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Genome wide identification of AGC kinase genes and their expression in response to heat and cold stresses in barley

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Abstract: AGC kinases are highly conserved regulators in a variety of cellular processes such as differentiation, proliferation, and growth. They are known to play important roles in stress and hormonal responses, including ROS signaling. AGC kinases are the main class of protein kinases in plants, having central functions in different stages of plant growth. In the present study, the analysis of phylogenetic relationships, gene structures, chromosomal locations, synteny analysis, gene ontology, subcellular localization, and gene expression of AGC kinase identified 28 AGC kinase genes in barley. Phylogenetic tree grouped them into seven subfamilies, as supported by exon-intron organization. Gene duplication and synteny indicated that tandom and block duplication events played an essential role in the expansion of AGC kinase gene families in barley. The Real-time quantitative reverse transcription PCR (qRT-PCR) analysis performed for *HvAGC kinase gene* were largely expressed in different tissues of roots, stems, and leaves in Azaran and Jolgeh cultivars under heat and cold stresses. The results of chromosomal localization showed that the AGC kinases were located on all chromosomes of barley except chromosome 1. Genome evolution of species was surveyed using identification of orthologous and paralogous genes. Identifying overlaps between orthologous clusters can enable us to study the function and evolution of proteins in different species. To our knowledge, this is the first detailed report of using AGC kinases for bioinformatics analysis in barley. Results revealed a broad understanding of the AGC kinase gene family in barley, which will be valuable for improving barley varieties' response to heat and cold stresses. Also, *HvNDR6.2* gene can utilized as molecular markers under cold stress in the three organs.

Key words: AGC kinase protein; protein model; synteny; gene duplication

Identifikacija genov na ravni celotnega genoma za kinaze AGC in njihovo izražanje kot odziv na vročinski in hladni stres pri ječmenu

Izvleček: Kinaze AGC so v veliki meri ohranjeni regulatorji različnih celični procesov kot so diferenciacija, proliferacina in rast. Znano je, da imajo pomembne vloge pri stresnih in hormonskih odzivih, vključno s signalizacijo ROS. Kinaze AGC so glavna skupina proteinskih kinaz v rastlinah, ki imajo osrednjo vlogo v razlilčnih fazah rasti rastlin. V tej raziskavi je bilo pri ječmenu na osnovi analize filogenetskih odnosov, genskih struktur, kromosomskih lokacij, analize sintenije in genske ontologije, njihove subcelularne lokalizacije in izražanja genov kinaz AGC identificiranih 28 genov kinaz AGC. Filogenetsko drevo jih je na osnovi organizacije intronov in eksonov porazdelilo v sedem poddružin. Podvojevanje genov in sintenija sta pokazali, da sta imela pri ječmenu tandemsko in bločno podvojevanje odločilno vlogo pri ekspanziji družin genov za kinaze AGC. Analiza kvantitativne reverzne transkripcije PCR v realnem času (qRT-PCR) opravljene za gene *kinase HvAGC je pokazala, da so se ti geni v veliki meri izrazili v različnih tkivih korenin, stebla in listov pri sortah* Azaran in Jolgeh v razmerah vročinskega in hladnega stresa. Rezultati kromosomske lokalizacije so pokazali, da so bili geni za kinase AGC pri ječmenu locirani na vseh kromosomih, razen na kromosomu 1. Evolucija genoma ječmena je bila preučena z identifikacijo ortolognih in paralognih genov. Prepoznavanje prekrivanj med skupinami ortolognih genov omogoča preučevanje funkcije in razvoja proteinov pri različnih vrstah. Glede na vedenje avtorjev je to prvo podrobnejše poročanje o uporabi kinaz AGC z analizo bioinfomatskih pristopov pri ječmenu. Rezulati so odkrili veliki pomen družin genov za kinaze AGC pri ječmenu, kar bo pomembno za izboljšanje sort ječmena pri odzivu na vročinski in hladni stres. Gen *HvNDR6.2* bi lahko uporabili kot molekularni marker odziva pri hladnem stresu v koreninah, steblu in listih.

