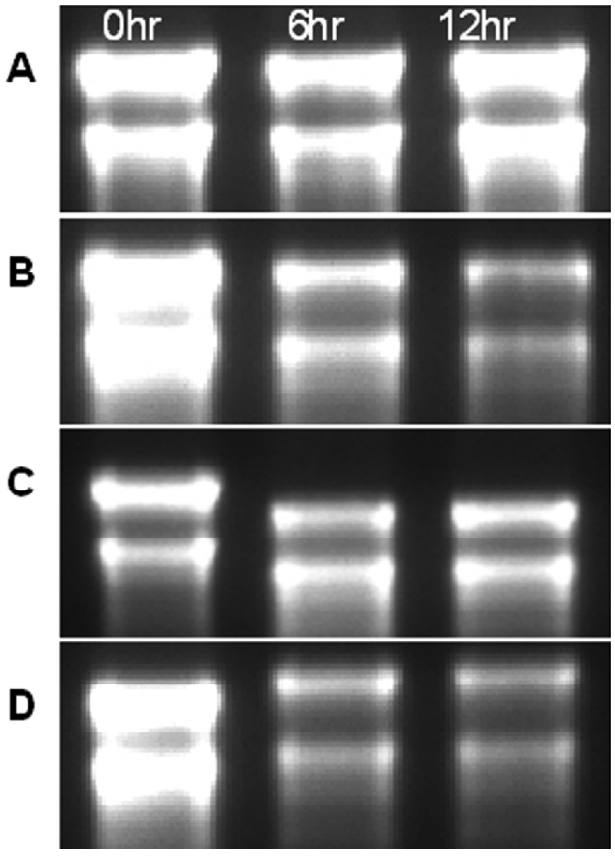

Figure 2: Isolation of RNA from salt treated flax plants. Total RNA was isolated from plants treated with (A)100mM, (B) 200mM, (C) 250mM, and (D) 300mM NaCl after 6 hours and 12 hours stress duration. 0 hour represents RNA isolated from unstressed (control) plants.

To understand the impact of saline stress on cellular RNA homeostasis, total RNA was isolated (Fig2A,B,C,D) from stress induced as well as control flax plants at different time points viz; 6 hours and 12 hours; while,0 hour samples served as unstressed control to be used for comparison. Since shoot does not come in direct contact with the salt solution it usually exhibitssalinity stress in a gradual manner [27]compared to roots that are the first organs to experience stress impact. To eliminate tissue specific (spatial) variation of yield of nucleic acids, we included root along with shoot tissues for isolation of total RNA and checked itsconcentration. As observed in Fig 2, the quantity of RNA visibly decreased with the increasing salt concentration and time. Quantitatively, there were variations in total RNA yield between control, 6-, and 12hours samples of 100mM, 200mM, 250mM, and 300mM (Table 1)NaCl. Precisely, mean RNA yield was 1593.3 ± 47.6 ng/µl after 6 hours and decreased to 1163 ± 46.4 ng/µl after 12 hours of stress treatment with 250mM NaCl (Table 1). At 300mM NaCl concentration, the mean total RNA yield decreased to 879.5 ± 27.4 ng/µl and 651.8 ± 27.1 ng/µl at 6 hours and 12 hours after stress (Table 1). This reduction in RNA yield can be accounted to the salinityinduced decrease in cellular nucleic acid metabolism. Additionally, this massive

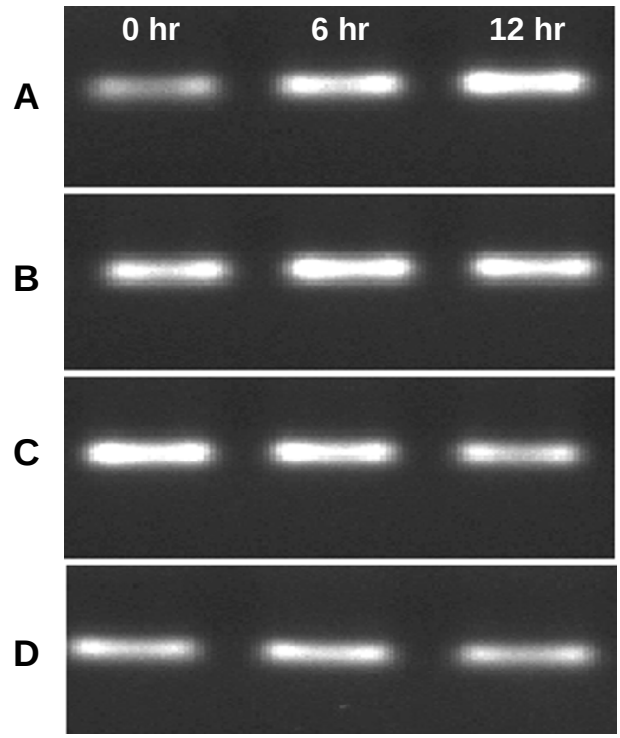

Figure 3: Amplification of actin gene by semi-quantitative PCR using cDNA synthesized from total RNA isolated from flax plants treated with (A)100mM, (B) 200mM, (C) 250mM, and (D) 300mM NaCl.

