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Identification, phylogenetic analysis of NAC transcription factor family in barely and expression pattern of *HvNAC* involved at salinity

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Abstract

Plants activate some mechanisms that increase their tolerance to stresses. The perception of stress signals and then signal transduction, leads to the activation of various physiological responses. In signal transduction networks, various transcription factors function as molecular switches for gene expression. NAC transcription factors are involved in various biotic and abiotic stresses, including stress-induced flowering and lateral root development. Considering that many of the NACs in barley will be revealed as key regulators of abiotic stresses, and also understanding the precise functions of the *NAC* genes can be of valuable importance for the improvement of barely, studies of this family are momentous. In the present study, a comprehensive analysis of *NAC* gene family in barely was performed. A total of 82 barely NAC proteins were identified, and phylogeneticly clustered into 5 subfamily and 13 distinct subgroups. The motif compositions were conserved among the subfamilies. It was resulted that high amounts of Proline and Alanine in the studied HvNAC proteins might have an association with the response of barely against salinity. The largest number of *NAC* genes (28%) belonged to chromosome 7 with 23 genes. Using qRT-PCR, It was investigated the expression profile of one *HvNAC* across two barley cultivars at two tissues and two saline treatments. Statistical analysis revealed a significant expression increase ($P< 0.01$) in response to salinity in tolerant cultivar Afzal.

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Introduction

In response to salinity, plants activate some mechanisms that increase tolerance to salinity. Osmotic regulation is an important mechanism for cellular homeostasis in salt stress conditions. The early occurrence of the adaptation of plants to stresses includes the perception of stress signals and subsequent signal transduction, leading to the activation of various metabolic and physiological responses (Valliyodan and Nguyen, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Tran *et al*., 2007). In signal transduction networks that are included in the alteration of stress signal perception to stress-responsive gene expression, various transcription factors (TFs) contained in stress-responsive promoters function not only as molecular switches for gene expression, but also as terminal points of signal transduction in the signaling processes. Regulation of gene expression plays an essential role in plant response to environmental stresses. Recently collected evidence demonstrates that many families of TFs, including the *MYB*, *zinc-finger*, *bZIP*, *DREB*, *WRKY* and *NAC* families, indirectly or directly regulate plant defenses and stress responses (Mukhopadhyay *et al*., 2004; Mao *et al*., 2011; Shen *et al*., 2012; Mao *et al*., 2012).

The NAC (NAM, ATAF, and CUC) transcription factors were originally identified from consensus sequences from petunia NAM, *Arabidopsis thaliana ATAF* and *CUC2*. The first reported *NAC* genes were *CUC2* from Arabidopsis (Aida *et al*., 1996) and *NAM* from petunia (Souer *et al*., 1996) that participate in shoot apical meristem development. The NAC is one of the important transcription factor families found only in plants. Members of this family are identified by the presence of the NAC domain. This domain consists of five subdomains that make up motifs for both DNA-binding and protein-protein interactions (Nuruzzaman *et al*., 2010). Proteins of this family are characterized by a highly conserved DNA-binding domain, known as the NAC domain, in the N-terminal region. The C-terminal of NAC proteins, which usually contains a TF domain, is highly different both in length and sequence (Ooka *et al*., 2003). The *NAC* gene family, represented by 152 genes in tobacco (Rushton *et al*., 2008), 163 in poplar (Hu *et al*., 2010), 117 genes in Arabidopsis and 151 in rice (Nuruzzaman *et al*., 2010), 152 gene in soybean (Le *et al*., 2011) and 74 gene in grape (Wang *et al*., 2013). NAC proteins are involved in different plant developmental processes and various biotic and abiotic environmental stresses, including stress-induced flowering and lateral root development (Xie *et al*., 2000; He *et al*., 2005), pattern formation in embryos and flowers, formation of secondary walls and leaf senescence (Guo and Gan, 2006; Zhong *et al*., 2006, 2007; Mitsuda and Ohme-Takagi, 2008). *ANAC*$_2$ is involved in response to ABA, salt stress, and lateral root development (He *et al*., 2005). *ATAF*$_1$ and *ATAF*$_2$, along with a barley gene known as *HvNAC6*, play negative roles in response to drought stress (Delessert *et al*., 2005; Lu *et al*., 2007). Many *NAC* genes, such as *AtNAC072*, *AtNAC019*, *AtNAC055* from *Arabidopsis* (Tran *et al*., 2004; Fujita *et al*., 2004), and *BnNAC* from *Brassica* (Hegedus *et al*., 2003) were reported to be involved in the response to environmental stresses. Thus, it is important to recognize and functionally characterize NAC families from economically important agricultural crops and to use functional NAC genes for producing these crops with improved tolerance to stresses.

