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IN SILICO GENE EXPRESSION ANALYSIS OF THE STRESS-RELATED NAC-A GENE SUBFAMILY TO DISSECT THEIR ROLE IN ABIOTIC STRESS TOLERANCE IN BREAD WHEAT (TRITICUM AESTIVUM L.)

Rabea Al-Sayaydeh [1*], Khaled Al-Habahbeh[1], Zahera Akkeh[2] , Farah Mbideen[2] and Randa N. Albdaiwi[3]

[1]Shoubak collage, Department of Agricultural Science Al-Balqa Applied University, Shoubak, Jordan
[2]Department of Horticulture and Crop Science, School of Agriculture, The University of Jordan, Amman 11942, Jordan.
[3]Department of Land, Water and Environment, School of Agriculture, The University of Jordan, Amman 11942, Jordan.

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ABSTRACT

Wheat is a major staple crop that is largely affected by different abiotic stresses that include heat, drought, and salinity. The main objective of this study was to identify wheat NAC transcription factors that are related to the NAC-a subfamily, which is involved in mediating stress tolerance in different plant species. Furthermore, in silico gene expression analysis was conducted to detect differential changes in wheat NAC-a subfamily members in different organs, developmental stages, and under various abiotic stress. Herein, using phylogenetic analysis for 488 NAC transcription factors, 41 proteins were identified as wheat NAC-a subfamily members. In silico gene expression analysis found that NAC-related wheat transcription factors are expressed exclusively at the anthesis stage till dough development with high expression levels detected in flag leaves. The in-silico gene expression analysis identified SNAC1-related members, which had high expression levels under drought, cold, and heat stresses. The identified stress-induced wheat NAC-a subfamily members can be utilized in the future to develop climate-smart wheat cultivars with improved tolerance against abiotic stresses.

Keywords: Abiotic stress, In silico gene expression, Phylogenetic analysis, Wheat.

INTRODUCTION

Bread wheat, *Triticum aestivum* L., is an allohexaploid plant that contains three homeologous genomes (2n=6x=42, AABBDD). It's considered a staple food for around 35% of the world population and globally it is classified as the second most important crop with a total estimated production of about 610 million tons (FAOSTAT, 2019). Wheat cultivation faces many challenges that are associated with multiple biotic and abiotic stresses (Ortiz *et al*., 2008). Poor water management and limited resources besides global warming and climate change are predicted to affect wheat productivity in many parts across the globe.

Plants are frequently exposed to different abiotic stress conditions including cold, drought, high temperature, floods, salinity, heavy metals toxicity, pathogens, and herbivores (Mahajan & Tuteja 2005). Plants can overcome such adverse conditions by

* Corresponding author. E-mail rabea.sayaydeh@bau.edu.jo

developing numerous biochemical and physiological strategies that are induced by key regulatory genes. To minimize the adverse effects caused by these stresses, plants induced the expression of different stress-responsive genes (Tardieu *et al*., 2010). These genes can be categorized into two groups: the first one includes genes related to cell metabolism and stress tolerance and the last one is composed of regulatory genes that encode protein phosphatases or kinases and transcription factors (Singh *et al.,* 2015). Transduction and perception of the stress signals in response to abiotic stresses lead to the expression of a large number of stress-related genes and eventually lead to different metabolic and physiological responses (Zhu *et al*., 2002). The ability to enhance crops' tolerance to salinity and drought stress, especially at the most sensitive stage of production during growth, can have a huge impact on the productivity of wheat.

In response to abiotic stresses, plant transcription factors play essential roles in regulating multiple gene expression pathways involved in their adaptation to different stresses (Hussain *et al.,* 2011). Transcription factors are defined as proteins that bind to specific regulatory elements found in the promoters of targeted genes to induce or repress their expression (Riechmann *et al*., 2000). In plants, transcription factors are classified into several families depending on their DNA binding domain structures (Hussain *et al*., 2011). More than 50 transcription factor families were identified, 12 of them were found to be specific for plants (Romani *et al*., 2020).

One of them is the NAC gene-family, which its members are distinguished by their highly conserved N-terminal DNA-binding domain and variable C-terminal domains needed for transcription activation or repression and protein-protein interactions (Ohbayashi *et al*., 2018). The abbreviation of NAC came from the initials of NAM from petunia (No Apical Meristem), the Arabidopsis Thaliana Activation Factor 1/2 (ATAF1/ATAF2), and the CUp Shaped Cotyledon (CUC) from Arabidopsis. *NAC* genes were found to have major roles in plant development such as the development of shoot apical meristem, development of roots and flowers, senescence, tiller number, cell wall, and wood formation, and the tolerance against different biotic and abiotic stresses (Puranik *et al.,* 2012).

