

Overexpression of the Transcription Factor *HvSNAC1* Improves Drought Tolerance in Barley (*Hordeum vulgare* L.)

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Abstract

NAC proteins constitute a family of plant-specific transcription factors that are involved in many plant cellular processes including responses to abiotic stress. In this study, a cDNA clone encoding the *HvSNAC1* transcription factor was isolated from drought stressed barley using a bioinformatics approach based on amino acid sequence data of the stress-related SNAC1 protein from rice. Phylogenetic analysis of the deduced amino acid sequence of *HvSNAC1* showed that this protein belongs to the stress clade of NAC proteins that include SNAC1 and TaNAC2. Expression analysis indicated that the *HvSNAC1* gene is strongly induced by different abiotic stresses including drought. Overexpression of *HvSNAC1* in barley under the control of a constitutive promoter produced plants that grew normally under well watered conditions when compared with wild type plants. Transgenic barley plants overexpressing *HvSNAC1* showed higher drought tolerance at different growth stages when compared with wild type plants. In addition, the constitutive overexpression of *HvSNAC1* resulted in improved water status, photosynthetic activity and reduced water loss rate when compared with wild type plants under drought conditions. Furthermore, the transgenic plants also showed significantly improved productivity as reflected by the increase in biological yield over the wild type plants under severe field drought conditions. In conclusion, the *HvSNAC1* gene

could be a useful tool to improve barley productivity under field drought conditions without impairment in growth under normal field conditions.

Keywords: Abiotic stress; Bioinformatics; drought; *Hordeum vulgare*; Stomatal conductance; transcription factor

Introduction

Barley (*Hordeum vulgare* L.) is an important cereal plant that is mainly cultivated under marginal lands where adverse environmental conditions such as drought and high temperatures are frequent. In such areas, there is an urgent need to develop drought-tolerant plants that can withstand such adverse conditions while maintaining high productivity (Habash et al. 2009). Plant breeding for improved drought tolerant crops has demonstrated that this complex trait is governed by multiple loci (Mir et al. 2012). This inherited genetic complexity and redundancy of tolerance mechanisms have made the identification of key regulatory genes governing drought tolerance in plants a priority.

Several studies were conducted to understand the mechanisms by which plants perceive environmental signals to activate cellular mechanisms involved in stress-tolerance responses (Bartels and Sunkar 2005). In this perspective, drought-related signals were found to activate large numbers of regulatory genes including many transcription factors and kinases (Zhu 2002). The activation of these genes will result eventually in the activation of numerous stress-related genes and the synthesis of diverse metabolites that improve plant tolerance to drought. For instance, studying gene expression changes associated with abiotic stresses in different plants revealed that several transcription factors were involved in plant responses to drought (Seki et al. 2001; Bhargava and Sawant 2013). Many of these transcription factors were found to be members of different families including AP2/EREBP, bZIP, NAC, MYB, MYC, zinc-finger and WRKY (Yamaguchi-Shinozaki and Shinozaki 2006; Nakashima et al. 2009). In many instances, the overexpression of these factors or their active forms in plants confers cold, drought and salt tolerance phenotypes (Umezawa et al. 2006; Xue et al. 2011; Mao et al. 2012).

One of these transcription factors families is the *NAC* gene family, which comprises over than 100 plant-specific transcription factors that are involved in various developmental and stress responses (Olsen et al. 2005; Puranik et al. 2012). *NAC* proteins are characterized by their highly conserved DNA binding domain in the N-terminal region that was originally identified from consensus sequences of petunia *NAM*, Arabidopsis *ATAF1*, *ATAF2*, and *CUC2* genes (Aida et al. 1997). *NAC* proteins play important roles in abiotic and biotic responses including viral resistance (Ren et al. 2000), bacterial infection and wounding (Collinge and Boller 2001; Mysore et al. 2002; Hegedus et al. 2003), salt stress and drought tolerance (Fang et al. 2008; Nakashima et al. 2012). For instance, a stress-inducible rice *NAC* gene (*SNAC1*) was found to play a major role in stress tolerance and its overexpression resulted in drought

tolerance of field growing rice plants at anthesis (Hu et al. 2006). The overexpression of another stress-inducible rice *NAC* gene (*SNAC2*) improved drought tolerance at seedling stage, however, testing the *SNAC2* overexpression rice lines in the field under drought conditions didn't have any significant effect on yield (Hu et al. 2008). The constitutive overexpression of another *NAC* gene (*OsNAC6*) in rice resulted in improved drought tolerance at the seedling stage, however, pleiotropic effects of vegetative growth were observed (Nakashima et al. 2007). On the other hand, overexpression of *OsNAC6* using stress-inducible promoter resulted in improved stress tolerance in transgenic rice plants while minimizing negative pleiotropic effects on plant growth. The roots specific expression of *OsNAC10* improved drought tolerance and yield in field grown rice plants (Jeong et al. 2010). In barley, *HvNAC6* was found to enhance resistance against pathogen penetration (Jensen et al. 2007), while rendering tolerance against drought stress (Lu et al. 2007). The characterization of 48 barley *NAC* genes suggested that they are functionally conserved with other related *NAC* genes from other plants species (Christiansen et al. 2011). Such reports indicate that *NAC* genes play a pivot role in mediating responses to different environmental stimuli and their potential utilization to enhance stress tolerance including drought. In many instances, the overexpression of the *NAC* transcription factors in different plant species resulted in improved stress tolerance toward freezing and dehydration tolerance (Fang et al. 2008). Such data suggest that stress-responsive *NAC* transcription factors overexpression has the potential to enhance drought tolerance in cultivated plants including barley. Accordingly, it seems feasible to utilize genetic information of previously characterized key regulatory stress-responsive *NAC* genes from model plants to clone candidate-genes in barley. The isolation of *NAC* candidate genes in barley can be utilized to generate transgenic plants with enhanced tolerance to drought stress. However, it is still not clear what would be the influence of the overexpression of these genes on the physiological processes under water deficit conditions.

To answer these questions, the current study was initiated in order to isolate and characterize a stress-related drought-tolerance *NAC* gene from barley. The *HvSNAC1* was cloned and found highly related to stress-responsive *NAC* genes from grasses. The *HvSNAC1* was induced in response to drought conditions indicating a potential role in mediating tolerance to dehydration. Transgenic plants overexpressing *HvSNAC1* showed normal growth and reduced water loss rate under drought conditions. In addition, *HvSNAC1* overexpression lines improved yield of barley under drought field conditions.

Materials and methods

Cloning of a stress-related *NAC* gene in barley

Based on the DNA sequence of the previously described *SNAC1* gene in rice (GenBank accession number: DQ394702), a TBLASTN search with the amino acid sequence of SNAC1 protein was performed against two nucleotide sequences databases (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi> or <http://pgrc.ipk-gatersleben.de/est/>). Using this approach, several expressed sequence tags (ESTs) and mRNA sequences were obtained and were found to encode a putative stress-related *NAC* gene in barley. Using the obtained DNA sequence of Genbank accession number of JF796130, a gene-specific primer pair of 5'-ATGGGGATGCCGGCGGCGAG-3' (HvSNACfwd) and 5'-TTAGAACGGGGCTGGCATGC-3' (HvSNACrev) was designed.