Barley is an important agricultural crop and has also been used as the model species for many researchers in small grain cereal crops. Also, barley has an extensive EST data base containing samples from a wide variety of developmental stages and tissues, as well as an increasing amount of microarray data. Considering that many of the NAC TFs in barley will most likely be revealed as key regulators of abiotic stresses, as they are in other species, studies of this family are appropriate. This work presents a genome-wide characterization of NAC TFs in *Hordeum vulgare*. The molecular features of *HvNAC* genes, such as genome distribution and conserved motif prediction, multiple alignments construction of phylogeny were performed. Furthermore,

we have analyzed the expression pattern of one candidate *HvNAC* gene for their tissue-specific expressions by quantitative real-time (RT-qPCR) analysis in responses salinity.

Materials and methods

Identification of HvNAC sequences

A total of 150 *HvNACs* encoding amino acid sequences belonging to *Hurdeum vulgar* were obtained from plant transcription factor database (http://planttfdb.cbi.pku.edu.cn). Decrease redundancy tools (web.expasy.org/decrease-redundancy) and elimdupes (http://hcv.lanl.gov/content/sequence/elimdupes/elimdupes.html) were utilized for removing of redundant sequences. Finally, 82 non-redundant amino acid sequences were identified and analyzed for the confirmation of conserved NAC domain by using pfam Sequence Search (http://pfam.xfam.org/) and MEME (http://meme-suite.org/).

Multiple alignments and construction of phylogeny

Multiple sequence alignment (MSA) of full-length protein sequences was performed with Muscle using CLC Genomics Workbench 5 software (CLC bio, Denmark) with default parameters. The *A. thaliana* NAC protein, *AtNAC2* (AED90739.1), was used as out-group for the phylogenetic tree. The phylogenetic tree of aligned HvNAC proteins was constructed using CLC Genomics Workbench via the Neighbor-Joining (NJ) method and bootstrap analysis. The constructed phylogenetic trees were visualized with FigTree v1.4.2 software.

Genome distribution and motif prediction

The amino acids of all HvNACs were used as query sequences for TBLASTN search of the *H. vulgar* sequences against the EnsemblPlants database (http://plants.ensembl.org/index.html) using default settings for position determination of their genes on barely chromosomes. Genes were distributed onto barely chromosomes according to their order from the first chromosome to seventh chromosome. Conserved motifs of HvNAC proteins were identified using Multiple Expectation Maximization for Motif Elicitation (MEME) program (http://meme-suite.org/) (Timothy *et al.*, 1994) with following parameters: any number of repetitions of a motif; maximum 10 motifs; and optimum width of the motif≥15. The motif profile for each protein is presented schematically.

Molecular and physicochemical parameters

The barely NAC proteins physicochemical parameters such as isoelectric point (PI) and molecular weight were determined using the ProtParam at ExPASy website (http://us.expasy.org/tools/protparam.html). The sub-cellular localization of barely NAC proteins was investigated using Multi-label protein mGOASVM database (http://bioinfo.eie.polyu.edu.hk/mGoaSvmServer2/mGOASVM_v2.html). Amino acid composition (AAC) of the HvNAC protein sequences was calculated using the ProtParam tool at ExPASy website (http://web.expasy.org/protparam). Subsequently, the AAC was subjected to one way analysis of variance. Then, the averages of the AACs were compared according to the least significant difference (LSD) method using SPSS software release 19.0.0 (α=0.05).

Plant materials and treatments

Two barely cultivars Afzal (as tolerant) and Fajr (as susceptible) were studied for expression of *HvNAC* (MLOC_37104.2 or JX855805.1) gene in different tissues under salinity stress. Two cultivars were grown at 28 ±2°C and 16 h light/8 h dark photoperiod in greenhouse conditions. They were watered two times a week. Salinity (300 mM NaCl) was carried out in two barely cultivars, at four-leaf stage for 10 days. The roots and leaves of both control and stressed plants from 45-day-old plants were harvested and stored at -80°C for RNA isolation.

RNA extraction and cDNA synthesis

Total RNA was extracted from leaves and roots of both control and stressed plants using extraction kit RNX-Plus solution (Inc. Sina clone. Iran). RNA

concentration was measured using nanodrop spectrophotometer. DNAase I (Inc. Fermentas) was used to remove genomic DNA contamination in samples of RNA, 1 µg RNA was used for the first strand cDNA synthesis using reverse transcription Kit (Cat. No. 205311, Co. Qiagen) followed by reverse transcription. Then, cDNA was used for gene expression analysis.