Shen *et al.* (2009) classified NAC proteins into eight distinctive subfamilies (NAC-a to NAC-h) that can be further divided into smaller subgroups according to their phylogeny. Each subfamily includes members that might have distinct functions when compared with other subfamilies. For example, membrane-associated NAC transcription factors involved in cell division or ER stress responses were grouped into the NAC-b subfamily. The NAC-a subfamily includes members that play a major role in stress responses and tolerance against abiotic stresses (Puranik *et al.,* 2012). For instance, the overexpression of three stress-related NAC proteins (ANAC019, ANAC055, and ANAC072) in the Arabidopsis plant improved tolerance against drought tolerance (Li *et al.,* 2012). Two Arabidopsis mutant lines for *ataf1-1* and *ataf1-2* genes showed higher drought tolerance responses when compared with wild-type plants indicating that they act as repressors (Lu *et al*., 2007). In wheat, the NAC family contains 488 members, whereas the phylogenetic trees of NAC domains indicated that wheat NACs divided into eight groups similar to rice and barley (Borrill *et al*., 2017). Recently, several members of the wheat NAC family were implemented in abiotic stress responses that highlighted their pivotal role in inducing tolerance against drought and other stresses. For instance, a drought-responsive allele of TaSNAC8-6A (NAM-A1) was found to improve wheat drought tolerance at the seedling stage and thus represent a valuable genetic resource for the improvement of drought-tolerant germplasm for wheat production (Mao *et al*., 2020). Transgenic wheat lines overexpressing the *TaSNAC8-6A* (*NAM-A1*) gene exhibited enhanced drought tolerance through induction of auxin and drought-response pathways by stimulating lateral root development and

improving water-use efficiency. TaNAC2, a wheat transcription factor was responsive to drought, salt, cold, and abscisic acid treatment and improved tolerance against these stresses in Arabidopsis plants (Mao *et al.*, 2011), which were simultaneously demonstrated by enhanced expression of abiotic stress-response genes and several physiological indices. Another wheat NAC member, *TaNAC29*, located on chromosome 2BS, was found to improve drought and stress tolerance in transgenic overexpression Arabidopsis plants (Huang *et al*., 2015).

This highlights the importance of *NAC* genes in breeding activity to produce climate-smart wheat varieties with improved resilience against abiotic stresses including drought. This study aimed to identify abiotic stress-responsive *NAC* genes that belong to the NAC-a subfamily using a comprehensive phylogenetic analysis and their expression profiling was analyzed using the Genevestigator software. The identified members and their expression profile can be used in future research to develop new climate-smart wheat plants with improved tolerance against multiple stresses including drought and high salt stress.

MATERIALS AND METHODS

PHYLOGENETIC ANALYSIS

To identify NAC members in the wheat plant, the Plant Ensemble database (http://plants.ensembl.org/Triticum_aestivum/Info/Index) and the first version and release of the wheat genome at IWGSC (International Wheat Genome Sequencing Consortium; the first reference genome of wheat (IWGSC Ref Seqv1.0: https://www.wheatgenome.org/News2/RefSeq-v1.0-URGI) was used to retrieve protein sequences of all *NAC* genes as described previously (Borril, *et al*., 2017). Using this approach, 488 unique wheat NAC proteins were retrieved and their final sequences were validated and crossed checked for further analysis.

To identify NAC-family stress-related members belonging to the NAC-a subfamily, a phylogenetic analysis was performed by using the 488-wheat retrieved amino acids sequences with a set of reference NAC proteins from different plant species that was retrieved NCBI GenBank and represent members with known functions belonging to different NAC subfamily members using the MEGA6 software (Tamura *et al*., 2013). The amino acid sequences of the NAC members were aligned using the embedded Muscle algorithm and the output was used to build a phylogenetic tree by calculating distance matrices for neighbor-joining (NJ) analysis with the Kimura two-parameter model and a bootstrapping analysis with 1000 replicates to test the robustness of internal branches. A separate phylogenetic tree that includes NAC-a gene subfamily and their reference proteins was constructed using MEGA6 software with the same parameters described above.

IN SILICO GENE EXPRESSION ANALYSIS

For *in silico* gene expression analysis, the Genevestigator software (Zimmermann *et al*., 2004; https://www.genevestigator.com/gv/plant.jsp/) was used to analyze the expression profile of the 41 selected *NAC-a* gene subfamily in response to drought, osmotic, and salt stress conditions of selected RNAseq data as described previously (Allimuthu *et al.,* 2020). Furthermore, the same software was used to analyze the expression profile in different plant organs and developmental stages.