Ključne besede: AGC proteinske kinaze; nabor proteinov; sintenija; podvajanje genov

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1 INTRODUCTION

Protein kinases catalyze the transfer of phosphoryl group from adenosine triphosphate (ATP) to some amino acids such as (serine, threonine) in their substrate proteins. Most protein kinases, like AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family) AGC kinase(, have seven subfamilies which have been reported in plants and bacteria. Protein phosphorylation is one of the most important post-translational modifications (PTMs) for cellular signaling, mediated by a group of enzymes called protein kinases (Bradley and Beltrao, 2019). The activity of these enzymes lead to the regulation of almost all cellular processes. This is achieved at any time by a network of different kinases that are transiently active. Therefore, control of cellular systems requires that each kinase targets only a limited set of substrates. AGC kinases are divided into seven groups based on sequence similarity, evolutionary conservation, and known functions (Bradley and Beltrao, 2019). Seven subgroups of AGC kinase (AGC1, AGC2, PDK-1, S6K, IRE, NDR, AGC2 related subfamilies) are common to eukaryotic genomes of animals, plants, and diatoms. AGC kinases are termed after the cyclic AMP dependent kinases (PKA), cGMP-dependent kinases, and the diacylglycerol-activated/phospholipid-dependent kinase PKC. Details and biochemical properties of 28 AGC kinase genes in barley are given in Table 1. The member of the 3-phosphoinositide dependent protein kinase 1 (PDK1) genes are highly conserved among eukaryotes (Mora et al., 2004). Furthermore, orthologous of the p70 ribosomal protein S6 kinase (S6K), the nuclear Dbf2-related (NDR) kinase subfamily identified in *Arabidopsis* (Galvan-Ampudia and Offringa, 2007). Based on amino acid sequence homology, AGC1-4 were placed in AGCVIII kinases in *Arabidopsis,* implicated in the regulation of cell polarity, growth, and cell division. AGCVIII kinases have conserved domains and are most associated to animal PKA and PKC, playing a key role in developmental stages and stress responses. In AGC2 related subfamily, two phototropin genes (PHOT1 and PHOT2) were expressed in plant seeds (Galvan-Ampudia and Offringa, 2007). Most protein kinases regulate cell growth and division in embryo, cotyledons, floral organs, and stress signaling (Rentel et al., 2004). In AGC1 subfamily, PINOID (PID) gene has been revealed to regulate the polarity of auxin transport by phosphorylating the large central hydrophilic loop of auxin efflux carriers (Dhonukshe et al., 2010; Huang et al., 2010). AGC kinases have multiple functions in different biological processes such as pollen germination and development and plant growth and development. Also, AGC kinase genes can be utilized in different abiotic and biotic stresses. Different function of proteins could be due to the presence of conserved domains or gene duplication (Saidi and Hajibarat 2020a). In this study, comprehensive analysis of phylogeny of AGC kinase genes, gene structures, gene duplications, synteny analysis, gene ontology, chromosomal distribution of AGC kinases were further performed. Gene expression of five AGC kinase genes were analyzed in response to heat and cold stresses in three tissues.

2 MATERIAL AND METHODS

2.1 PHYSICOCHEMICAL CHARACTERISTICS, PHYLOGENETIC ANALYSIS, AND GENE STRUCTURE OF AGC KINASE PROTEINS

The ensemble plant database was utilized to download the sequences of AGC kinase family genes from barley (*H. vulgare L), w*ild cabbage *(B. oleraceae L.), r*apeseed *(B. napus L.), f*ield mustard *(B. rapa L.), maize (Z. mays L.), Arabidopsis, and rice (O. sativa L.)*. ExPASy server (https://www.expasy.org/) was used to predict the theoretical isoelectric point (pI) and the molecular mass (Mm) of each of the AGC kinase proteins. AGC kinase protein sequences were aligned in seven species using the *MUSCLE* and phylogenetic tree was drawn using MEGA 7, applying the Neighborjoining algorithm. The Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) was used to obtain information on the exon – intron of AGC kinase proteins. The prediction subcellular localization of AGC kinase was performed using CELLO database.