To isolate the full length cDNA of the corresponding stress-related *NAC* gene, two weeks old barley cv. Golden Promise seedlings were subjected to drought treatment by water withholding for 7 days. Whole leaf tissue was harvested from stressed plants and flash frozen in liquid nitrogen until total RNA extraction. Total RNA was isolated from stressed tissue using SV Total RNA Isolation System Kit (Promega, Madison, USA) following the manufacturer's instructions. The isolated RNA was used to synthesize first strand cDNA library using the SuperScript® First-Strand Synthesis System (Invitrogen, USA) and oligo T₍₁₈₎ primer following the manufacturer's instructions. The full-length cDNA of stress-related barley *NAC* was amplified using PCR in a 25 µL reaction mixture containing 5 µL of cDNA as a template, 2.5 µL of dNTPs (100 µM), 5 µL of 5× PCR buffer, 0.5 µM of each primer and 0.25 µL of 5 U/µL GoTaq DNA polymerase (Promega, Madison, Wisconsin). The PCR conditions were 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 min, 55 °C for 1 min, and 72 °C for 1 min, and a final 10 min extension at 72°C. The amplified PCR products were separated in 1% agarose gel stained with ethidium bromide. Positive PCR products were extracted from agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin) and cloned into pGEM®-T Easy Vector System (Promega, Madison, Wisconsin) following manufacturer's instructions. The pGEM®-T Easy Vector System allows the selection of positive recombinant plasmids based on the insertional inactivation of the α -peptide of the enzyme β -galactosidase. Positive recombinant plasmids that contained the full-length *HvSNAC1* cDNA were fully sequenced using the M13 reverse and forward sequencing primers by ABI 3730XL machine by Macrogen (Seoul, Korea).

Bioinformatics analysis

Sequence analysis and annotation predication was performed using Gramene database (Youens-Clark et al. 2010) and MIPS barley genome database (<http://mips.helmholtz-muenchen.de/plant/barley/index.jsp>). For promoter analysis, PlantCARE (Lescot et al. 2002) and PLACE/Signal Scan (Higo et al. 1999) databases were used to mine for stress-related *cis* regulatory elements. *In silico* gene expression analysis was based on microarray expression data from various datasets by using GeneVestigator software (<https://www.genevestigator.com/gv/>; Zimmermann et al. 2008). For multiple sequence alignments analysis, the Clustal_X program (version 2.0; Thompson et al. 1997) was used. A phylogenetic analysis was carried out using MEGA5 (Tamura et al. 2011). The protein sequence of the barley stress induced NAC, together with selected NAC protein sequences from wheat and rice retrieved from the GenBank, were aligned using the ClustalW algorithm in the MEGA5. The alignment was used to calculate distance matrices for neighbor-joining analyses with the Kimura two-parameter model. Bootstrap analysis with 1000 replicates was performed to test the robustness of the internal branches.

Semi-quantitative and quantitative Real-time RT-PCR analysis

Semi-quantitative RT-PCR analysis was performed using first-strand cDNA prepared from barley plants using gene-specific and internal reference gene primers. For this purpose, a *HvSNAC1* gene-specific primer pair of 5'-GGATTCCGCTTCCACCCCAC-3' and 5'-ATCATGGTCTCCCCTTCCCC-3' was designed for expression analysis. A fragment of barley *HvActin* gene (Accession number: AY145451) was amplified with primer pair of 5'-CTCCTTCACAACCTCAGCTG-3' and 5'-CAAGGTCGTCGCTCCACCTG-3' as an internal control for the relative amount of RNA. For semi-quantitative RT-PCR, the PCR conditions were as described above and modified as follows: denaturation for 5 min at 95 °C, followed by 30-35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55°C, and elongation for 60 s at 72°C and a final elongation at 72°C for 10 minutes.

For quantitative real-time PCR analysis, total RNA was isolated from leaf samples taken from drought-stressed for three and five days and non-stressed Golden Promise seedlings. Specific primers pairs for *HvSNAC1*, *HvActin* (used as the reference gene), *HVA1*, *HVA22*, *DHN8* and *HvCor14b* (considered as drought-inducible genes) were designed using Primer 3 Software. The amplification of the targeted genes were carried out using the GoTaq® qPCR Master Mix Kit (Promega, Madison, USA), and real-time detection of products was performed in a Mini Opticon Real Time PCR System (BioRad, Hercules, CA). All cDNA samples were analyzed in triplicate, and the cDNA was

derived from at least two biological replicates. Thermal cycling conditions consisted of 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, plus a final extension at 72 °C for 5 min. The relative changes in gene expression were quantified as described in Vandesompele et al. (2002).

Plant transformation

To generate transgenic barley plants overexpressing the stress-related *HvSNAC1* gene, the verified coding sequence of *HvSNAC1* was inserted downstream of the maize *Ubi* promoter in the pBRACT211 binary plasmid. In addition, the coding sequence of *HvCBF3* (Accession number AF239616), a drought responsive transcription factor conferring tolerance in overexpression transgenic barley plants (Choi et al. 2002) was introduced into the same binary plasmid to generate positive control lines. The cloned plasmids were introduced into barley cv. Golden Promise via *Agrobacterium tumefaciens*-mediated transformation as described previously (Bartlett et al. 2008). Positive transgenic plants were verified using PCR and antibiotic resistance screening assay. T1 plants were further analyzed for transgene presence using PCR as described in Bartlett et al. (2008), screening for antibiotic resistance plants as described in Wang and Waterhouse (1997) and gene expression levels using quantitative real-time PCR analysis using first strand cDNA library prepared from total RNA isolated transgenic plants as described above. Two T1 plants showing high level of *HvSNAC1* expression were selected to obtain homozygous T2 plants by screening for antibiotic resistance plants as described in Wang and Waterhouse (1997). The T2 homozygous plants from both lines were further selected to produce T3 homozygous plants that were then used for the stress experiments described below.

Greenhouse Experiments

Drought tolerance at the seedling stage was evaluated in sand culture in small pots. For this purpose, fifteen seeds from each positive homozygous line and non-transgenic control were planted in small pots (10 cm length and 10 cm diameter). After seedling emergence, the seedlings were fed with Hoagland solution for two weeks. After two weeks, drought condition was imposed by continuous water withholding for 10 days followed by re-watering. The wilting behavior of the treated plants and the survival rates of transgenic lines and the non-transgenic control were monitored.

For continuous drought stress treatment on plants at flag leaf stage, seeds of both transgenic and wild-type barley plants were grown in pots. The pots were filled with a mixture of soil and perlite and one seed of each genotype was

sown in the pot. The pots were then irrigated to bring initial soil moisture to field capacity. After seedling emergence, the pots were exposed to two water regimes; "non-stressed" in which irrigation water was applied when soil moisture reaches a level of 20% water depletion of field capacity and "stressed" in which irrigation was applied after 7 days from soil moisture reached 50% water depletion of the field capacity. The treated plants were counted for final tiller number at the flag leaf stage.