RESULTS AND DISCUSSION

The phylogenetic relationships and the functional relatedness of the *NAC* gene super-family in wheat were analyzed. Phylogenetic analysis was performed by using the 488-wheat retrieved NAC amino acid sequences from selected plant species (Fig. 1). The phylogenetic analysis grouped the 488 *T. aestivum* NAC proteins (TaNAC) into several sub-groups with the selected reference NAC proteins from other plant species. The first sub-group

includes 50 TaNAC proteins that are closely related to ANAC046, which is involved in the regulation of senescence and chlorophyll degradation (Oda-Yamamizo *et al*., 2016). A second sub-group included six members that were closely related to two reference NAC domains, including ANAC058-related and ANAC038-related and that is predicted to function in RNAi-mediated pathways in the Arabidopsis plant (Hisako *et al*., 2003). Sub-group-3 included six members that are highly related to ANAC054, which is known as cup-shaped cotyledon (CUC) and involved in meristem identity in Arabidopsis plant (Lee *et al*., 2015). Sub-group-4 included 13 members that were clustered with ANAC074, which is known as KIR1 and it's involved in regulating the programmed cell death of stigmatic tissue in the Arabidopsis plant (He *et al*., 2018). Sub-group-5 included 18 members that were closely related to ANAC022, which is involved in shoot apical meristem formation and auxin-mediated lateral root formation (He *et al*., 2005). Sub-group-6 included 21 members that are related to ANAC043, also known as NTS1, and involved in secondary wall thickening in Arabidopsis (Zhong *et al*., 2015). Sub-group-7 included 6 members that are closely related to ANAC033 (SMB) with a major role in root cap development in plants (Fendrych *et al*., 2014). Sub-group-8 included 10 members related to ANAC007, which is also involved in secondary wall thickening in xylem tissue (Zhou *et al.,* 2014). Sub-group-9 included 20 members that are closely related to ANAC072 (VDN2), which is also involved in secondary cell wall growth (Zhou *et al*., 2014). Sub-group-10 included five members that are closely related to ANAC011, which contains a transmembrane motif and potential function in ROS production during drought-induced leaf senescence (Ooka *et al*., 2003). Sub-group-11 included 14 members that are closely related to ANAC053 with unknown functions to date. Sub-group-12 included 12 members that are closely related to ANAC083 and Os08g44820, where ANAC083 is involved in inhibiting xylem vessel formation (Hu *et al.,* 2010). Sub-group-13 included 18 members that are closely related to ANAC082, also known as VND-INTERACTING 1 (VNI1), which might be involved as a ribosomal stress response mediator that causes growth defects in Arabidopsis (Ohbayashi *et al*., 2017). Sub-group-14 included three members that were closely related to ANAC016, which are involved in mediating abiotic-stress responses in plants is associated with NAP1 (Abdelrahman *et al*., 2017). Sub-group-15 included 19 members that were closely related to ANAC094, which are involved in mediating resistance against viruses in plants (Ooka *et al*., 2003). Sub-group-16 included 11 members that were closely related to ANAC042, which is a NAC transcription factor induced by hydrogen peroxide (H_2O_2) and has a role in delaying senescence (Saga *et al*., 2012). Sub-group-17 included 15 members that were closely related to ANAC036 and Os03g04070, which are involved in leaf and inflorescence stem morphogenesis and its mRNA has a cell-to-cell mobile activity (Kato *et al*., 2010). Sub-group-18 included nine members that were closely related to ANAC034, which is known as Long Vegetative Phase One and involved in leaf morphogenesis (Hu *et al*., 2010). Sub-group-19 included 11 members that were closely related to ANAC090, which is involved in delaying senescence (Hu *et al.,* 2010). Sub-group-20 included six members that were closely related to ANAC052, which is also known as the SUPPRESSOR OF GENE SILENCING 1, which is a NAC protein that physically associates with the histone H3K4 demethylase JMJ14 to repress the transcription of flowering time genes (Ning *et al*., 2015). Sub-group-21 included 10 members that were closely related to ANAC060, a transmembrane protein involved in ABA-mediated sugar metabolism in Arabidopsis (Li *et al*., 2014). Sub-group-22 included 15 members that were closely related to ANAC067, a transmembrane protein involved in regulating cell division in Arabidopsis (Ooka *et al*., 2003). Sub-group-23 included 11 TaNACs that were not affiliated with any

reference NAC protein. Sub-group-24 included 18 members that were closely related to ANAC064 that have no clear function to date. Sub-group-25 included 62 members that were not affiliated with any reference NAC protein. Sub-group-26 included 21 members that were closely related to ANAC008, which is an encoded suppressor of gamma response 1 (SOG1) and involved responses to DNA damage (Hu *et al*., 2010). Sub-group-27 included eight members that were closely related to ANAC073, a secondary wall-associated NAC protein (Hu *et al.*, 2010). Sub-group-28 included 17 members that were closely related to ANAC075, a flowering time repressor (Fujiwara *et al*., 2016). Sub-group-28 included 3 members that were not affiliated with any reference NAC protein. Sub-group-29 included three wheat-specific members (TraesCS2B02G376900.1; TraesCS4A02G065000.1 and TraesCS4D02G24200).

