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Semi-quantitative analysis of transcript accumulation in response to drought stress by *Lepidium latifolium* seedlings

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Cross-amplification of five *Arabidopsis* abiotic stress-responsive genes (*AtPAP, ZFAN, Vn, LC4* and *SNS*) in *Lepidium* has been documented in plants raised out of seeds pre-treated with potassium nitrate (KNO_3) for assessment of enhanced drought stress tolerance. cDNA was synthesized from *Lepidium* plants pre-treated with KNO_3 (0.1% and 0.3%) and exposed to drought conditions (5% and 15% PEG) at seedling stage for 30 d. Transcript accumulation of all the five genes were found suppressed in set of seedlings, which were pre-treated with 0.1% KNO_3 and were exposed to 15% PEG for 30 d. The present study establishes that different pre-treatments may further enhance the survivability of *Lepidium* plants under conditions of drought stress to different degrees.

Introduction

The genus *Lepidium* is one of the largest genera of the monophyletic Brassicaceae family with more than 150 species distributed worldwide.[1] *Lepidium latifolium* (commonly called as Pepperweed, Pepperwort or Peppergrass) is an invasive plant[2] of western Asia and southeastern Europe and currently distributed from Norway in the west to up to western Himalayas in the east.[3] In high-altitude cold-arid Ladakh area of Western Himalayas, leaves of *Lepidium latifolium* are consumed as vegetable and salad by the natives.[4] In addition to its agronomic, economic and ecological importance, *Lepidium* is also known for its high medicinal value and used in treatment of stomach related disorders.[5]

Each mature plant of *Lepidium* produces thousands of seeds each year,[6] however only a small percentage of seeds survive.[7] The inherent dormancy in the *Lepidium* seeds further delays the onset of advanced generation by a few months. Dormancy may be exogenous or endogenous in nature and a number of molecular to environmental signals may be required to overcome it. However, several physio-chemical pre-germination treatments are known that can break the dormancy by acting as direct or indirect artificial endo/exogenous signals for seed germination.[8] Interestingly, most of these methods are simple, low-cost and can be handled by relatively unskilled manpower. Seed pre-treatments not only improve seed germination rate, they also result into faster and synchronous seed germination and have often reported to improve the matured plant's ability to tolerate environmental stress.[9] *Lepidium*, despite its occurrence in conditions of abiotic stress, has escaped the focus of the scientific community. Till date, there are only a few reports on *Lepidium* pre-germination seed treatments[10-12] or characterization of the stress responsive genes.[4,13-15] To improve the efficiency of seed germination and characterize the gene expression behavior of *Lepidium* plants away from their natural habitat, we have applied a number of seed pre-treatments and subsequently assessed the performance of plants under drought stress conditions. As little molecular data of *Lepidium* exists in public domain, known stress responsive genes from *Arabidopsis thaliana* have been cross-amplified in *Lepidium*.

Results and Discussion

Pre-treatments. Pre-treatments were classified on the lines of types of dormancy. Certain treatments like ionic solutions, salts, acids, etc. are primarily used to overcome exogenous dormancy, while phytohormones like GA3, hot and cold water stratifications were generally used to overcome endogenous dormancy. Among the various methods used to overcome exogenous dormancy, seed pre-treatment with salts could improve seed germination rates, as they stick to cell surfaces and thereby induce osmotic pressure on the cytosol.[16] Most saline pre-treatments of the seeds have been based on NaCl and KNO_3.[8,17,18] We found that concentrations of KNO_3 improved both seed germination by 5-fold, as well as the germination velocity (Timson's index) in comparison to control (**Fig. 1**). Timson's index is effective in evaluation of germination