For physiological measurements, leaf relative water content was measured under control (well-watered plants) and drought stress conditions (described above) for transgenic and wild-type plants. For this purpose, fully expanded leaves were taken from transgenic and wild-type plants grown in the same pot at two different growth stages (onset of stem elongation and flag leaf emergence) and their fresh weights were immediately recorded after leaf excision. The excised leaves were soaked in distilled water for 6 h at room temperature in darkness and the turgid weight (TW) was recorded. Total dry weight (DW) was then recorded after drying for 24 h at 70 °C. R.W.C was calculated according to Barrs & Weatherley (1962): $R.W.C (\%) = [(FW - DW)/(TW - DW)] \times 100$. Stomatal resistance was measured in greenhouse grown seedlings at approximately midday after starting water stress treatments using porometer (AP4, Delta-T Devices, Cambridge, UK) on three selected leaves per barley genotype. Chlorophyll fluorescence parameters were also measured after starting water stress treatments with a pulse-modulated fluorometer (OS1-FL modulated chlorophyll fluorometer, ADC BioScientific Ltd., Hertfords, UK). Fluorescence measurements were made after 20 min of acclimation to darkness under leaf clips (FL-DC; Opti-Sciences). The maximum quantum efficiency (F_v/F_m) of photosystem II was calculated as $F_v/F_m = (F_m - F_0)/F_m$; where F_m and F_0 were the maximal and minimal fluorescence measured in darkness-adapted leaves, respectively (Maxwell and Johnson, 2000).

Field Trials

Transgenic plants and wild-type control were grown under rainfed conditions at the experimental station of the Jordan University of Science and (JUST) in Ramtha, Jordan in the 2011-2012 growing season. The JUST station represents a drought prone area in the northern part of Jordan with an annual precipitation of 200 mm. The experimental layout was prepared according to randomized complete block design with four replications. In each block, seeds of the tested genotypes were sown into 2 m rows at a density of 50 seeds per meter and 50 cm row spacing. The field experiment was managed following the standard agricultural practices including: fertilizer

application, weed control and pesticide use against main pathogens. At the end of the growing season, plants were harvested and yield parameters such as tiller number, total biological yield, spikes weight, straw weight, spikes number, grains per spike, grains number and grains yield were recorded.

Results

Isolation of stress related *NAC* gene in barley

To identify stress-related *NAC* gene in barley, bioinformatics analysis was carried out based on the full-length sequence data of a previously described stress-related rice SNAC1 protein (SNAC1; GenBank ID: ABD52007). For this purpose, a TBLASTN search was performed against the GenBank nucleotide database (www.ncbi.nlm.nih.gov/blast) and the IPK Crop expressed sequence tags (EST) Database (<http://pgrc.ipk-gatersleben.de/est/>). As a result, several EST and mRNA sequences were retrieved and found to encode a putative SNAC1 protein in barley (*HvSNAC1*; GenBank ID: JF796130). The deduced complete amino acid sequence of HvSNAC1 has identity of 69% with the rice SNAC1 protein and 97% to TaNAC2 (AAU08786). Using a gene-specific primer pair designed from the JF796130 nucleotide sequence, a 993 bp fragment containing the coding sequence of *HvSNAC* gene was obtained from drought-stressed barley tissues.

Mining the sequence data of the released barley genome in MIPS barley genome database (The International Barley Genome Sequencing Consortium 2012: <http://mips.helmholtz-muenchen.de/plant/barley/index.jsp>) and Gramene database (http://www.gramene.org/Hordeum_vulgare/Info/Index) indicated that the *HvSNAC1* gene was located on chromosome 5H and it is annotated as *MLOC_37104*. Two splice variants were predicated where *MLOC_37104.1* encodes a truncated protein (186 amino acids) while *MLOC_37104.2* encodes a functional HvSNAC1 protein (330 amino acids). In addition several natural SNP variants were identified in the DNA sequences of the 5' UTR, exons, intron and 3'-UTR (data not shown). The genomics structure of the *MLOC_37104.2* variant revealed that *HvSNAC1* has 1,769 bps that contained two exons and one intron, a 115 bp 5' UTR and a 213 bp 3'-UTR. The protein architecture of HvSNAC1 indicated the presence of NAC domain (DNA binding domain) in the N-terminal region (19-172 amino acid residues) that could be further divided into five highly conserved sub-domains (Fig. 1a).

Phylogenetic analysis grouped the isolated HvSNAC1 protein with SNAC1 and TaNAC2 proteins in the stress NAC proteins clade (Fig. 1b).

***HvSNAC1* is expressed in response to drought conditions**

To analyze the expression patterns of *HvSNAC1* under different conditions, *in silico* expression analysis using the gene-expression search engine, GeneVestigator (<https://www.genevestigator.com/gv/user/gvLogin.jsp>), was performed. As shown in Fig. 2a, the *in silico* gene expression showed up-regulation in response to different abiotic stresses including drought-treated barley plants. In addition, gene expression induction was observed in barley challenged with *Fusarium graminearum*. Interestingly, a significant down-regulation of *HvSNAC1* expression was observed in barley plants treated with gibberellin, aphid and *Blumeria graminis*, the fungal agent that causes powdery mildew in cereals.

In order to find *cis*-acting elements involved in abiotic responses, the *HvSNAC1* promoter region (2000 bp upstream of 5' UTR obtained from morex_contig_2547787 based on the draft barley genome sequence (<http://mips.helmholtz-muenchen.de/plant/barley/index.jsp>)) was mined using two plant *cis*-element databases: PlantCARE (Lescot et al. 2002) and PLACE (Higo et al. 1999). Using this strategy, several stress responsive elements were identified in *HvSNAC1* promoter region that include *ABRE* (*Abscisic Acid Responsive Element*), *DRE* (*Dehydration Responsive Element*), *LTR* (*low temperature responsive*) *MYBR* (*Myeloblastosis Recognition site*) and *MYCR* (*Myelocytomatosis Recognition site*) (Fig. 2b).

To further investigate the role of the *HvSNAC1* gene in response to drought stress, expression analysis of *HvSNAC1* and selected drought-inducible genes was performed in Golden Promise seedlings exposed to drought conditions for three and five days. These selected genes included *HVA1*, *HVA22*, *DHN8* and *HvCor14b* known for their induction under drought in barley (Morran et al. 2011). Under drought conditions, the expression of *HvSNAC1* was induced after three days and showed higher levels of induction after five days of treatment when compared with wild type plants under normal conditions (Fig. 2c). The expression patterns of *HvSNAC1* were similar to the drought inducible genes where increased levels were observed after three days of drought treatment.

Overexpression of *HvSNAC1* in barley enhanced drought tolerance

To test whether *HvSNAC1* can improve tolerance to drought stress in barley plants, an overexpression construct with *HvSNAC1* CDS under the control of maize *Ubiquitin* promoter was introduced into barley cv. Golden Promise.