2.2 CHROMOSOMAL DISTRIBUTION AND GENE ONTOLOGY (GO) ANALYSIS AND DETECTION OF ORTHOLOGOUS AND PARALOGOUS OF AGC KINASE GENES

For locating the AGC kinase genes on barley chromosome, AGC kinase genes were placed on each chromosome according to the physical location of the gene. The AGC kinase genes were distributed on all chromosomes and depicted with MapChart (Voorrips, 2002). For functional annotation using default parameters, the nucleotide sequences of DEGs were submitted to the online annotation tool of Mapman (http://www.plabipd.de/portal/mercator-sequence-annotation) (Thimm et al., 2004). The orthologous and paralogous genes among species were identified using Blastp of proteins (Altschul et al., 1990). When the protein sequence identity exceeded 70 %, it was considered orthologous genes whereas, when the AGC kinase protein sequences identity exceed-

Table 1: Details and Biochemical properties of AGC kinase genes in barley

Protein Name	Genomic Locus	Duplication	Chromosome location	Number of amino acids	Molecular weight	Theoretical pI	Subcellular localization
HORVU2Hr1G072580	HvAGC1.8		chr2H : 520772898-520775368	384	42.4	5.61	Cytoplasmic
HORVU3Hr1G082260	HvPDPK1		chr3H:598502260-598506773	481	53.7	6.17	Mitochondrial
HORVU3Hr1G024030	HvAGC1.10		chr3H : 90576271-90577182	1315	145	6.36	Cytoplasmic
HORVU4Hr1G062610	HvAGC1.11		chr4H : 523887114-523888691	417	47.7	6.03	Cytoplasmic
HORVU3Hr1G031930	HvPHOT1		chr3H : 160765754-160767689	527	60.4	5.9	Cytoplasmic
HORVU4Hr1G007540	HvIREH1		chr4H : 19756134-19766838	320	45	5.7	Mitochondrial
HORVU4Hr1G062610	HvPK3		chr4H : 523887114-523888691	464	53.8	8.05	Cytoplasmic
HORVU5Hr1G108690	HvAGC1.5	Block duplicate	chr5H : 628434675-628436329	692	75.4	6.39	Cytoplasmic
HORVU5Hr1G041960	HvAGC1.12		chr5H : 318743447-318747958	670	71.6	5.99	Cytoplasmic
HORVU1Hr1G030190	HvNDR6		chr1H : 172906058-172908519	427	45.2	9.09	Cytoplasmic
HORVU1Hr1G077430	HvNDR7		chr1H : 517244865-517251727	281	30	5.8	Cytoplasmic
HORVU1Hr1G031420	HvAGC1.7		chr1H : 188829523-188831273	470	51.6	9.78	Cytoplasmic
HORVU2Hr1G072580	HvKIPK		chr2H : 520772898-520775368	465	50.6	9.64	Cytoplasmic
HORVU2Hr1G093580	HvAGC1.3.1		chr2H : 658836435-658839125	123	13.7	9.99	Cytoplasmic
HORVU5Hr1G035610	HvKIPK.1		chr5H : 248570403-248571724	337	37.3	9.19	Cytoplasmic
HORVU5Hr1G072930	HvAGC2.4		chr5H : 537104020-537105417	512	55.2	9.27	Cytoplasmic
HORVU0Hr1G005020	HvAGC2.2		chr6h : 28702476-28703321	337	37.3	9.19	Cytoplasmic
HORVU6Hr1G062330	HvAGC1.3		chr6H : 417602550-417605458	340	38	9.2	Cytoplasmic
HORVU5Hr1G009390	HvPID		chr5H : 21969701-21971152	555	60.3	6.23	Cytoplasmic
HORVU6Hr1G054770	HvAGC2.1		chr6H : 347811467-347812777	783	85.9	9.25	Cytoplasmic
HORVU7Hr1G050240	HvAGC2.4		chr7H : 180272159-180273571	790	86	9.3	Cytoplasmic
HORVU7Hr1G050240	HvAGC2.3		chr7H : 180272159-180273571	529	58.3	7.1	Cytoplasmic
HORVU3Hr1G020020	HvNDR4	Block duplicate	chr3H : 60417083-60425196	525	57	9.22	Cytoplasmic
HORVU3Hr1G020020	HvNDR6.1	Block duplicate	chr3H : 60417083-60425196	527	56.5	8.07	Cytoplasmic
HORVU3Hr1G031930	HvAGC1.6		chr3H : 160765754-160767689	525	56.9	9.22	Cytoplasmic
HORVU4Hr1G050660	HvS6K2		Chr3 : 2648625-2650407	483	52	9.46	Mitochondrial
HORVU3Hr1G031930	HvAGC1.5.1		chr3H : 160765754-160767689	783	85.4	9.3	Cytoplasmic
HORVU1Hr1G027770	HvNDR6.2	Block duplicate	chr1H : 143842779-143854406	350	37.6	6.33	Cytoplasmic