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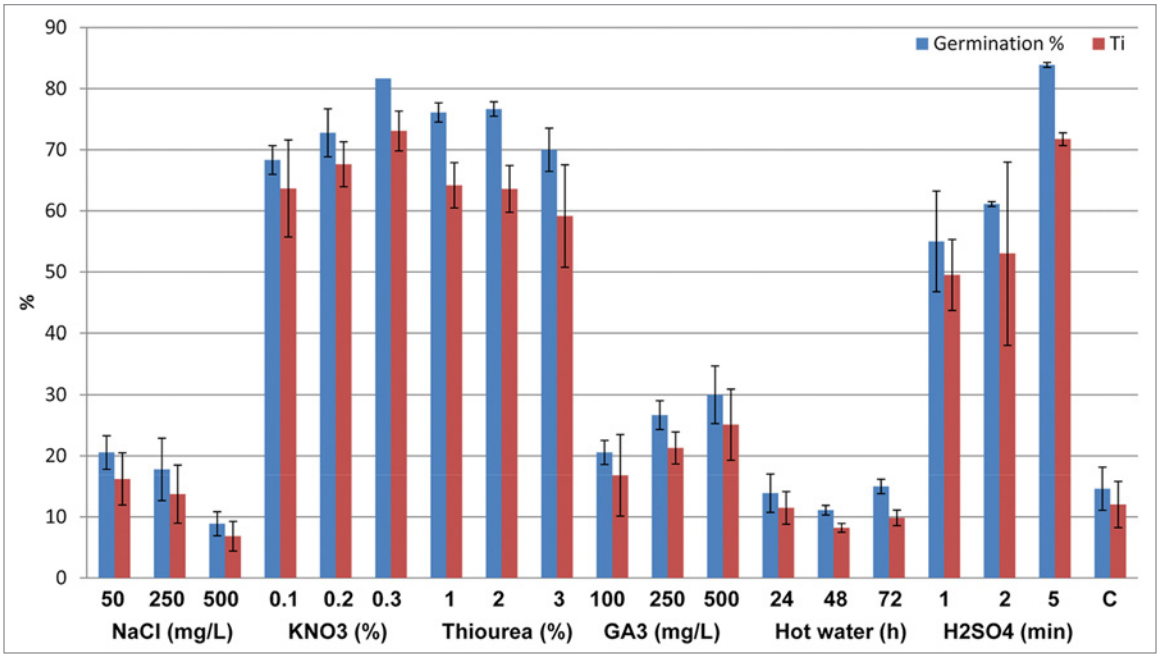

Figure 1. Germination percentage and Timson Index (Ti) of *Lepidium latifolium* L. seeds as influenced by various pre-sowing seed priming treatments including NaCl (50, 250, 500 mg/L), KNO_3 (0.1, 0.2, 0.3%), thiourea (1, 2, 3%), GA3 (100, 250, 500 mg/L), hot water (65°C) stratifications (24, 48, 72 h) and sulphuric acid (98% for 1, 2 and 5 min). A set of seeds without pre-sowing treatments was considered as control (C).

considering the time to 50% germination and the final percentage of germination obtained. Pre-treatments with NaCl (50 and 250 mg/L) did not show any major improvement in the germination percentage and seed treatment with 500 mg/L NaCl had rather a negative effect on seed germination. In a previous study from this laboratory, effects of thiourea treatments on germination percentage were found comparable to KNO_3 treatments[8] on seeds of *Hippophae salicifolia*. The same has been found true in the present study for *Lepidium latifolium* as well (**Fig. 1**). However, the germination velocity was more profound in case of KNO_3 treatment as compared with thiourea treatments. Pre-soaking of *Lepidium* seeds in 98% H_2SO_4 for 5 min was found as the best pre-germination treatment in terms of obtaining the highest germination percentage (**Fig. 1**). Shorter soaking times in 98% H_2SO_4 i.e., 1–2 min. had lesser effect on improvement of germination percentage.