Quantitative Real-Time PCR Assay and data analysis

Quantitative RT-PCR was performed using SYBR [Premix Ex TagII, Takara Co, Japan) in the Bio-Rad real time PCR system according to the manufacturer's instructions. Each 20 µl PCR reactions contained 10 µl SYBR Premix Ex Tag, 0.5µl each of primers (10 µM), 2 µl DNA templates, and 7 µl ddH2O. Gene-specific primers for candidate *HvNAC* (MLOC_37104.2 or JX855805.1) were designed using Primer3 software (Forward: 5-GACCCCCTTAGGCGCCTTG-3, Reverse: 5-TACCCCAACGGCTCCCGACC) and used for qPCR analysis. The housekeeping gene *18SrRNA* was used as the internal control for normalization of the amount of cDNA in each qPCR reaction. QRT-PCR was performed with thermal conditions: 94°C for 3 min, 40 cycles of 94°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. Finally the data were analyzed using the method of $2^{-\Delta\Delta CT}$ according to Livak *et al* (2001). For each individual sample in real-time PCR, two technical replications from the mean of three biological replications were conducted. One-way ANOVA and Duncan's test (P-value≤0.05) were used to determine the significance differential expression patterns between tissues and/or treatments.

Results and discussion

Amino acid composition of HvNAC proteins

In silico recognition and characterization of the genes in different organisms under various conditions got importance due to growing data (Barozai and Abdul-Wahid, 2012). Study of the amino acid composition revealed that the frequencies of amino acids were statistically ($p<0.01$) different among HvNAC proteins (Table 2).

Comparisons between mean percentages of the amino acids (Fig. 4) showed that Alanine and Glycine (class a) were the most frequent residues followed by serine (b), Proline (bc), Leucin (c) and Arginine (d), respectively, while, Cysteine and Tryptophan were in the lowest frequency. Nakashima and Nishikawa (1992) reported that the amino acid composition was different between cytoplasmic and extracellular peptides of membrane proteins.

Alanine and Arginine were preferentially sited on the cytoplasmic side, while the Threonine and Cysteine were sited on the extracellular side. Researches have shown that plants significantly accumulate Proline when subjected to environmental stresses (Routley, 1966), Also, Thomas and Shanmugasundaram (1991) believe that as an osmoregulation, Alanine is able to reduce the damage caused by salt stress. Therefore, high amounts of these two amino acids (Proline and Alanine) in the studied HvNAC proteins might have an association with the response of barely against salinity stress. Also, It was reported that Glycine-rich proteins have important roles in plants under abiotic and biotic stresses by regulating the gene expression post-transcriptionally (Mousavi and Hotta, 2005).

Physicochemical and biochemical properties of HvNAC proteins

The coding sequence lengths (CDs) of genes, protein amino acid No., protein MWs, PI values, and subcellular localization of these putative genes were analyzed and presented in Table 1. Gene CDs lengths ranged from 315 bp (MOLC_37364.2) to 2115 bp (MOLC_50406.5) and protein amino acid number ranged from 104 aa (MOLC_37364.2) to 704 aa (MOLC_50406.5). All deduced proteins had a predicted molecular mass from 12.03 kDa (MOLC_37364.2) to 78.25 kDa (MOLC_50406.5) and their predicted isoelectric points were very divergent, ranging from 3.86 (MOLC_20905.1) to 10.6 (MOLC_25708.2 and MOLC_67296.1).

Cellular localization is often an important factor in determining the protein function (Thamilarasan *et al*., 2014). Subcellular localization prediction made by mGOASVM showed that all of 82 HvNAC proteins were located in nucleus. It was reported that Transcription Factors are located only in the nucleus (Liu *et al*., 2013).

Chromosomal location and gene clusters of HvNAC genes

The physical position analysis of *HvNAC* genes showed that 82 barely *NAC* genes were distributed across all 7 linkage groups (7 chromosomes). Five *NAC* genes were situated on chromosome 1. Also, eighteen genes were assigned to chromosome 2, which fifteen of them were on q arm of chromosome as cluster.

Table 1. List of NAC transcription factor proteins in barely (*Hordeum vulgar* L.) along with their CDs and protein length, gene localization in chromosome, theoretical isoelectric point, and molecular weight.