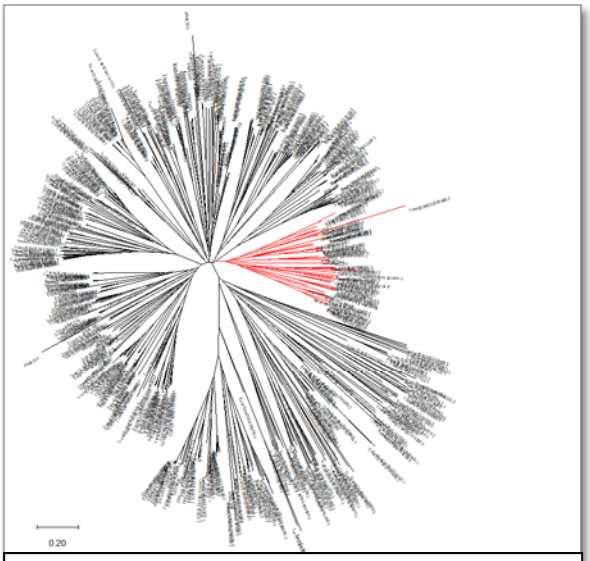

FIGURE 1. Phylogenetic analysis of 488 wheat NAC proteins and selected reference proteins from different plant species. The position of TaNAC sub-family A in the tree is indicated by red colour.

Based on NAC subfamilies calcifications by Shen *et al*. (2009), the NAC-a subfamily was identified and was found to include seven subgroups based on their clustering with reference NAC proteins (Fig. 2): sub-group-1 included eight members that are closely related to ANAC025 (NAM), which is involved in stress-responses and promoting senescence (Hu *et al*., 2010) and this group also included the *NAM-B1*, a major gene involved in leaf senescence and protein content in wheat (Uauy *et al*., 2006). Sub-group-2 included three members that are closely related to ANAC029 (NAP1) that is known to be involved in abiotic stress responses (Ooka *et al*., 2003). Sub-group-3 included 12 members that are closely related to Os03g0327800 (OsNAC047) that has a potential function in abiotic stress-responses (Mito *et al*., 2011). Sub-group-4 included three TaNACs (TraesCS3A02G162900,TraesCS3B02G194000, TraesCS3D02G170000) that were not affiliated with any reference NAC protein and seems to be specific to wheat. Sub-group-5 included nine TaNACs that were closely related to the Arabidopsis ATAF1 and two stress-related rice transcription factors (Os11g0184900 and SNAC2), which are involved in mediating abiotic stress responses in plants (Hu *et al*., 2008; Wu *et al*., 2009). Sub-group-6 included three members that were related to OsNAC4, which is known to promote drought tolerance in rice plants (Kaneda *et al*., 2009). Sub-group 7 included eight members that are closely related to SNAC1, a stress-responsive NAC in rice (Hu *et al*., 2006).