Twenty-six independent transgenic lines were obtained based on hygromycin selection, PCR analysis and expression analysis of *HvSNAC1* (data not shown). The majority of the transgenic lines grew normally with no stunting when compared with non-transformed plants (Supplement Fig. 1a). The T3 generations of two homozygous lines overexpressing *HvSNAC1* (line OE3 and line OE11) (Supplement Fig. 1b) were selected and further used for drought tolerance assay and physiological measurements.

To test drought tolerance in the selected transgenic lines at early growth stages, two weeks old plants grown in small pots filled with sand were subjected to withholding of water for a period of 10 days prior to re-watering. The drought subjected plants were monitored for growth and survival at different set points of the treatment. Wild-type plants were not able to survive under such adverse conditions and the majority of the stressed plants wilted and turned yellow after 10 days of treatment (Fig. 3a, b). However, the *HvSNAC1* transgenic lines were able to survive such adverse conditions and over 90% of the tested OE plants showed improved drought tolerance when compared with wild-type plants. In addition, measuring the R.W.C. of stressed plants after 5 days of drought treatment revealed that the transgenic plant had higher values when compared with wild type plants (Fig. 3c). Furthermore, under water deficit conditions (5 days of drought treatment) more stomata were found to be closed in transgenic lines when compared with wild type (Fig. 3d). Interestingly, no differences in stomatal resistance were observed between transgenic and wild type plants under normal conditions. Measuring the maximum yield of PSII (F_v/F_m) in well-watered plants showed no major differences among the wild type and transgenic lines (Fig. 3e). After 5 days of drought stress, the F_v/F_m values were decreased in wild type plants while transgenic lines showed no reduction in their values.

To test the effect of drought stress on mature plants at the flag leaf stage under greenhouse conditions, wild type plants and T3 homozygous transgenic plants were subjected to two water regimes as described in materials and methods. Under well watered conditions, the OE lines and wild type plants showed normal vegetative growth, which was almost similar between the tested genotypes (Fig. 4a). Under prolonged drought conditions, both wild type and transgenic lines suffered from the adverse conditions with a severe reduction in growth (Fig. 4a). However, under severe drought conditions, the transgenic lines showed a higher number of tillers when compared with wild type plants (Fig. 4b). Measuring plant R.W.C. of non-stressed plants indicated that the well-watered wild type and transgenic plants had no significant difference (Fig. 4c). Under drought stress conditions, the R.W.C in the wild-type plant was significantly lower when compared with the OE lines (Fig. 4c). These results indicate that the OE

transgenic lines had better water status when compared with wild-type plants after exposing them to severe drought conditions.

The performance of the OE lines and wild type plants was evaluated under drought conditions in the field. T3 seeds of homozygous lines of OE#3 and OE#11 together with wild type controls were sown under rainfed conditions in a field location characterized with low annual precipitation (215 mm), silty soil and prevalent drought conditions. In addition, the T3 homozygous lines of OE#3 and OE#11 were compared with two T4 homozygous transgenic lines overexpressing *HvCBF3*, a key transcription factor of cold and drought regulation in barley (Choi et al. 2002). The overexpression of CBF/DREB factors in wheat and barley was shown to improve tolerance under severe drought conditions (Morran et al. 2011). At the end of the growing season, yield parameters were recorded from four replicates. As shown in Fig. 5a, the OE#3 and OE#11 transgenic plants showed improved growth at the tillering stage in the field when compared with the wild type plants. Comparing the yields of the tested genotypes showed that all of the transgenic lines (*HvSNAC1* OE and *HvCBF3* OE) produced significantly higher tiller number that resulted in higher total weight, spikes number, grains/spike, grains number and grains weight when compared with the wild type plants (Fig. 5b). These findings clearly demonstrate that *HvSNAC1* overexpression can improve yield under drought conditions in the field.

Discussion

In this study, a stress-related *NAC* gene was isolated, characterized and showed significant improved tolerance to drought stress when overexpressed in barley plants. Recently, 48 *NAC* genes were identified in barley and found to have conserved functions with related *NAC* genes from other plant species including developmental processes and responses to environmental stimuli (Christiansen et al. 2011). Phylogenetic analysis of *HvSNAC1* showed a close relationship with previously characterized stress-related *NAC* genes such as *SNAC1* from rice (Hu et al. 2006) and *TaNAC2* from wheat (Mao et al. 2012). *HvSNAC1* protein exhibited maximum sequence identity (97%) to *TaNAC2* (AAU08786), which was found to be involved in response to drought, salt, cold, and abscisic acid and to improve stress tolerance when expressed in *Arabidopsis* plants (Mao et al. 2012). This similarity of *HvSNAC1* to other stress-related *NAC* genes in grasses indicates a major role in mediating stress tolerance in barley. The *HvSNAC1* is located

on the lower arm of chromosome 5H and it is annotated as *MLOC_37104*. This region is well-known to contain many drought tolerant candidate genes in barley plant (Tondelli et al. 2006). Identifying of *HvSNAC1* beside other candidate genes involved in drought tolerance in this important region in barley genome, highlights the need to develop an efficient marker assisted selection strategy to improve breeding activities related to the enhancement of barley productivity in drought-prone areas.

In silico expression analyses showed that the *HvSNAC1* gene was induced by several abiotic stresses including drought, cold and heat. *HvSNAC1* expression induction in response to dehydration stress was confirmed in drought stressed barley seedlings. This is consistent with the *in silico* promoter analysis where several stress-responsive regulatory elements were identified in *HvSNAC1* promoter region. Such induction patterns are similar with previously characterized stress-related *NAC* genes from other plant species (Hu et al. 2006; Mao et al. 2012) suggesting a role of *HvSNAC1* in mediating drought tolerance in barley plants. Besides its role in abiotic tolerance, the isolated gene is expected to function in responses against biotic stresses. This assumption is supported by GeneVestigator data, where *HvSNAC1* expression is altered by several pathogens. *HvSNAC1* was induced in plants challenged with *Fusarium graminearum* and repressed in plants challenged with *Blumeria graminis*. Several *NAC* genes were found to have an important role in mediating responses against pathogens. For instance, a novel wheat *NAC* transcription factor was found to play a major role in mediating responses to both biotic and abiotic stresses (Xia et al. 2010). The *HvNAC6* gene was found to act as a positive regulator of penetration resistance against *Blumeria graminis* in barley (Jensen et al. 2007). In Arabidopsis, the overexpression of *ATAF2*, a stress-related *NAC* gene, resulted in higher susceptibility to the soil-borne fungal pathogen *Fusarium oxysporum* (Delessert et al. 2005). Furthermore, the *HvSNAC1* homolog in rice (*SNAC2*) was found to play a major role in tolerance responses against biotic and abiotic stresses (Hu et al. 2008). This suggested that *HvSNAC1* might play a major role in mediating plant responses to different kinds of stresses besides drought. In the future, the role of *HvSNAC1* in mediating responses of barley plant to different pathogen infection will be studied.