Protein	AA	MW (KDa)	PI	CDs	Subcellular localization	Chr
MLOC_11017.1	193	21.69	10.1	582	Nuc	2q
MLOC_11732.1	442	49.46	6.77	1329	Nuc	2q
MLOC_12466.2	310	34.66	6.79	933	Nuc	2q
MLOC_12466.4	128	14.14	10.5	387	Nuc	2q
MLOC_13932.1	332	36.7	6.93	999	Nuc	7p
MLOC_15184.1	291	32.38	9.28	876	Nuc	7q
MLOC_15681.2	199	22.51	6.3	600	Nuc	2q
MLOC_15746.1	339	38.78	7.1	1020	Nuc	2q
MLOC_17128.1	370	41.37	6.96	1113	Nuc	7p
MLOC_19175.1	336	37.08	6.96	1011	Nuc	2q
MLOC_19933.1	313	34.35	6.51	942	Nuc	7q
MLOC_20905.1	521	56.63	3.86	1566	Nuc	6q
MLOC_21966.2	296	32.7	5.38	891	Nuc	2q
MLOC_25708.2	254	29.27	10.6	765	Nuc	7q
MLOC_36501.1	205	22.97	6.52	618	Nuc	2q
MLOC_36942.2	147	16.96	10.05	444	Nuc	2q
MLOC_37104.2	330	36.94	6.8	993	Nuc	5q
MLOC_37364.1	391	43.3	9.6	1176	Nuc	7p
MLOC_37364.2	104	12.03	6.5	315	Nuc	7p
MLOC_37843.1	262	29.28	4.8	789	Nuc	4q
MLOC_37844.1	217	25.16	6.7	654	Nuc	4q
MLOC_40602.1	144	15.69	8.7	435	Nuc	7p
MLOC_43934.1	401	43.56	5.3	1206	Nuc	2q
MLOC_4747.1	354	39.3	6.5	1065	Nuc	5p
MLOC_4952.1	347	38.74	6.25	1044	Nuc	2p
MLOC_50406.5	704	78.25	4.6	2115	Nuc	7p
MLOC_50895.3	655	72.27	4.44	1968	Nuc	4q
MLOC_5165.1	310	34.17	6.9	933	Nuc	6q
MLOC_53086.2	336	39.04	6.7	1011	Nuc	7p
MLOC_5374.1	346	38.94	7.24	1041	Nuc	7p
MLOC_53744.1	307	34.06	8.55	924	Nuc	7q
MLOC_55957.2	225	24.77	10.4	678	Nuc	5q
MLOC_56103.1	356	40.6	7.2	1071	Nuc	6q
MLOC_56769.2	323	36.04	7.6	972	Nuc	4q
MLOC_56943.2	365	41.08	6.56	1098	Nuc	7q
MLOC_57215.1	410	44.29	9.5	1233	Nuc	3q
MLOC_57427.1	350	38.96	7.8	1053	Nuc	4q
MLOC_58272.1	354	38.97	7.8	1065	Nuc	7p
MLOC_5832.1	405	43.82	5.7	1218	Nuc	5q
MLOC_58950.3	335	36.79	6.1	1008	Nuc	1
MLOC_59162.2	174	19.74	10.4	525	Nuc	3q
MLOC_59591.1	229	24.8	8.6	690	Nuc	7p
MLOC_60079.1	362	40.68	8.57	1089	Nuc	2p
MLOC_60153.1	167	18.38	9.8	504	Nuc	5p

MLOC_6066.1	482	53.3	6.3	1449	Nuc	3q
MLOC_61270.5	229	25.15	10.6	690	Nuc	4p
MLOC_63121.1	357	40.46	6.1	1074	Nuc	3p
MLOC_63743.3	163	18.25	10.3	492	Nuc	1
MLOC_64057.2	346	37.98	6.23	1041	Nuc	4q
MLOC_64240.2	304	33.3	9.4	915	Nuc	5p
MLOC_64644.3	122	13.51	4.35	369	Nuc	2q
MLOC_65101.1	355	38.7	9.36	1068	Nuc	4q
MLOC_65286.1	366	39.1	8.5	1101	Nuc	5q
MLOC_65400.2	187	21.3	9.5	564	Nuc	2p
MLOC_65456.1	356	39.01	8.2	1071	Nuc	4q
MLOC_65480.1	296	31.15	6.7	891	Nuc	7p
MLOC_65714.1	235	26.2	5.7	708	Nuc	7p
MLOC_66083.4	168	19.37	8.66	507	Nuc	2q
MLOC_66348.3	296	32.1	9.45	891	Nuc	5q
MLOC_66537.1	292	33.1	8.7	879	Nuc	3q
MLOC_66924.2	398	43.2	7.1	1197	Nuc	7p
MLOC_67250.4	272	30.8	10.1	819	Nuc	2q
MLOC_67296.1	237	25.6	10.6	714	Nuc	7p
MLOC_68185.2	313	34.5	9.8	942	Nuc	5q
MLOC_68284.1	299	33.2	7.2	900	Nuc	4q
MLOC_6901.1	394	42.4	6.4	1185	Nuc	1
MLOC_69621.2	392	42.3	6.7	1179	Nuc	1
MLOC_70929.1	316	35.6	6.6	951	Nuc	3q
MLOC_71128.1	139	16.5	9.6	420	Nuc	4q
MLOC_72553.2	318	34.7	9.2	957	Nuc	7q
MLOC_72737.1	217	24.4	9.4	654	Nuc	4q
MLOC_74840.1	349	37.3	7.9	1050	Nuc	3q
MLOC_75297.2	199	21.1	10.5	600	Nuc	7p
MLOC_75524.1	446	48.4	6.6	1341	Nuc	7p
MLOC_75524.4	242	27.04	9.5	729	Nuc	7p
MLOC_75795.1	469	52.3	7.7	1410	Nuc	3p
MLOC_75795.5	211	24.4	8.86	636	Nuc	3p
MLOC_75795.6	416	46.3	7.98	1251	Nuc	3p
MLOC_7757.1	268	29.7	6.94	807	Nuc	1
MLOC_79696.1	317	33.6	4.56	954	Nuc	6q
MLOC_8116.1	274	29.8	7.98	825	Nuc	2q
MLOC_81739.1	184	21	8.6	555	Nuc	6q