For many species, the seeds buried under soil germinate by themselves after remaining dormant for a particular season i.e., winter or summer months, as part of their seasonal patterns of dormancy behavior.[7] Such seeds show a form of dormancy called as endogenous dormancy, which is either controlled by environmental signals or by relative levels of phytohormone concentrations inside the cells. In laboratory, these conditions can be easily mimicked by soaking the seeds in hot or cold water. Alternatively, applications of phytohormones may produce the desired effect. In the present study, hot water treatments had no positive effect on seed germination (**Fig. 1**) and cold water treatments completely inhibited the seed germination, which strongly suggested that dormancy in *Lepidium* is controlled by exogenous factors and there is little, if at all, effect of high or low temperatures under natural conditions.[12] Our results were further strengthened by the observation that different GA_3 concentrations had relatively small effect on seed germination (**Fig. 1**).

Morphometric analysis of drought stress tolerance. Among all the treatments described above, concentrations of KNO_3 made most uniform impact on the germination characteristics of *Lepidium* seeds. It is well established that its application impact the performance of plants in a positive manner and also increase their water use efficiency (WUE).[19,20] We transplanted the plantlets obtained after KNO_3 treatment to pots filled with vermiculite and further assessed for their ability to tolerate drought stress. From each set of KNO_3 treated seedlings, three subsets were subjected to drought stress by 5 and 15% PEG. A control set of equal number of seedlings were irrigated with distilled water. These conditions were maintained for 15 d, after which further subsets were drawn and exposed to 5 or 15% PEG and control subset irrigated with water for another 15 d, as detailed in **Figure 1**. The state of survivability of the plants was assessed based on leaf counts and the lengths of their midribs. The control set (Experimental set up series 1; **Table 1**) did not either survive the duration of the experiment or wherever some plants survived, they showed stunted growth. Interestingly, plants showed better adaptability to stress in initial period of stress after transplanting. As the latter 15 d stress of 5% PEG only seemed to have been acclimated by the plants (**Table 1**). The behavior of plants in terms of elongation of aerial parts in response to increasing PEG concentrations vary with species and evidences exist that point such responses to be age-dependent as well.[21] While certain plants like tomato show positive correlation with increase in lengths of aerial parts under increasing PEG concentrations,[22] others like salvia show negative correlation.[23] Interestingly, in case of *Lepidium*, 5% PEG treatments showed increase in shoot

length, but higher concentrations resulted in antagonist response. In general, the drought stress inhibits cell enlargement and affects various physiological and biochemical processes.[21] Among different concentrations of seed pre-treatments, 0.1% KNO_3 primed seeds showed better adaptability to drought stress in later stages.

Molecular analysis of drought stress tolerance. Drought is a complex stress, in whose response many bio-molecules interact including nucleic acids, proteins, carbohydrates, lipids, hormones, free radicals, etc. In fact, drought affects almost all aspects of plant cell metabolism. As the plant responses to different stresses have cross-talk among themselves at more than one level of metabolism, studies targeted onto any one may have direct implications in understanding other stresses as well.[24] Evidences exist, wherein drought stress has been found related to stresses caused by salinity, temperature extremes, pH extremes, pathological reactions, senescence, growth, development, UV-B damage, wounding, embryogenesis, flowering, signal transduction and so on.[25] Thus, the signals of water scarcity drive the regulation of a number of plant genes. A good number of the genes are induced within a few minutes of receiving the signal and have been called as "early responsive genes." Transcription factors that further effect expression of other genes are mostly categorized into this category. The late or delayed responsive genes, in contrast are activated by stress more slowly and their expression is sustained. Fourteen primer pairs were designed for known stress responsive genes in *Arabidopsis* to amplify homologous genes in closely related *Lepidium*. Five of these genes, i.e., zinc finger protein coding gene (*ZFAN*), LHCA4 (*LC4*) encoding PSI type IV chlorophyll in *Arabidopsis*, vernalization related gene (*Vn*), plastid lipid associated protein (*AtPAP*) and senescence related gene (*SNS*), were successfully amplified in *Lepidium*. In order to assess the efficiency with which KNO_3 treated plants acclimatize to varying degrees of drought stress, the expression levels of each of these five genes were studied based on end-point fluorescence and using cDNA from ten experimental set ups exposed to varying degrees of stress.