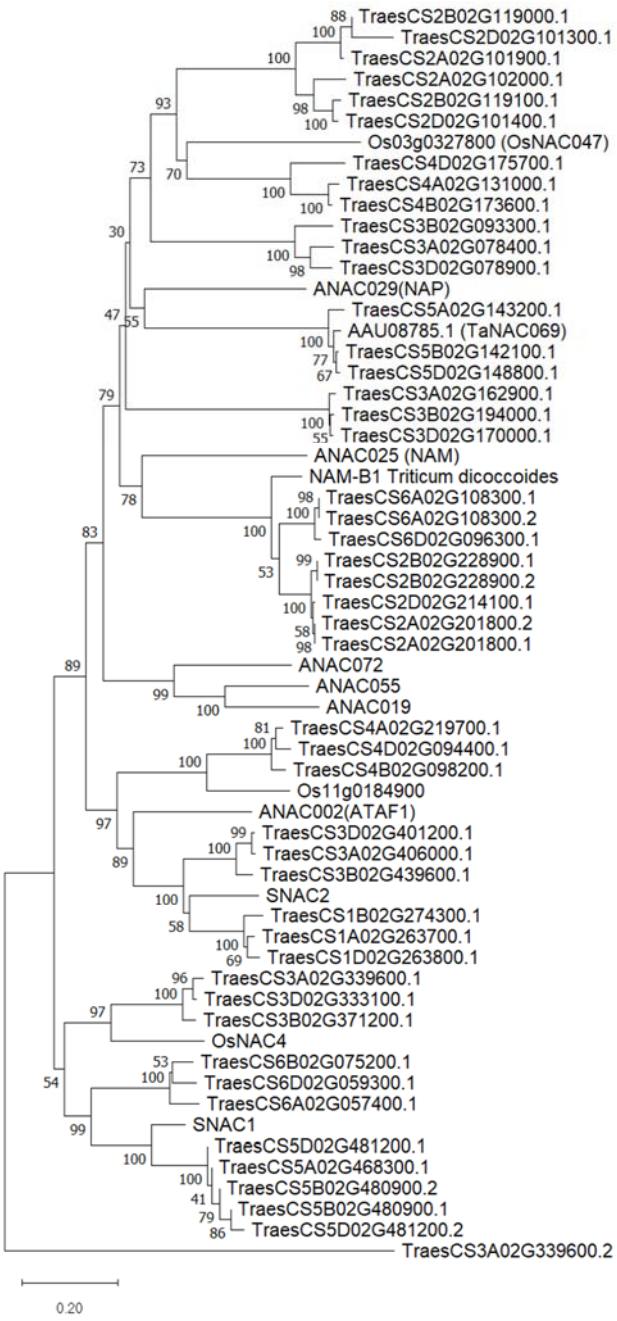

FIGURE 2. Phylogenetic analysis of wheat TaNAC-a sub-family proteins and selected reference stress-related proteins from different plant species.

The *in-silico* gene expression analysis for 41 members of the TaNAC-a subfamily was carried out with RNA-seq data associated with anatomy, development, and abiotic stresses (Fig. 3-5). Three members related to ANAC016 were included as checks for comparison. The expression analysis in seven anatomical parts, showed a clear induction of NAM-related members in the flag leaf of wheat plants (Fig. 3). This is consistent with data reported in Borrill *et al.* (2017), who observed an increased expression of NAM-related members in wheat flag leaf at senescence. Similarly, other members showed a high level of expression in flag leaf that might highlight their potential role during the flowering and grain-filling period. The three specific members (sub-group-4), showed no expression in the seven anatomical parts when compared with other members (Fig. 3).