Chr, gene localization in chromosome; CDs, length of CDs; AA, protein length (number of amino acid); PI, theoretical isoelectric point; MW, molecular weight, KDa, Nuc, nucleus.

The largest number of *NAC* genes (28%) belonged to chromosome 7 with 23 genes (17 genes on 7p and 6 on 7q), whereas only five *NAC* genes were mapped to chromosomes 1 and 6. Moreover, nine genes were mapped to chromosome 5 and ten and twelve *NAC* genes were found on chromosomes 3 and 4, respectively (Table 1).

As suggested by Holub (2001) a gene cluster is defined as a chromosome region with two or more genes located within 200 kb sequence. Accordingly, based on the Holub's criterion, we found six *NAC* gene clusters.

Chromosome 7 contains the maximum number of clusters (one on 7q and two on 7p) comprising of 23 *HvNAC* genes. Only one gene cluster was found on chromosome 4 (4q), whereas chromosomes 2 contain two gene clusters (on 2q).

Genes belonging to a family are often distributed in clusters at certain chromosomal regions. *NAC* family genes in rice (Nuruzzaman *et al.*, 2010), poplar (Hu *et al.*, 2010) and soybean (Le *et al.*, 2011) were also found to be distributed in clusters. Gene cluster analysis showed that the number of genes on chromosomes was positively correlated with the number of gene clusters.

Table 2. Analysis of variance for amino acid composition pattern in different *Hordeum vulgar* NAC proteins.

Effect	SS	DF	MS	F
Amino acids	7081.1	19	372.6	146.6**
Residual	4167.7	1640	2.5	
Total	11248. 8	1659	6.7	
LSD (p<0.05)	0.49		C.V. (%)	31.88
LSD (p<0.01)	0.64		S.E.M.	0.17

Phylogenetic analysis and conserved motifs of HvNAC family

Classification of genes and phylogeny are important for the functional analysis of a gene family. Multiple sequence alignment of full-length HvNAC proteins revealed that all of the HvNAC proteins contain highly conserved N-terminal NAC domain and a highly variable C-terminal NAC domain. Phylogenetic tree made from multiple sequence alignment of 82 HvNAC proteins divided them into 5 subfamily and 13 distinct subgroups (Fig. 1).