ed 85 %, it was considered paralogous genes. The analysis of synteny of AGC kinase genes were performed using Circos program (http://mkweb.bcgsc.ca/tableviewer/visualize/). PLAZZA was used to detect the duplication patterns containing segmental/tandem duplications (Wang et al., 2009). Identification of orthologous clustering between AGC kinase members of *Arabidopsis thaliana*, (L.) Heynh. rice, and barley was performed using OrthoVenn2 (https://orthovenn2.bioinfotoolkits.net/home) webserver.

2.3 BARLEY GROWTH UNDER HEAT AND COLD TREATMENTS

This study was done based on a randomized complete block design (RCBD) with three replications at the Seed and Plant Improvement Institute, Karaj (latitude 35 °, longitude 50 ° and altitude 1313 m above sea level) during 2021. Azaran and Jolgeh cultivars were cultured in pots at 25 °C for two weeks. Young roots, stems, and leaves from 2-week-old seedlings were harvested for tissue-specific

expression analysis under cold or heat stress, treated for four hours at 4 °C or 42 °C, respectively.

2.4 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (QRT-PCR) ANALYSIS

Total RNA was extracted from root, stem, and leaf under stress conditions using RNA-Plus kit (*Sinaclone*) based on the manufacturer's instructions. For the preparation of tissue-specific RNA, the organ samples were collected separately two week-old seedlings under cold (4 °C) and heat (42 °C) stresses for 4 hrs. To remove residual genomic DNA contamination in RNA samples, DNase I (Fermentase Company) was utilized. The purity and concentration of RNA were determined by nanodrop as well as the quality of which was validated using 1 % agarose gel analysis. Then, cDNA synthesis was performed according to Easy cDNA Synthesis Kit instructions. Three replications were performed for the analysis of each gene, with the barley Actin gene utilized as the reference gene. All primers used in gene expression analysis are listed in Table 2. Primers were designed using the Oligo program. Real time (qPCR) was done on ABI 7500 using SYBR Green Supermix as described in the manufacture's guidelines. Relative expression was determined via $2^{-\Delta\Delta Ct}$ technique after normalization of the Ct value for individual genes versus Actin as the reference gene. Analysis of gene expression was performed using the REST software (according to the Pfaffl method). RT-qPCR was conducted to determine the expression profile for the five AGC kinase genes using various tissues under heat and cold stresses. RT-qPCR expression analysis was carried out using our established protocol.

Table 2: Primers used for AGC kinase genes in this study

No.	Primer Name	Sequence 5'→ 3'
1	HvNDR4F	TGGCTTCGTCTGGACAACCTGCT
	HvNDR4R	CTTGGTTGGAACACGCTACACC
2	HvNDR6F	GAGGAGTTGTATACACCACC
	HvNDR6R	ACGTCGCATCCCTTTGCTCA
3	HvNDR6.2F	CCGATGAGTCCATAACCCGGCG
	HvNDR6.2R	GGATTTCTACAAACTGCTCGCC
4	HvAGC1.5F	AAGCAACACCCCTTCTTCGAG
	HvAGC1.5R	GTTCTAGAAATACTCGAACTGGCC
5	HvAGC1.5.1F	AGATCAAGCAGCACCCCTTC
	HvAGC1.5.1R	CGCTGTGGTCTAAAAGAACTCG
6	Actin	F: GGTCCATCCTAGCCTCACTC
	Actin	R: GATAACAGCAGTGGAGCGCT