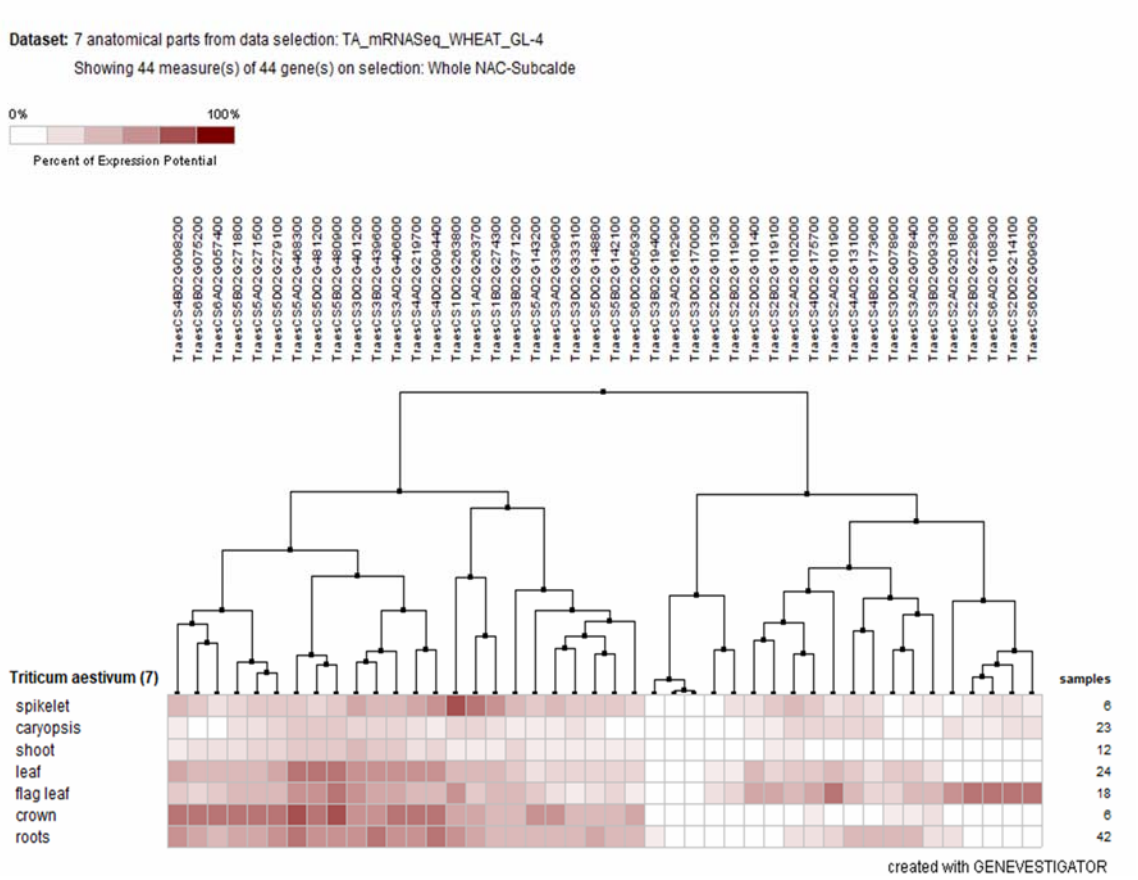

FIGURE 3. ***In silico*** **gene expression analysis of 41 TaNAC-a sub-family genes in seven anatomical parts by using Genevestigator. ANAC016-related members (*TraesCS5A02G271500*, *TraesCS5B02G271800* and *TraesCS5D02G279100*) were used for comparison.**

The expression patterns were also analyzed at six different development stages of wheat and as shown in Fig. 4. The three specific members (sub-group-4), showed no expression at any stage confirming their low expression patterns in the wheat plants. Inconsistent with their role in promoting senescence in wheat plants (Borrill *et al.,* 2019), the NAM-related members showed higher expression levels at the late stages starting from anthesis with increasing levels at the dough developmental stage. Except for NAM-related and sub-group-4 members, the expression of the remaining members was detected at the seedling stage. At the anthesis stage, three members belonging to sub-group-5 and closely related to SNAC2 (*TraesCS1A02G263700*, *TraesCS1B02G274300*, and *TraesCS1D02G263800*), were found to be expressed at high levels, which might indicate a potential during heading for these members.