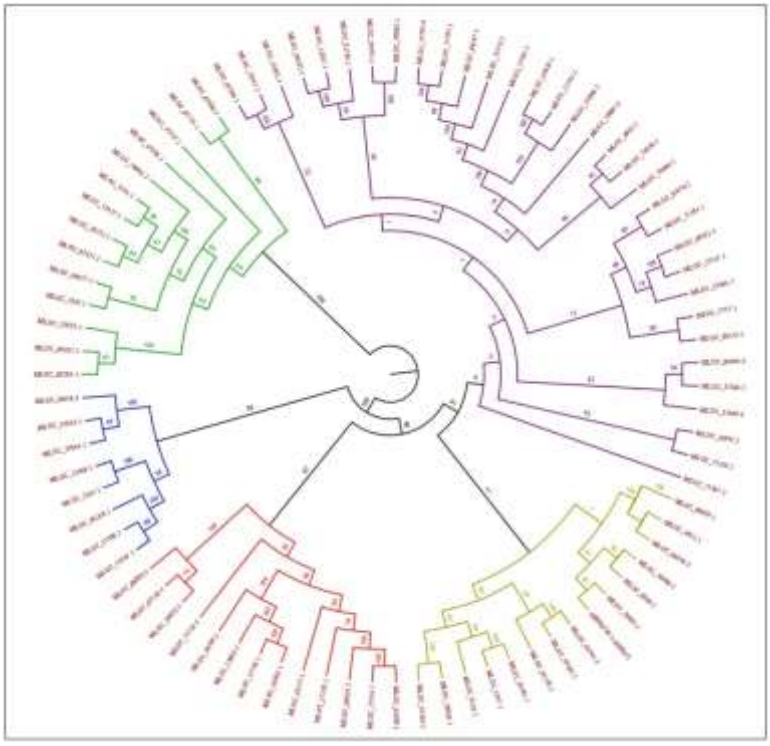

Fig. 1. Phylogenetic tree of 82 *Hordeum vulgar* NAC proteins. The phylogenetic tree constructed using CLC Genomics Workbench via the Neighbor-Joining (NJ) method and bootstrap analysis. The different subfamily of NAC proteins are highlighted in different colors.

Subfamily I (with 33 proteins) consisted of the maximum number of HvNAC proteins, while subfamily II, III, IV and V each contained 14, 13, 8 and 14 HvNAC proteins, respectively. Subfamily I comprised five subgroups with 3, 5, 12, 7 and 6 members, respectively. Each of subfamilies II, III, IV and V comprised of two subgroups with (6, 8), (10, 3), (5, 3) and (11, 3) members of HvNACs. The phylogenetic tree might serve as a framework to show the genetic diversity and distance in different biological species and other entities (Shi *et al.*, 2013). In similar studies, phylogenetic trees divided soybean (Le *et al.*, 2011) and poplar (Hu *et al.*, 2010) NACs into six and ten subgroups, respectively.

These observations indicate that barely NAC proteins have more diversity than soybean and poplar. Also we can see from the phylogenetic tree that MLOC-20905.1 was closest to out-group protein (AtNAC2.1) and MLOC_20905.1 was closest to MLOC_50406.5 and MLOC_6066.1. In *A. thaliana*, *AtNAC2* expression was induced by salt stress. Also, *AtNAC2* was highly expressed in roots and flowers, but less expressed in other organs examined.