Interestingly, the constitutive expression of *HvSNAC1* in barley resulted in normal vegetative growth when compared with wild-type plants. In the contrary to *HvSNAC1*, the overexpression of other drought responsive transcription factors in other plants usually resulted in pleiotropic effects such as reduction in plants growth and delayed flowering (Morran et al. 2011). Such adverse growth retardation will be reflected in the final yield and plant performance under field conditions. Such results are in general agreement with previous studies where the

expression of *NAC* genes in different plant species resulted in normal growth without major yield penalties (Hu et al. 2006; Hu et al. 2008; Mao et al. 2012; Saad et al. 2013). Such improved tolerance without major pleiotropic effects of *HvSNAC1* overexpression is considered promising for crop improvement for dry areas.

The induction of *HvSNAC1* to different abiotic stresses indicates the possibility of conferring enhanced tolerance to drought in *HvSNAC1* overexpressing transgenic plants. Testing drought tolerance in transgenic lines indicates that *HvSNAC1* overexpression enhanced drought tolerance at seedling and reproductive stages under greenhouse and field conditions. Under drought field conditions, the overexpression of *HvSNAC1* in barley plants enhanced several yield components including tiller number, spike number, grain number, grains weight and grains number per spike when compared with non-transgenic plants. Similar results were obtained with the overexpression *SNAC1* in rice where no obvious differences between the transgenic and wild type plants were observed (Hu et al. 2006) indicating a conserved role of *SNAC1* orthologs in drought tolerance in grasses. Under greenhouse conditions, the overexpression of *HvSNAC1* in barley plants resulted in higher survival rates and reduced water loss from stressed leaves when compared with wild type plants. This was reflected in higher R.W.C, *Fv/Fm* and stomatal resistance values of stressed transgenic plants when compared with wild type plants under the same conditions. These results are in general agreement with previous data of overexpression of stress-responsive *NAC* genes in different plant species. For instance, the overexpression of *HvSNAC1* highly similar homolog, *TaNAC*, in *Arabidopsis* plants resulted in improved plant survival after drought conditions, reduced leaf water loss rate, and better photosynthetic capabilities when compared with wild type plants. Similarly, the overexpression of *SNAC1* in rice resulted in increased stomatal closure without affecting the photosynthetic rate. Recently, the characterization of *OsSRO1c*, which is a direct target of *SNAC1* and involved in H₂O₂-induced stomatal closure, revealed an indirect role of *SNAC1* in regulating stomatal closure under stress conditions.

In conclusion, a stress-related *NAC* transcription factor gene, *HvSNAC1*, which plays a major role against drought stress in barley was identified and characterized. The overexpression of *HvSNAC1* in barley plants resulted in lower water loss rate and yield improvement under drought conditions in the field. The significantly enhanced drought tolerance of the *HvSNAC1*-overexpressing barley lines indicates its potential for crop improvement under marginal lands.

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Figure captions:

Fig. 1 a Multiple sequence alignment analysis of HvSNAC1 (AEG21060) and selected NAC proteins from wheat (AAU08786 (TaNAC2) and ADE34586) and rice (ABD52007 (SNAC1) and ACX71077). The five subdomains (A-E) of the NAC domain are underlined. **b** Phylogenetic analysis of selected NAC protein sequences from barley, rice and wheat. The position of HvSNAC1 protein (AEG21060) in the tree is indicated by the dashed box.

Fig. 2 a *In silico* expression analysis of *HvSNAC1* by GeneVestigator. **b** Distribution of major known stress-related *cis*-elements in *HvSNAC1* promoter region. **c** Expression analysis of *HvSNAC1*, *HVA1*, *HVA22*, *HvCor14b* and *DHN8* genes in responses to drought treatment after three and five days compared with well-watered samples (0 day). Values are means \pm SD of three technical replicates.

Fig. 3 Growth performance of wild type, transgenic line OE#3 and transgenic line OE#11 at stem elongation stage. **A** after 5 of drought stress. **b** after 10 days of drought stress. **c** R.W.C in leaves of two weeks old wild type, OE#3 and OE#11 seedlings after 5 days of water withholding. **d** Stomatal resistance of two weeks old wild type, OE#3 and OE#11 seedlings after 5 days of water withholding. Values are the means \pm S.E of 10 plants. **e** Maximum quantum yield of PSII (F_v/F_m) of two weeks old wild type, OE#3 and OE#11 seedlings after 5 days of water withholding.

Fig. 4 a Growth performance of OE#3 and wild type at flag leaf stage after continuous drought stress treatment. **b** Tiller number of wild type, OE#3 and OE#11 plants at flag leaf stage after subjecting them to two different watering regimes. **c** R.W.C in leaves of wild type, OE#3 and OE#11 plants at flag leaf stage after subjecting them to two different watering regimes. Values are the means \pm S.E of three replicates.

Fig. 5 a growth of wild type and *HvSNAC1* OE transgenic lines #3 and #11 in the field with drought conditions. **b** Agronomic performance of *HvSNAC1* and *HvCBF3* OE plants grown in the field under drought conditions. Each

data point represents the percentage of the mean values of an agronomic trait from plants harvested from a single row.

Supplement Fig. 1 a Phenotype of one positive transgenic plant (OE#3) compared with wild-type plant grown under well-watered conditions. **b** Semi-quantitative gene expression analysis of *HvSNAC1* in Golden promise (wt) and in different transgenic overexpression lines (OE); Barley *HvActin* gene was used as an internal control.