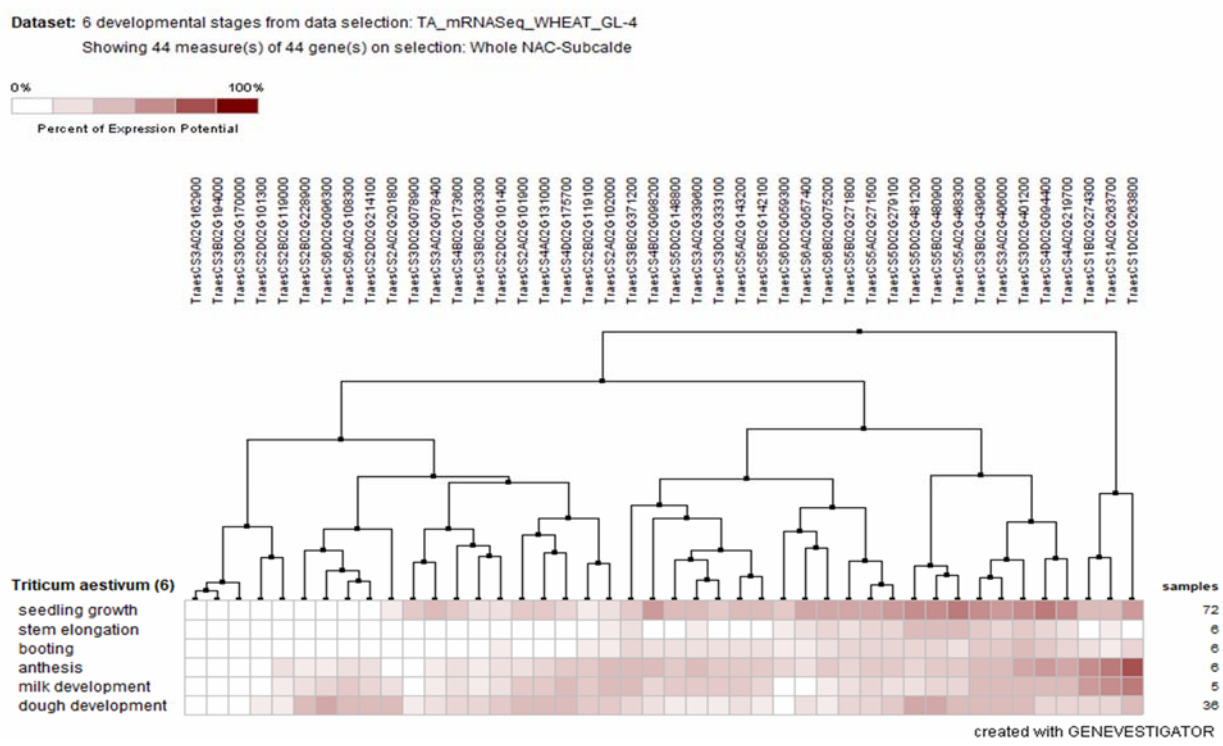

FIGURE 4. *In silico* gene expression analysis of 41 TaNAC-a sub-family genes in seven anatomical parts by using Genevestigator. ANAC016-related members (*TraesCS5A02G271500*, *TraesCS5B02G271800 and TraesCS5D02G279100*) were used for comparison.

To analyze the expression patterns of the 44 selected stress-related genes (41 TaNAC-a subfamily and three ANAC016-related) under abiotic stress conditions, eight RNA-seq experiments were used. As shown in Fig. 5, the SNAC1-related members (*TraesCS5A02G468300*, *TraesCS5B02G480900*, and *TraesCS5D02G481200*) were induced at high levels in response to different stresses including, cold, heat, and drought. This is consistent with previous reports that showed inducible expression in response to different abiotic stresses in rice (Hu *et al*., 2006) and barley (Al-Abdallat *et al.,* 2014) plants. Members that were related to OsNAC4, showed also high levels of induction in response to drought and cold stress (Kaneda et al., 2009). Under drought stress, the three members belong to sub-group-5 and are closely related to SNAC2 (*TraesCS1A02G263700*, *TraesCS1B02G274300*, and *TraesCS1D02G263800*), were found to be expressed at high levels, however, no induction was observed in response to cold and heat stresses. This is not in general agreement with their rice orthologue, which showed inducible expression in response to cold and heat stresses (Hu *et al*., 2008). Excluding the SNAC2-related members, the remaining six TaNACs of sub-group 5 were found to be highly expressed in response to cold treatment (Fig. 5). Interestingly, the NAM-related members showed reduced expression levels in the flag leaf in response to heat stress, which highlights a potential role of heat stress on senescence, and future studies are needed to uncover their role under heat stress. Another member that showed reduced expression in response to heat and drought was

TraesCS2A02G102000, which might indicate a potential role in the wheat plant. Several NAC proteins were found to act as negative regulators of drought response in plants (Sakuraba *et al*., 2015; Kim *et al*., 2018).