The zinc finger protein coding gene (ZFAN) acts as a transcription repressor during abiotic stress and is an early responsive gene. The effect of 0.3% KNO_3 could be clearly seen in the ability of the plants to accumulate its transcripts during drought stress induced by PEG treatments (**Table 2**). The vernalization (*Vn*) and *LC4* genes, known to be responsive to cold stress, were found to have no significant differential expression in response to the drought stress. Only a minute amount of transcript accumulation was recorded for *Vn* gene in the plants matured out of 0.3% KNO_3 treated seeds, whereas, no net significant difference could be observed in accumulation of *LC4* gene in differently treated plants samples under study. *AtPAP* is known to get up-regulated during abiotic stress. Interestingly, we observed that this gene overexpressed by three times in the plants maturing out of 0.3% KNO_3 treated seeds compared with the plants that were matured out of 0.1% KNO_3 treated seeds (**Table 1**). However, in a given set of water vs. PEG treatment analysis, no change could be observed in the finally accumulated amplified transcripts. *SNS* is expected to get up-regulated during the stress, but its expression was found more or less constant in all the samples (**Table 2**).

The abilities of the plants to perform well under stress after treatment with KNO_3 might have a direct relation with the plant's ability to utilize the constituent ions (K^+ and NO_3^-) separately. At cellular level, Potassium (K^+) substantially affects enzyme activation, protein synthesis, photosynthesis, stomatal movement and water-relation (turgor regulation and osmotic adjustment), signal transduction, intracellular movement of macromolecules (especially sugars) and many other processes.[26] It is known to enhance plant growth, yield and drought resistance in different crops under water stress conditions.[27-30] Further, Nitrate ions promote water use efficiency (WUE), photosynthesis and biomass production rates.[31] Applied together, potassium and nitrate ions produce synergistic effect,[32] enhancing survival of seeds and subsequent drought tolerance.

Pre-treatment of *Lepidium* seeds with KNO_3 (0.1–0.3%) has thus been found an effective method to enhance the germination percentage and ensure its growth under arid conditions. For a cold arid climate like that of Ladakh, where *Lepidium* leaves are cooked as vegetables, such practices might be important to ensure the availability of food material for the local population. Similar applications in other food crops can also be applied to ensure their harvest even under conditions of water deficiency, especially in countries with lesser acceptability of genetically modified crops and foods.

Material and Methods

Pre-germination treatments. Mature seeds of *Lepidium latifolium* L. were collected from Leh, India (34° 8' 43.43" N, 77° 34' 3.41", 3505 m asl) and transported to our laboratory in Haldwani, India (29° 13' 11.946" N, 79° 31' 11.9244" E, 443 m asl) in zip lock bags. Seeds were disinfected by immersing in 0.5% sodium hypochlorite solution for 2 min followed by rinsing thoroughly with distilled water four times. Various pre-treatments, as described earlier[8] and depicted in **Figure 2** were performed in triplicate sets of 60 seeds each. Emergence of radicle was recognized as the event of seed germination and the plants were grown for 30 d in 16/8 h light/dark cycle at 25°C. Besides final germination percentages, the rate of germination (or germination velocity) was also calculated according to a modified Timson's velocity index or Timson Index:[32] $\Sigma G/T$, where "G" is the percentage of seeds germinated after 2 d interval and "T" is the total time of germination. The overall line of work is described in **Figure 2**.

Analysis of gene expression. Primers of 14 genes (**Table 3**) from *Arabidopsis* were used in PCR with cDNA of *Lepidium latifolium* as template. Out of these, five genes were amplified successfully, which were assessed further using semi-quantitative PCR analysis to study transcript accumulation. *Actin* gene was used as an internal control for assessment of differential regulation of the five genes under analysis.