Fig. 1 a

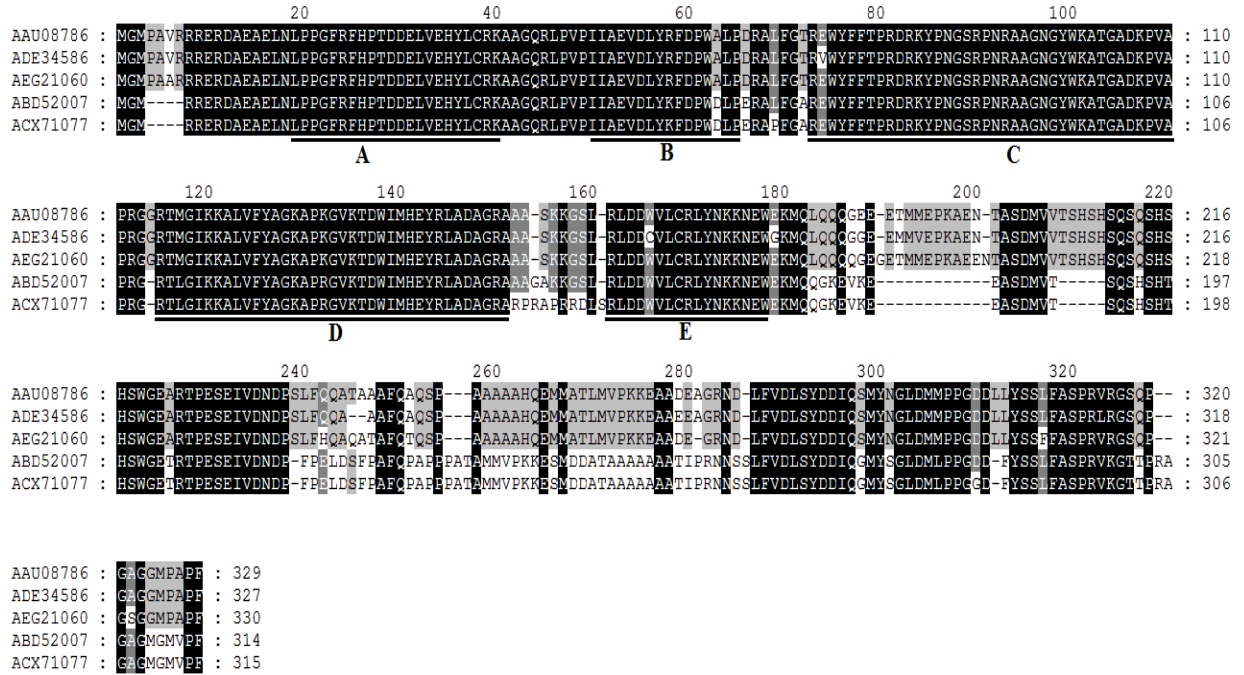


Fig. 1 b

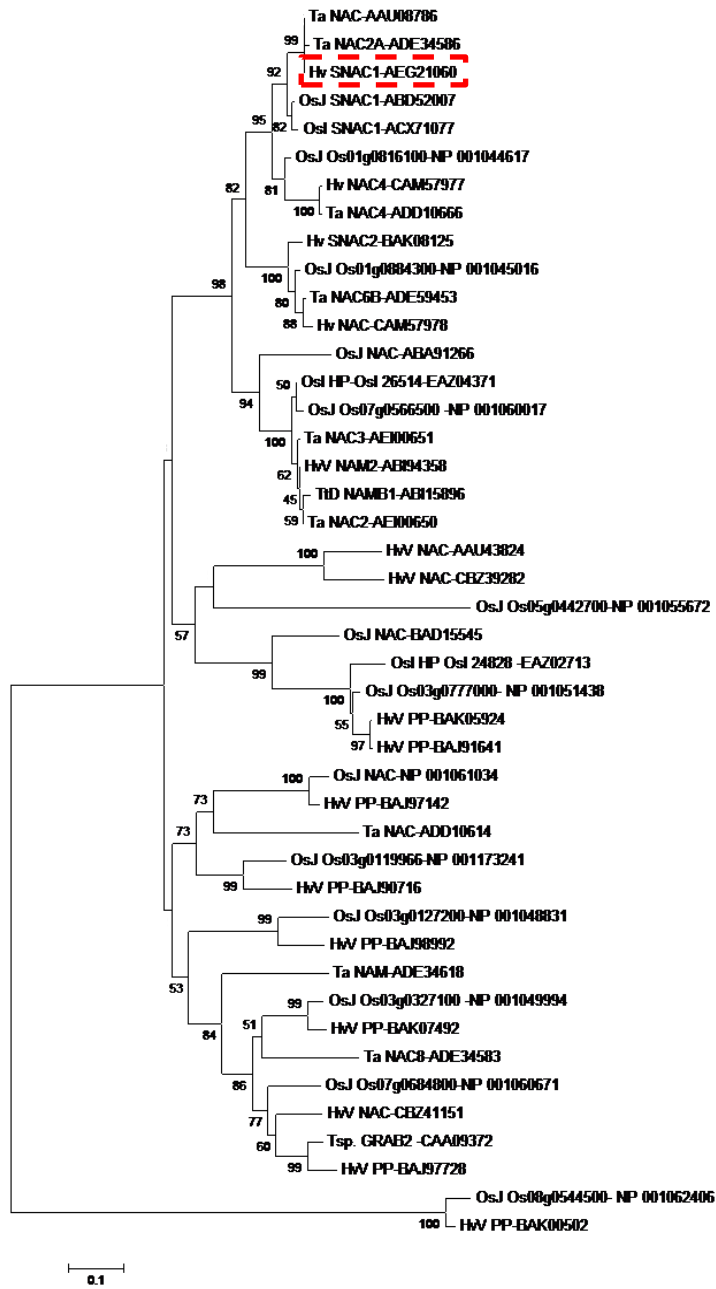


Fig. 2 a

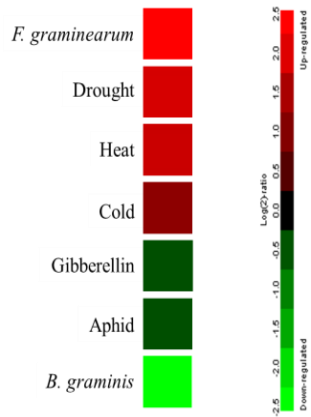


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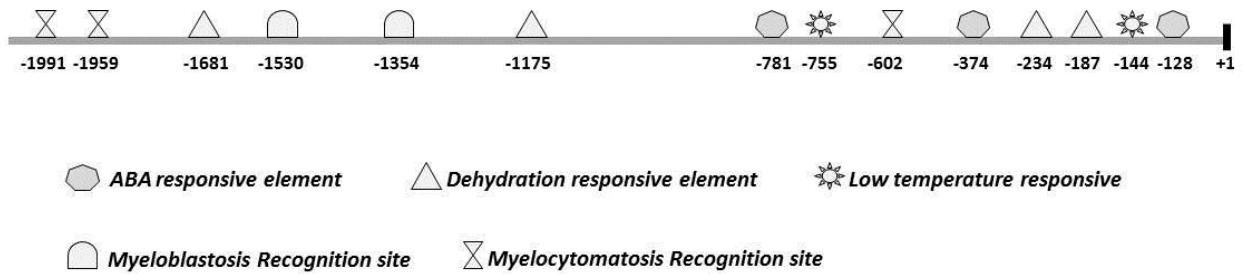