3 RESULTS AND DISCUSSION

In the present study, AGC kinase genes in barley were surveyed using genome-wide identification, chromosomal distribution, evolutionary relationships, synteny analysis, and gene structure. Detailed information including the biochemical properties of the 28 AGC kinase genes are listed in Table 1. Sequence analysis showed that the lengths of the deduced AGC kinase proteins varied from 123 amino acids (HvAGC1.3.1) to 1315 amino acids (HvAGC1.10). The predicted molecular weights (MW) and isoelectric points (pI) ranged from 13.7 kDa (HvAGC1.3.1) to 85.9 kDa (HvAGC2.1) and from 5.61 (HvAGC1.8) to 9.99 (HvAGC1.3.1) (Table 1). AGC kinase proteins grouped into the same subfamily exhibited similar motif distributions, suggesting functional similarities for the members in the same subfamily. In addition, same roles of AGC kinases in various plant species that showed differential aspects of AGC kinase functionality in species. For instance, in contrast to the reported conserved functions of many AGC kinases, INCOMPLETE ROOT HAIR ELONGATION (IRE), a kinase of the "AGC other" group, seems to have acquired a new function in *Medicago Truncatula* Gaertn.. In *Arabidopsis,* IRE kinase has been revealed to control root hair elongation, while in*Medicago* a role in the formation of nodules has been indicated (Pislariu and Dickstein, 2007; Oyama et al., 2002; Saidi and Hajibarat, 2021a). Most of AGC kinase genes were located in the cytoplasm, but HvPDPK1, HvIREH1 and HvS6K2 genes were located in the mitochondria.

3.1 PHYLOGENETIC ANALYSIS AND GENE STUCRURE

To assess the evolutionary relationships of AGC kianse proteins in *H. vulgare, B.oleraceae, B.napus. B.rapa, Z.mays, Arabidopis, and O.sativa.* used to discribe phylogenetic analysis using MEGA7 based on protein sequences (Figure 1). The phylogenetic relationships and exon/intron analysis of the barley were showed in Figure 1. The HvAGC from barley was distributed in all groups, proposing that the expansion of HvAGC occurred in barley genome. Many researchers have revealed that the AGC kinase genes from monocots, based on their domains, could be grouped into 7 subfamilies (Kong et al., 2021). The genes within each subfamily showed similar gene structures. Gene structure of AGC kinase genes in barley was grouped into 7 subfamilies, with the largest cluster related to AGC-1 subfamily. The smallest cluster was related to PDK-1 and SK6 subfamilies. The num-