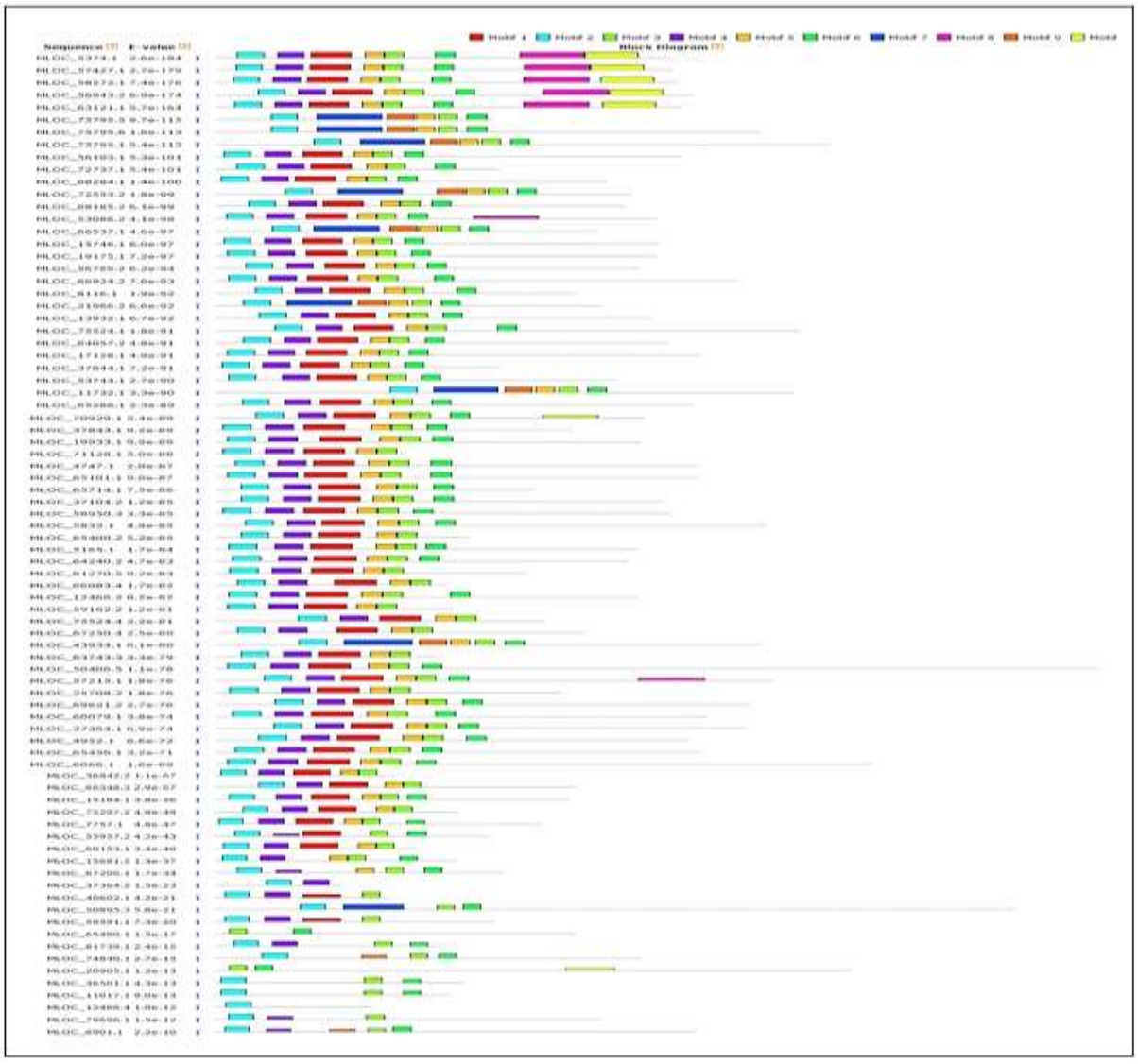

Fig. 2. Hidden Markov Model consensus sequences obtained using MEME for Motifs of HvNAC protein sequences. The motifs obtained via MEME analysis were plotted according to their position within the protein sequences. The heights of the symbols at each motif position indicate sequence conservation.

These results indicate that *AtNAC2* may be a transcription factor combine the environmental and endogenous stimuli into the process of plant lateral root development (He *et al.*, 2005). To further examine the diversity of barely *NAC* genes, the conserved motifs and motifs architecture (Fig. 2) were predicted by using MEME program. In general, NAC proteins clustered in same subgroups, share similar motif composition, indicating functional similarities among the proteins of the same subgroup (Fig. 2). The most of conserved motifs were found within the N-terminal NAC domain, indicating that these motifs may be essential for the function of NAC proteins.

Continued Fig. 2. Hidden Markov Model consensus sequences obtained using MEME for Motifs of HvNAC protein sequences.

The result of motif analysis showed that motifs 2 (LPPGFRFHPTDEELITHYLCR) and 3 (PKGEKTNWIMHEYRL) were more conserved followed by motifs 5 (LIGMKKTLVFYKGRA), 6 (KDEWVVCRIFKKTQI), 4 (IIPEVDLYKCEPWDLPEKCCI) and 1 (YFFCHRDRKYPTGIRTNRATTAGYWKATGKD), which were presented in 80, 79, 67, 63, 62 and 53 proteins of all HvNAC proteins (82), respectively. While, two conserved motifs 8 (YQLHNIEVGSSMMGSVVLPMMNNHYFGNHHHQ MMVAPPQPLISFYHHNQH) and 10 (QMMMHMSADQGFMVGVEPGSGPSSIVSQEDVVT GLNNNNQG) were found at the C-terminal ends of NAC proteins. Generally, there is low conservation in the C-terminal region of the NAC proteins.

Gene expression profiling of HvNAC

To test whether HvNAC is stress responsive gene, we performed a qPCR analysis on total RNA isolated from the roots and leaves of seedlings subjected to salinity stress. Result of One-way ANOVA indicated that there were highly significant differences ($P< 0.01$) among the studied gene in terms of expression at stress conditions and tissues. QRT-PCR analysis performed to monitor the expression levels of *HvNAC* gene in cultivars Afzal (as tolerant) and Fajr (susceptible) showed a greater level of expression in Afzal (4.9 fold) compared to the Fajr (Fig. 3).

Statistical analysis revealed a significant expression increase ($P< 0.01$) in response to salinity in cultivars Afzal and Fajr (Fig. 3).

The observed expression increase following the salt treatment was higher in cultivar Afzal (940%) than Fajr (115%) with statistically significant difference (Fig. 3). According to the results of Duncan test for the interaction of organs × cultivar, the relative expression levels of *HvNAC* gene in leaves (120%) was slightly higher than roots in Afzal but it was higher in roots (265%) compared to leaves of Fajr under both conditions.

The expression of *HvNAC* gene at leaves and root tissues of the cultivar Afzal showed a bigger increase in response to salt stress (10.67 and 8.16 folds, respectively) compared to the normal conditions, while in the cultivar Fajr the salinity stress caused an increase only in root by 207% showing significantly different response of two cultivars to salinity stress at both tissues.