Fig. 2 c

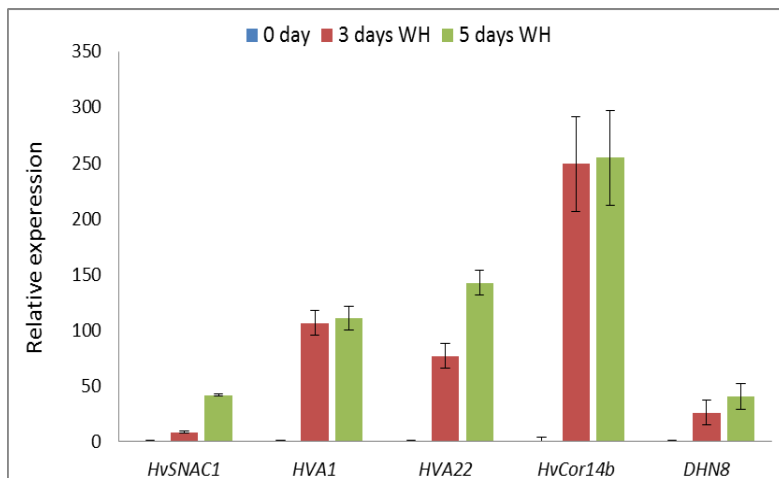


Fig. 3 a

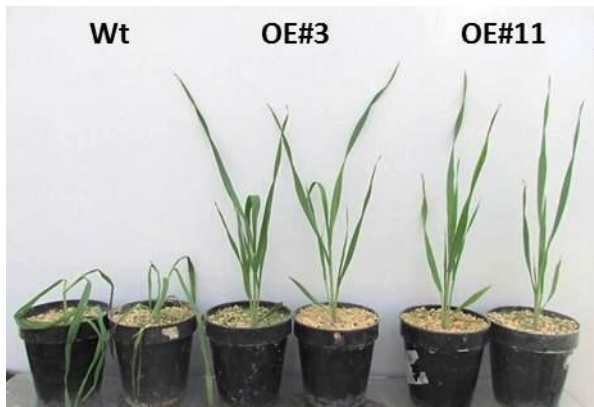


Fig. 3 b

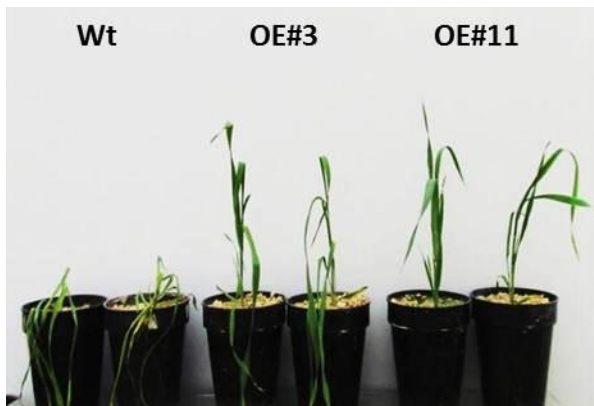


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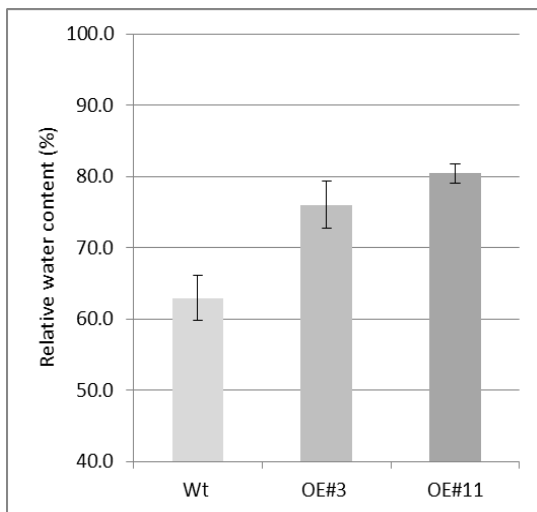


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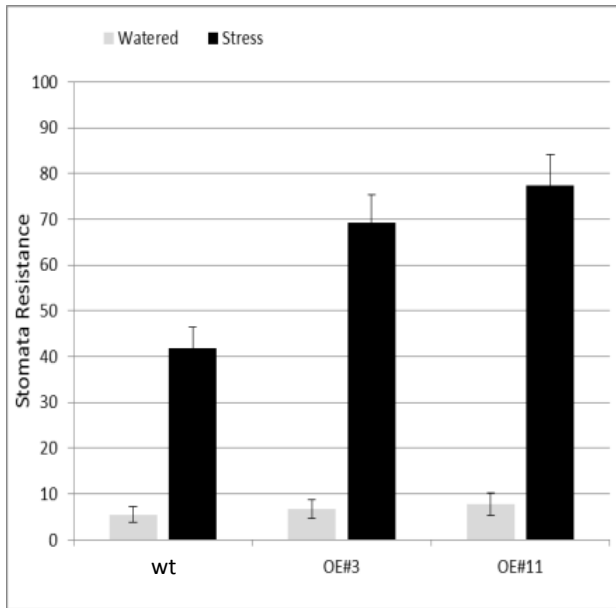


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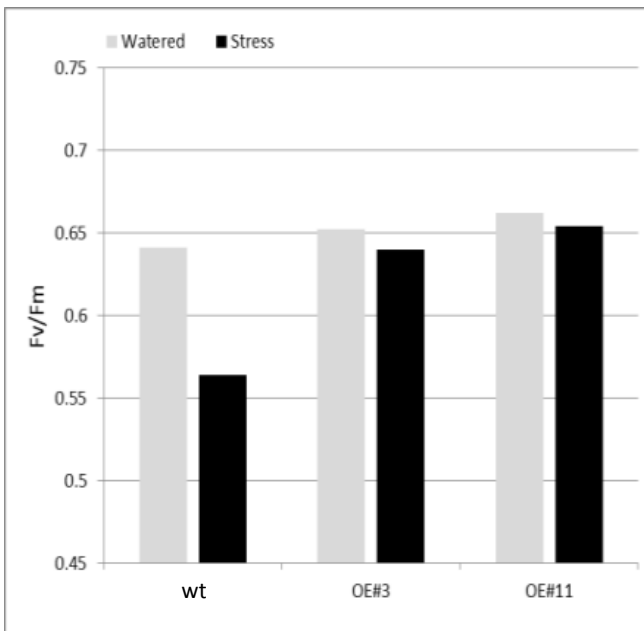