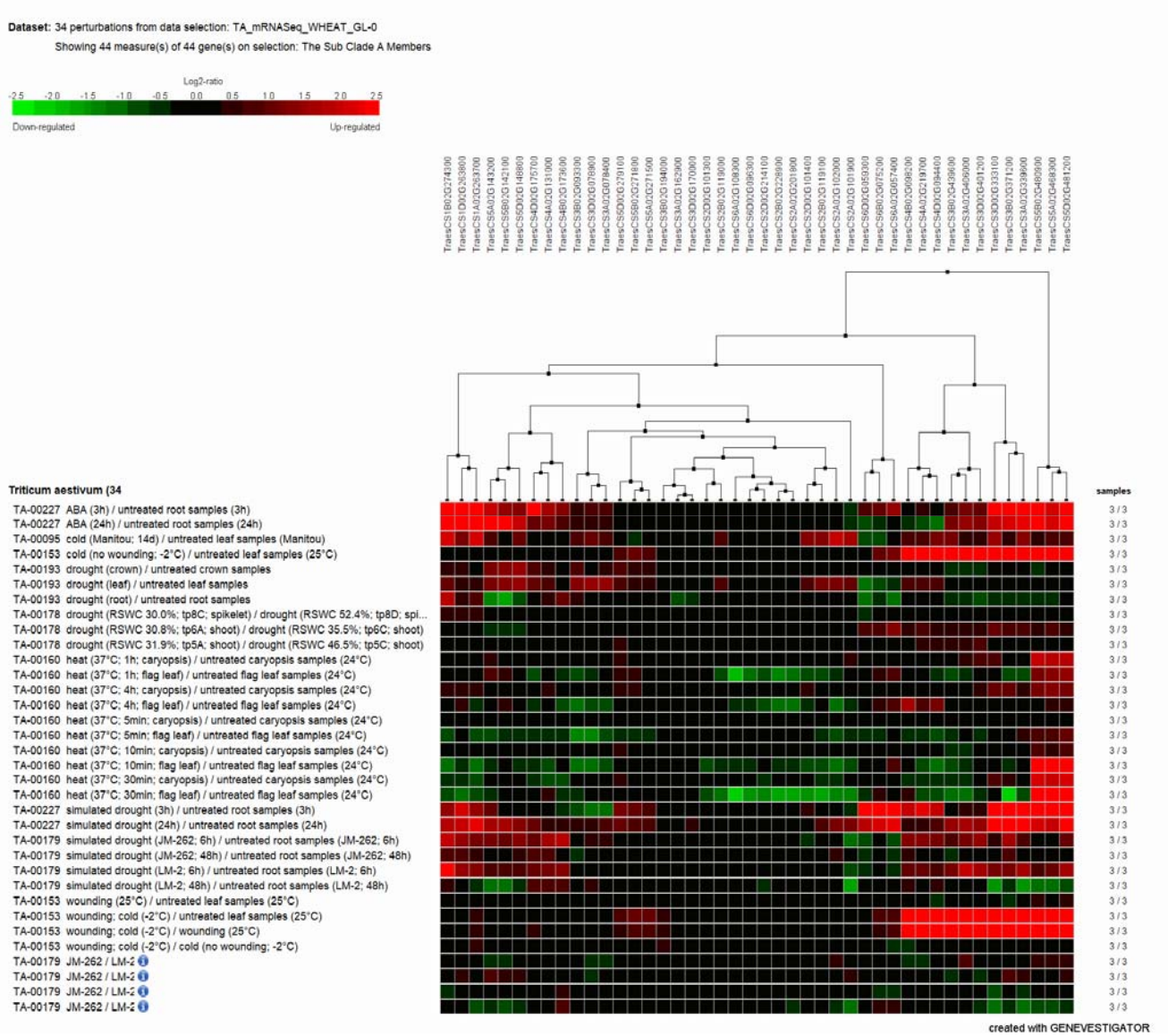

Figure 5. ***In silico*** **gene expression analysis of 41 TaNAC-a sub-family genes in eight selected abiotic stress experiments by using Genevestigator. ANAC016-related members (*TraesCS5A02G271500*, *TraesCS5B02G271800* and *TraesCS5D02G279100*)**

CONCLUSION

In conclusion, the presented data of this study identified 41 members of TaNACs that belong to the TaNAC-a subfamily, with a putative role in stress tolerance mechanisms in wheat. The differential expression of these members highlights the presence of divergent functions under different stress conditions and developmental stages. Future studies should be carried out using transgenic and mutant lines to elucidate their role in wheat tolerance against different abiotic stresses.

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تحليل تعبيري لجين السيليكو للعائلة الفرعية لجين NAC-aالمرتبط بالاجهاد لتشريح دورة في تحمل الاجهاد اللاحياتي في قمح الخبز
(TRITICUM AESTIVUM L) .

ربيع السيايدة[1*]، خالد الهباهبة[1]، زهرة عكا [2]، فرح مبيضين[2]، رندة البدوي[3]

[1] كلية الشوبك، جامعة البلقاء التطبيقية
[2] كلية الزراعة، الجامعة الردنية
[3] كلية الزراعة، الجامعة الردنية

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الملخص

يعتبر القمح من المحاصيل الاساسية التي تتأثر الى حد كبير بالاجهادات المختلفة التي تشمل الحرارة والجفاف والملوحة. كان الهدف الرئيسي من الدراسة هو نسخ عوامل القمح NAC ذوات العلاقة بالعائلة الفرعية NAC–a, والتي تشارك في التوسط في تحمل الاجهاد في انواع نباتية مختلفة. علاوه على ذلك, تم اجراء تحليل تعبيري لجيني السيليكو للكشف عن المتغيرات التفاضلية لافراد عائلة القمح NAC–a في الاعضاء المختلف, ومراحل التطور, وتحت مختلف الضغوط اللاحياتية. وباستخدام تحليل النشوء والارتقاء ل 488 عامل نسخ تم تحديد 42 بروتينا على انها افراد عائلة القمح NAC–a .ووجد في تحليل تعبيري لجين السيليكو ان عوامل نسخ القمح ذوات العلاقة ب NAC يتم التعبير عنها حصريا في مرحلة التخليق حتى تطور العجين بمستويات تعبير عالية تم اكتشافها في اوراق القمح. اظهر تحليل التعبير لجيني السيليكو ان الاعضاء ذوي العلاقة ب SNACI لديهم مستويات عالية من التعبير تحت ضغوط الجفاف والبرد والحرارة. يمكن مستقبلا استخدام افراد العائلة الفرعية للقمح المحسن NAC–a في تحمله للاجهاد لتطوير اصناف قمح ذكية مناخيا ومحسنة ضد الاجهادات اللاحياتية

الكلمات الدالة: الضغط اللاحياتي، التعبير لجيني السيليكو، تحليل النشوء والارتقاء، القمح.