Table 1
Quantitative and qualitative estimation of isolated total RNA from flax tissues after 0 hour, 6 hours and 12 hours of salt stress

NaCl Conc. (mM)	*Time (Hrs)*	*Replicate*	*Concentration (ng/µl)*	*Mean ± SE (ng/µl)*	*Absorbance (OD 260/280)*	*Absorbance (OD 230/260)*
100 mM	0 hr	S1	1932.2	1957.6 ± 27.6	2.18	2.43
		S2	1927.8		2.19	2.36
		S3	2013		2.18	2.43
	6 hrs	S1	1784.6	1716.7 ± 42.4	2.18	2.45
		S2	1638.5		2.16	2.44
		S3	1727		2.16	2.46
	12 hrs	S1	1664	1669.3 ± 9.02	2.18	2.38
		S2	1657		2.2	2.43
		S3	1686.9		2.19	2.39
200 mM	0 hr	S1	1459.4	1473.3 ± 10.04	2.18	2.43
		S2	1492.8		2.21	2.45
		S3	1467.7		2.18	2.4
	6 hrs	S1	1235.5	1294.1 ± 71.7	2.19	2.4
		S2	1437		2.18	2.39
		S3	1210		2.17	2.31
	12 hrs	S1	1119.6	1176.2 ± 28.9	2.18	2.37
		S2	1194		2.11	2.32
		S3	1215		2.1	2.23
250 mM	0 hr	S1	1784	1800.3 ± 41.5	2.1	2.46
		S2	1879		2.19	2.44
		S3	1738		2.19	2.36
	6 hrs	S1	1589	1593.3 ± 47.6	2.18	2.3
		S2	1513		2.17	2.39
		S3	1678		2.21	2.34
	12 hrs	S1	1101	1163 ± 46.4	2.18	2.21
		S2	1254		2.19	2.24
		S3	1134		2.11	2.23
300 mM	0 hr	S1	1787	1847.6 ± 35.5	2.16	2.43
		S2	1845.8		2.19	2.38
		S3	1910		2.18	2.46
	6 hrs	S1	828	879.5 ± 27.4	2.18	2.44
		S2	889		2.17	2.45
		S3	921.6		2.16	2.43
	12 hrs	S1	681	651.8 ± 27.1	2.15	2.36
		S2	597.5		2.17	2.43
		S3	677		2.11	2.45

reduction in RNA homeostasis due to high osmotic stress of 300mM NaCl might be the reason for which plants were unable to recover from this high saline shock (Fig. 1-4A,B,C).

Besides quantity of RNA, the quality of isolated total RNA from stressed as well as control samples was checked by spectrophotometric analysis (Table 1). The quality of total RNA extracted from stressed samples as estimated by ratio of absorbance at 260/280 and 260/230 was comparable to that of control samples. Mean absorbance ratio at 260/280 and 260/230 for control samples was observed to be 2.18 and 2.41 respectively whereas for stress induced samples it was 2.16 and 2.36 respectively (Table 1). This shows that though the yield of RNA decreased with increasing duration of stress, the isolated RNA was of good quality and amenable for down-stream molecular studies such as cDNA synthesis and semi-qRT-PCR (Fig 3).

To further check the amenability of stressed samples for downstream molecular applications, cDNA was successfully synthesized from isolated total RNA of stressed as well as control samples. Subsequently, the synthesized cDNA was used to amplify the house-keeping gene "Actin" using gene specific PCR. The rationale of using actin gene was that it has been reported to be the most common housekeeping gene used for normalization in qRT-PCR analysis [28] as the expression of this gene is not altered by any stress. Since, equal amount of total RNA was used for cDNA synthesis, amplifications corresponding to actin with equal intensity were obtained with both stressed and control RNA samples. Such pattern of amplification was expected owing to

housekeeping activity of actin gene and its transcripts are present in the same amount irrespective of the tissue type and stress induction. Additionally, it also reflects that the stress induction process in flax was optimum and RNA quality remained good over a range of salt concentrations as evidenced by successful cDNA synthesis and semi qRT-PCR (Fig 3 A,B,C,D).

Salinity is an increasingly important agricultural problem. Globally, salinization is expected to cause devastation of more than 50% of all arable lands by the year 2050 [29]. It inflictsa combination of dehydration/osmotic-related cellular stress and damage due to high sodium ions that eventually affects plant growth and crop production. In addition to epigenetic changes [30], many a times drought and salt stress inflict common cellular damage as both stressesconverges at one point i.e. reduce the ability of plants to uptake water. However, salt stress is more deleterious as it causes salt specific damage in addition to water deficit. The targets of saline shock are membrane proteins involved in transport, metabolism, photosynthesis,cell structure, and signal transduction [31]. To get a deep insight into the biology of these processes in flax, we reported here the methodology of salt stress infliction and stage of symptom elicitation. The salinity treatment induced massive changes in the flax transcriptome but did not deteriorate the RNA quality. Our results suggest that 250 mMNaCl concentration of 12 hours duration is optimum for imparting saline shock in flax.The same can further be used to capture novel candidate genes differentially expressed during salt stress in flax.

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