The PCR reaction was performed in a total volume of 25 μl, which was constituted of 10 pmol of gene-specific primers (**Table 2**), 20 μM of dNTPs mix, 1.5 mM $MgCl_2$, 1× *Taq* polymerase buffer and 1 U *Taq* polymerase (Genei, Merck Millipore). Thermal cycling conditions constituted of initial denaturation

Table 1. Pre-treatment of lepidium seeds and subsequent exposure to drought stress

Pre germination treatment	Post-transplant treatment-1 (1–15 d)	Post-transplant treatment-2 (16–30 d)	Performance	Experimental set up No.
H_2O	H_2O	H_2O	Death	1.1.1
		5% PEG	Stunted growth	1.1.2
		15% PEG	Stunted growth	1.1.3
		30% PEG	Death	1.1.4
	5% PEG	H_2O	Death	1.2.1
		5% PEG		1.2.2
		15% PEG		1.2.3
		30% PEG		1.2.4
	15% PEG	H_2O	Death	1.3.1
		5% PEG	Stunted growth	1.3.2
		15% PEG	Stunted growth	1.3.3
		30% PEG	Death	1.3.4
0.1% KNO_3	H_2O	H_2O	Good growth	2.1.1
		5% PEG	Good growth	2.1.2
		15% PEG	Stunted growth	2.1.3
		30% PEG	Death	2.1.4
	5% PEG	H_2O	Good growth	2.2.1
		5% PEG	Good growth	2.2.2
		15% PEG	Stunted growth	2.2.3
		30% PEG	Death	2.2.4
	15% PEG	H_2O	Good growth	2.3.1
		5% PEG	Death	2.3.2
		15% PEG	Stunted growth	2.3.3
		30% PEG	Death	2.3.4
0.2% KNO_3	H_2O	H_2O	Good growth	3.1.1
		5% PEG	Good growth, but poor health	3.1.2
		15% PEG	Good growth, but poor health	3.1.3
		30% PEG	Death	3.1.4
	5% PEG	H_2O	Death	3.2.1
		5% PEG		3.2.2
		15% PEG		3.2.3
		30% PEG		3.2.4
	15% PEG	H_2O	Death	3.3.1
		5% PEG		3.3.2
		15% PEG		3.3.3
		30% PEG		3.3.4

Note 1. The visible inspection of "good growth" and "stunted growth" is relative to each other. A statistically significant difference of average length of midrib of longest leaf > 7 cm was called "good growth", while average length of midrib of longest leaf < 5 cm was called "stunted growth." In none of the data sets, average lengths of midrib of longest leaves were found in the range 5–7 cm. 2) "Poor health" describes the condition of relatively more number of yellowing and senescent leaves on average in a particular data set compared with healthy and green leaves.

of 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, re-annealing for 30 sec at 55°C followed by elongation at 72°C for 30 sec. A final elongation for 5 min was performed at 72°C. Amplicons were run on 2% agarose gel and stained with ethidium bromide (0.1 ppm) in 1× TAE buffer. The gel was documented on a phosphoimager (Typhoon Trio+ Imagers, GE Healthcare) and analyzed using ImageQuant software (GE Healthcare). Molecular sizes and their quantities were calibrated against the known values of various DNA bands of the GeneRuler™ 100 bp Plus ladder (Fermentas). The background intensity of the gel material was suitably subtracted prior to analysis.