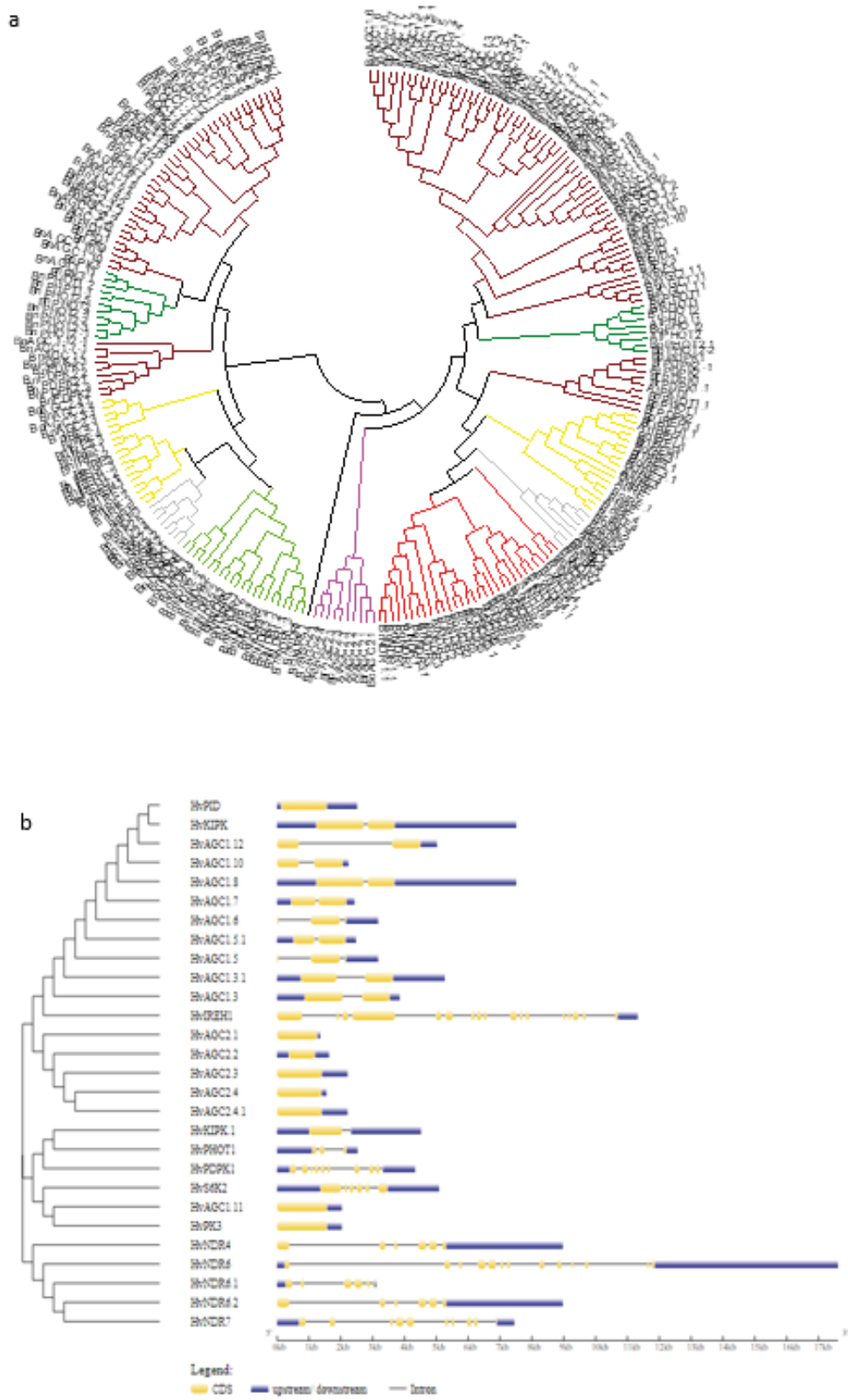

Figure 1: Phylogenetic tree of AGC kinase genes created by the neighbor-joining (NJ) method (a) in MEGA7.0 software in *Arabidopsis,* rice, barley, *Brassica napus, Brassica rapa,* and *Brassica oleracea*. The tree was constructed using the MEGA 6.0 software by the Neighbor joining method. Distributions of the exon- intron pattern in AGC kinase proteins in barley (b)

ber of exons in AGC kinase genes ranged from one to 17. The HvNDR6 and HvIREH1 genes had the highest number of exons. Some AGC kinases such as HvAGC2.1 and HvKIPK.1 genes contained only one exon, indicating that these genes have conserved domains. The gene structure of the AGC kinase genes in the same subfamily is highly consistent (Figure 1b). Most of the AGC kinase genes were grouped in the same subfamily. Most genes had many exons with introns, indicating that patterns of exons and introns, which correlate well with the phylogenetic tree, support their close evolutionary relationships between the AGC kinase genes within the same subfamilies.

The AGC2.1, also known as OXI1, was revealed to be a prerequisite for ROS-mediated responses in *Arabidopsis* like root hair elongation and for resistance to biotrophic pathogens. The activity of OXI1 was enhanced through H_2O_2, wounding, and various elicitor treatments mimicking pathogen attack (Rentel et al., 2004; Petersen et al., 2009). Also, as *oxi1* mutant plants are impaired in the activation of mitogen-activated protein kinase (MPK) and MPK6 in response to cellular injury and oxidative stress, OXI1 is a regulator of stress-responsive MPKs although its mechanism is still unclear. *PDK1* is a key factor involved in stress signaling (Petersen et al., 2009). *PDK1* encodes a gene of the AGC protein kinase family and a significant regulator of AGC kinases. PDK1, detected in mammalian cells, has an essential role in relating lipid signaling to a comprehensive range of cellular signaling and processes (Kyoko et al., 1989). Also, it is involved in the advancement of cell proliferation and survival and is overexpressed in many different tumors (Toda et al., 1988; Saidi and Hajibarat, 2021b). PDK1 contains an N-terminal kinase domain and a C-terminal pleckstrin homology (PH) domain through which it binds to phospholipids (Krupnick et al., 1998).