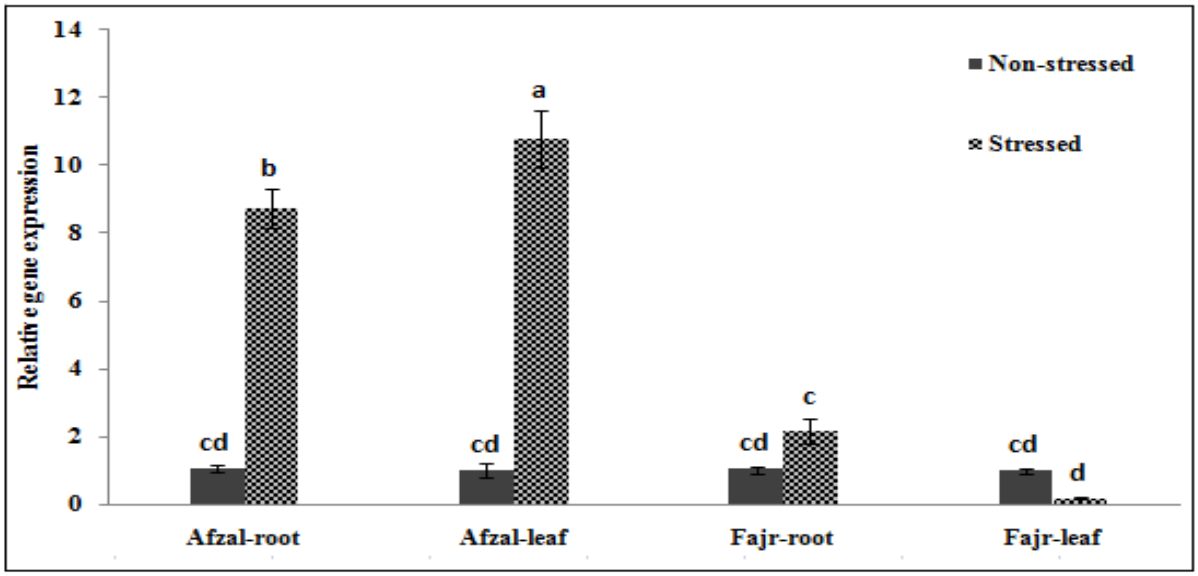

Fig. 3. The cultivar × stress × tissue triple interaction mean comparison of *HvNAC* gene expression in Afzal and Fajr cultivars under salt stress. Bars indicate standard errors and different letters indicated statistically significant differences at (P< 0.01).

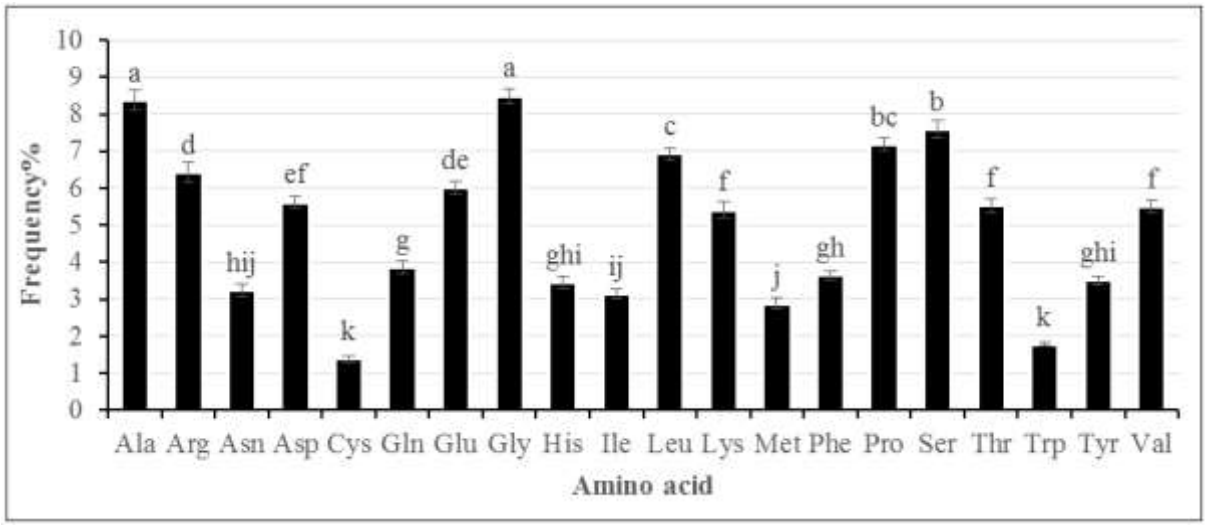

Fig. 4. Amino acid composition in different *Hordeum vulgar* NAC proteins. Comparisons were performed according to LSD method (α=0.05). (Means that do not share any letter are statistically different).

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