Table 1. Pre-treatment of lepidium seeds and subsequent exposure to drought stress

0.3% KNO_3	H_2O	H_2O	Good growth	4.1.1
		5% PEG	Good growth, but poor health	4.1.2
		15% PEG	Good growth, but poor health	4.1.3
		30% PEG	Death	4.1.4
	5% PEG	H_2O	Good growth	4.2.1
		5% PEG	Good growth, but poor health	4.2.2
		15% PEG	Stunted growth	4.2.3
		30% PEG	Death	4.2.4
	15% PEG	H_2O	Good growth	4.3.1
		5% PEG	Stunted growth	4.3.2
		15% PEG	Death	4.3.3
		30% PEG	Death	4.3.4

Note 1. The visible inspection of "good growth" and "stunted growth" is relative to each other. A statistically significant difference of average length of midrib of longest leaf > 7 cm was called "good growth", while average length of midrib of longest leaf < 5 cm was called "stunted growth." In none of the data sets, average lengths of midrib of longest leaves were found in the range 5–7 cm. 2) "Poor health" describes the condition of relatively more number of yellowing and senescent leaves on average in a particular data set compared with healthy and green leaves.

Table 2. Transcript accumulation of abiotic stress related genes in response to different post-transplant treatments

Pre-germination treatment	Post-transplant treatment-1 (1–15 d)	Post-transplant treatment-2 (16–30 d)	Transcript accumulation (Folds)				
			AtPAP	*SNS*	*ZFAN*	*Vn*	*LC4*
0.1% KNO_3	H_2O	H_2O	1.00	1.01	2.37	0	1.01
		15% PEG	0.83	0.99	1.62	0	1.00
	5% PEG	H_2O	0	1.05	1.59	0	1.11
		15% PEG	0.87	0.98	1.56	0	1.12
	15% PEG	H_2O	1.68	1.27	1.96	0	1.21
		15% PEG	0	1.01	0	0	0.93
0.3% KNO_3	H_2O	H_2O	0	0.92	0	0	0
		15% PEG	2.97	1.26	3.39	0.28	1.21
	5% PEG	H_2O	2.52	1.22	0.90	0.16	1.02
		15% PEG	2.75	1.05	3.61	0.21	1.26

Actin transcript accumulation was recorded 41.21 ng under control conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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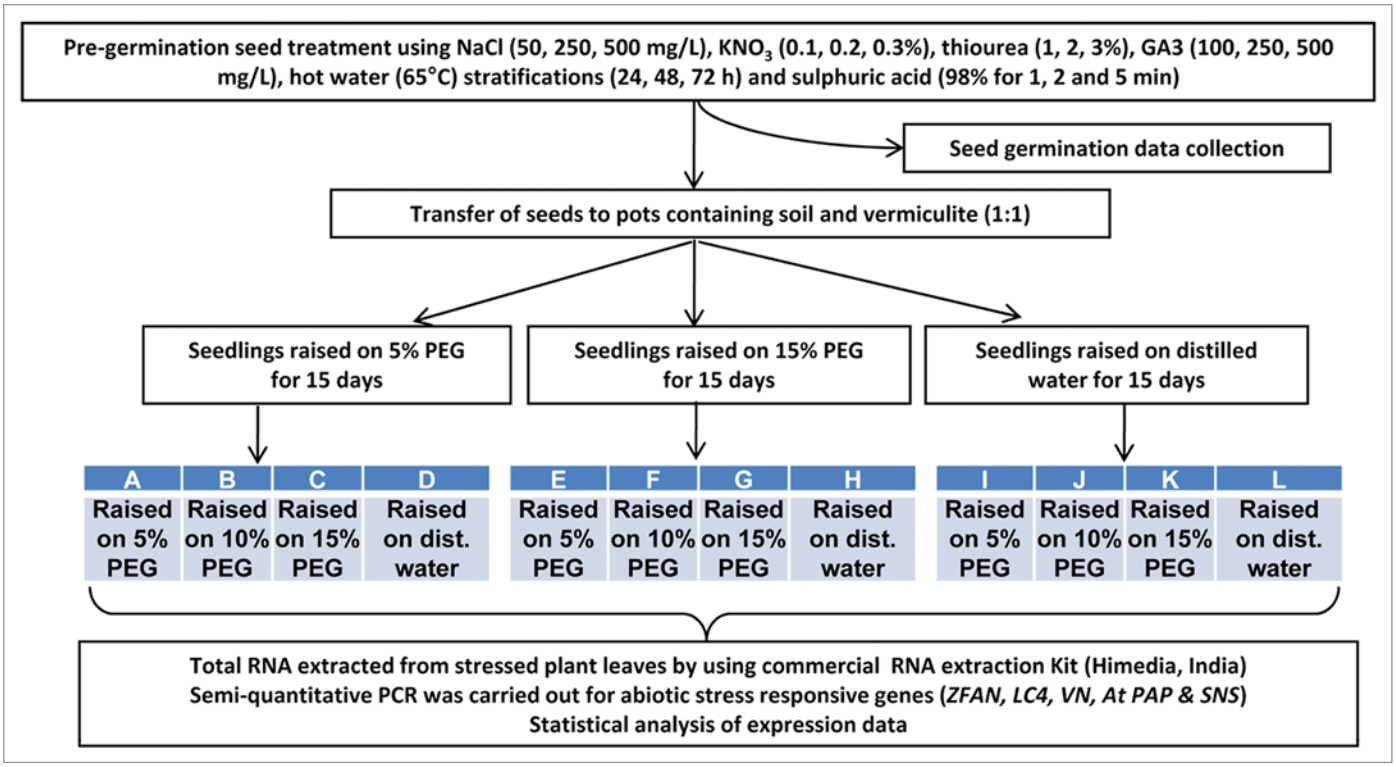