3.2 CHROMOSOMAL DISTRIBUTION AND DUPLICATION OF AGC KINASE GENES

Anslysis of physical locations on barley chromosomes preseneted that 28 AGC kinase genes were drawn using Mapchart software (Figure 2). In barley, three AGC kinase genes were located on both chromosomes 2 and 6. Six and 10 HvAGC were located on the chromo-

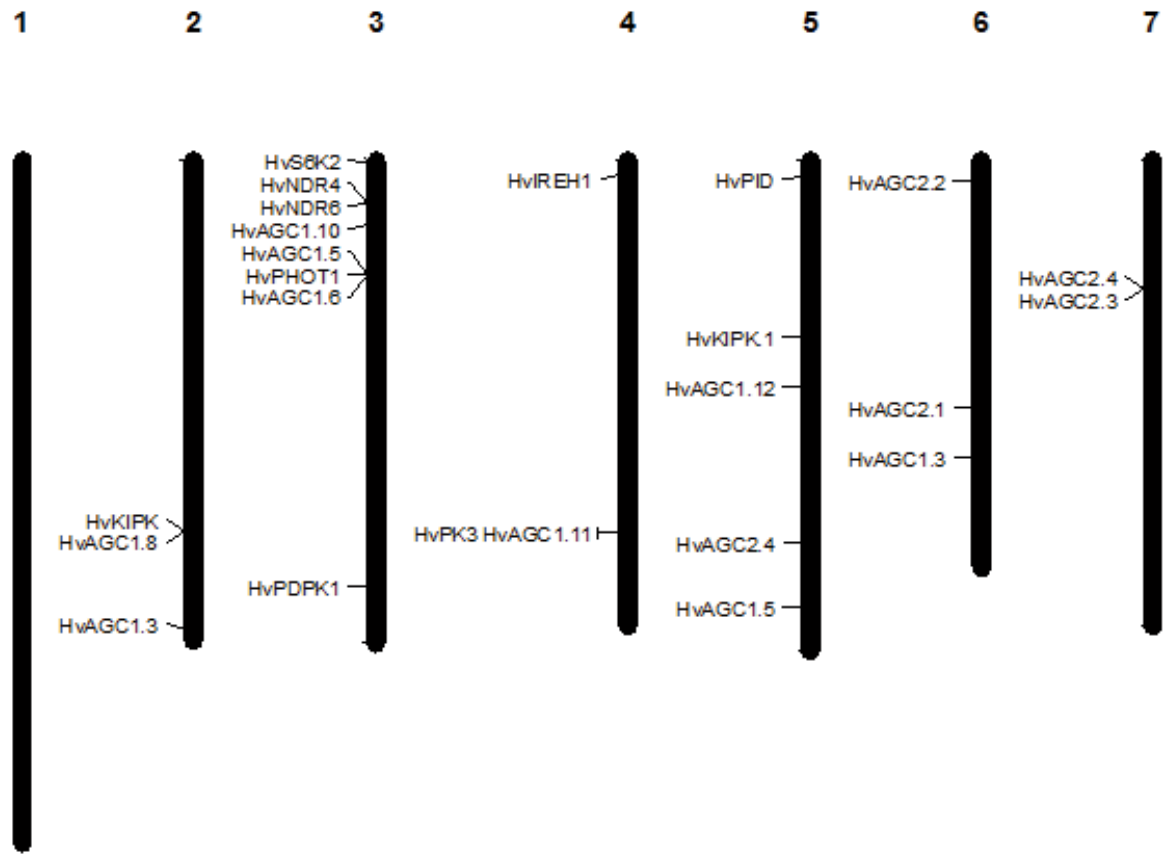

Figure 2: Chromosomal distribution and expansion patterns of AGC kinase genes in barley, drawn using Mapchart software

somes 2 and 3, respectively. Finally, nine HvAGC were distributed on chromosome 5. Our findings showed that the *Hv*AGC genes are unevenly distributed on different chromosomes. Based on the research findings on rice, *Arabidopsis* and *Brachypodium distachyon* (L.) P.Beauv., it has been shown that AGC kinase gene families mainly expanded through whole-genome and chromosomal segment duplications (Xue et al., 2008; Yang et al., 2008). Genes located within a distance of less than 200 kb on the same chromosome are defined as tandem duplications, otherwise they are segmental duplications (Cheung et al., 2003). In barley, 11 pairs of HvAGC duplication genes were involved in tandem duplication events and no gene segmental duplication pairs were found (Figure 2).