Fig. 4 a

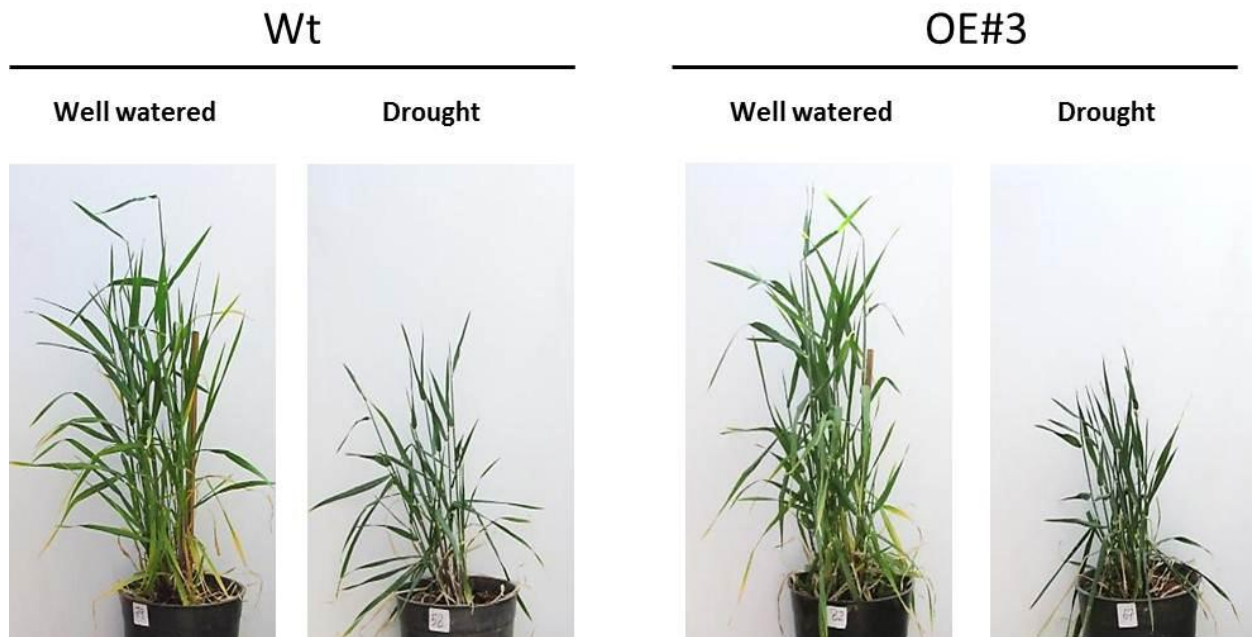


Fig. 4 b

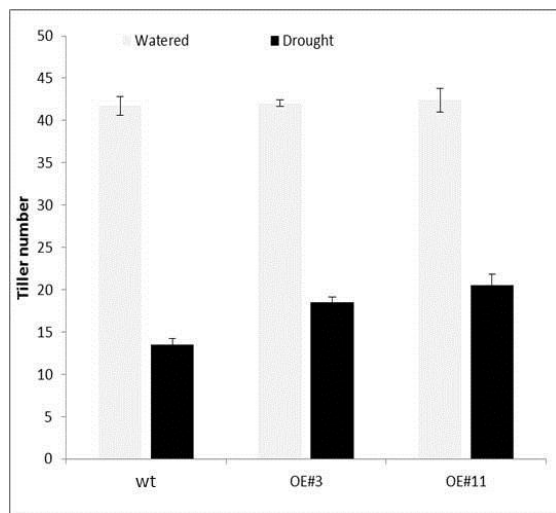


Fig. 4 c

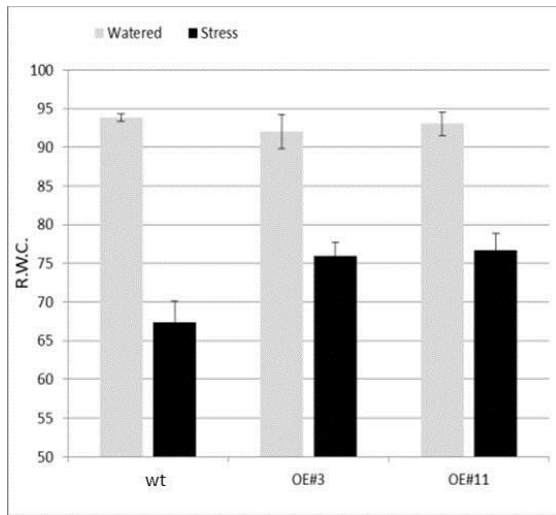
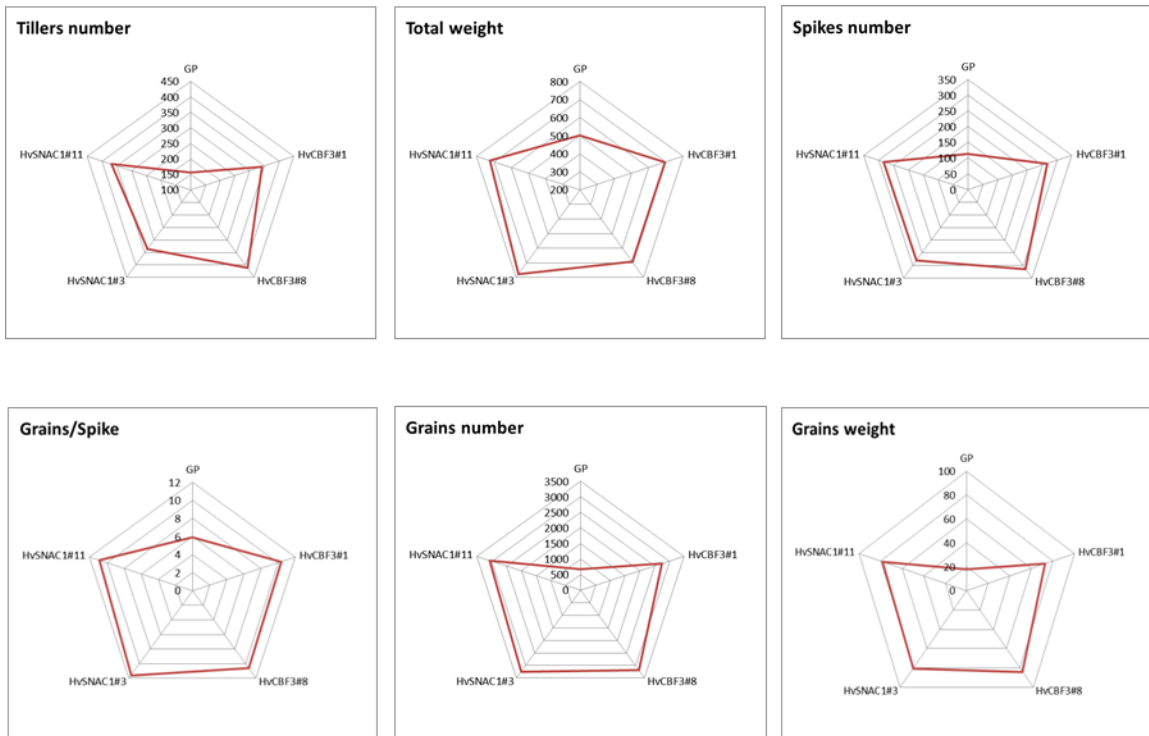


Fig. 5 a



Fig. 5 b

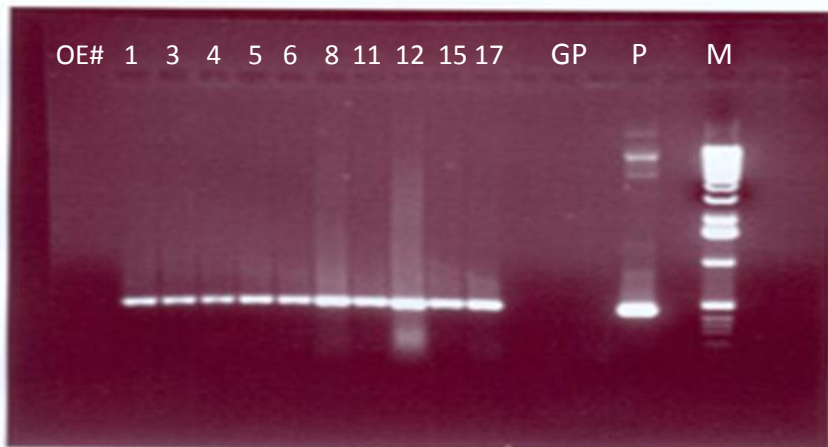


Supplement Fig. 1 a Phenotype of one positive transgenic plant (OE#3) compared with wild-type plant grown under well-watered conditions. **b** PCR analysis of *hpt* gene in Golden promise (wt) and in different transgenic overexpression lines (OE); P: pBRACT211/HvSNAC1 binary plasmid. **c** Real-time gene expression analysis of *HvSNAC1* in Golden promise (wt) and in different transgenic overexpression lines (OE). Values are means \pm SD of three technical replicates.

Supplement Fig. 1 a



Supplement Fig. 1 b



Supplement Fig. 1 c