Figure 2. Experimental out line of pre- and post-germination treatments in *Lepidium latifolium*.

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Table 3. List of primers used for cross-amplification of *Arabidopsis* genes and in semi quantitative transcript accumulation of different abiotic stress responsive genes

Gene name	Description	Forward primer (5'-3') Reverse primer (5'-3')
CABP	Calcium binding protein coding gene	ATGAAGGGGA GGATTTCGAA G TCATGAAACC AACTGAGAGC
ZFAN	Zinc finger protein coding gene	AGTGGCTCAG AGGAAAACAA CTCA AATCCT CTGAACTTTA TCC
CAL	Calcinurin like gene	ATGAAGGATC CGAAACTAAG TTAGTCGATC TCCACAATTT GA
LC4	LHCA4 gene encodes the photosystem 1 type IV chlorophyll a/b-binding protein complex	ATGGCTACTG TCACTACTCA TGTTA GCCTC TGAGAGTCTG GAC
SEN	Senescence related gene	CCTCTCGAAT CTGCTTTTCG CAGTC GCCAGGACGA GAGTTCCTGT CCGT
LEY	Leafy gene	ATGGATCCTG AAGGTTTCAC CAG CAGAGCTCCT AGAATCGCAA AGTCGTCG
ZIP	Zinc transporter gene	CCTGAGGGAA AATGCAGAC CCGAGAAATC CCCTCGAAGA
SDR	Short chain dehydrogenase related gene	TGGATGGTGC TGGCTCTGGA CTCGTCCTGT TCTCTGCCCA GTTCC
VN	Vernalization related gene	ATGTGTAGGC AGAATTGTCG C TTACTTGTCT CTGCTGTTAT TG
At PAP	Plastid Lipid associated protein coding gene	TTGCGGCATT TAGATTCTCC AGCT AAATTT CTCGGATGTG CAC
SNS	Senescence related gene	ATGGCTTCGT ATTACTCTGG T CTAAGCCACG ACGAGAGTTC
AP2	APE2 transporter gene	ATGGAGTCAC GCGTGCTGT TCAGCTGCTT TCTATGCTTT C
C4	Cbf4 gene	ATGAATCCAT TTTACTCTAC ATTCC TTACTCGTCA AAACTCCAGA GTC
SK	Serine/Threonin kinase gene	ATGACTGATG AGGTAGACGG TTAGTCGAAG CTCGAAGAAC G