3.3 ORTHOLOGOUS AND PARALOGOUS GENES STUDY IN AGC KINASE

In this study, a comparative analysis was done to detect the orthologous of AGC kinase genes in barley genome (Figure 3). Based on the gene identity, orthologous (exceeding 70 %) and paralogous (exceeding 85 %) gene pairs were revealed. Among 28 genes, one gene was paralogous. According to our results, high similarity in barley genes suggests genome duplication (polyploidy), playing a key role in the evolution of AGC kinase genes. The comparative analysis to identify orthologous of AGC kinase genes in barley genome showed that the HvNDR4 with HvNDR6 genes had high similarity (identity 70 %) was orthologous one gene pair and HvNDR6.2 with HvNDR4 genes was paralogous (Figure 3). The syntenic analysis indicated that duplications as main elements for the diversity in AGC kinase genes, suggesting the structural and functional conservation of the genes underlying the origins of evolutionary with conserved domains (Altenhoff and Dessimoz, 2009). Often orthologous genes have similar expression among various species, while paralogous genes have the same basic but slightly different functions. It has been shown that gene duplication is collectively deemed from single-gene duplications (Saidi et al., 2020b). The structural conservation of NDR proteins indicates that they may had similar functions and regulatory mechanisms in various species. Surveys have suggested that NDRs are the main factors of the signaling mechanism in yeast and human (Hergovich, 2016).

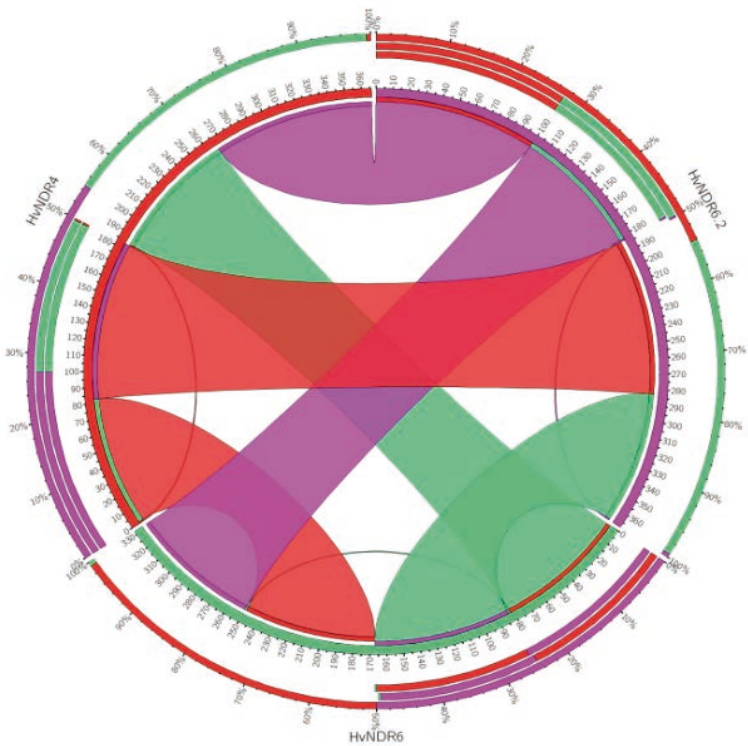

Figure 3: Orthologous and paralogous relationships of AGC kinase genes with three genomes visualized by Circos database in barley

3.4 ORTHOLOGOUS GENE CLUSTERING OF AGC KINASE GENE FAMILY IN *ARABIDOPSIS*, RICE, AND BARLEY

OrthoVenn2 web server was utilized to identify the evolutionary relationship of AGC kinases between dicot (*A. thaliana*) and monocot (*O. sativa*, and *H.vulgare*) plants using orthology analysis. In this study, 86 AGC kinase proteins from three species were clustered in 18 orthologous groups. One monocot-specific cluster was observed containing two AGC kinase proteins, whereas seven AGC kinase proteins were found to be present in two dicot-monocot orthologous clusters in *Arabidopsis* and barley (Fig 4). Also, 18 AGC kinase proteins were found to be present in nine dicot-monocot orthologous clusters in *Arabidopsis* and rice. One cluster containing two AGC kinase proteins has been identified between rice and barley (Figure 4). Interestingly, the OrthoVenn2 analysis also indicated the presence of an *Arabidopsis*-specific orthologous cluster containing two AGC kinases (Figure 7a). A singleton is a rare variant for which genetic difference is performed by a single chromosome in a genome. Among the three species, *Arabidopsis* had the highest number of singletons (54). Singletons usually are dispersed in the genome, indicating that they have been developed independently.

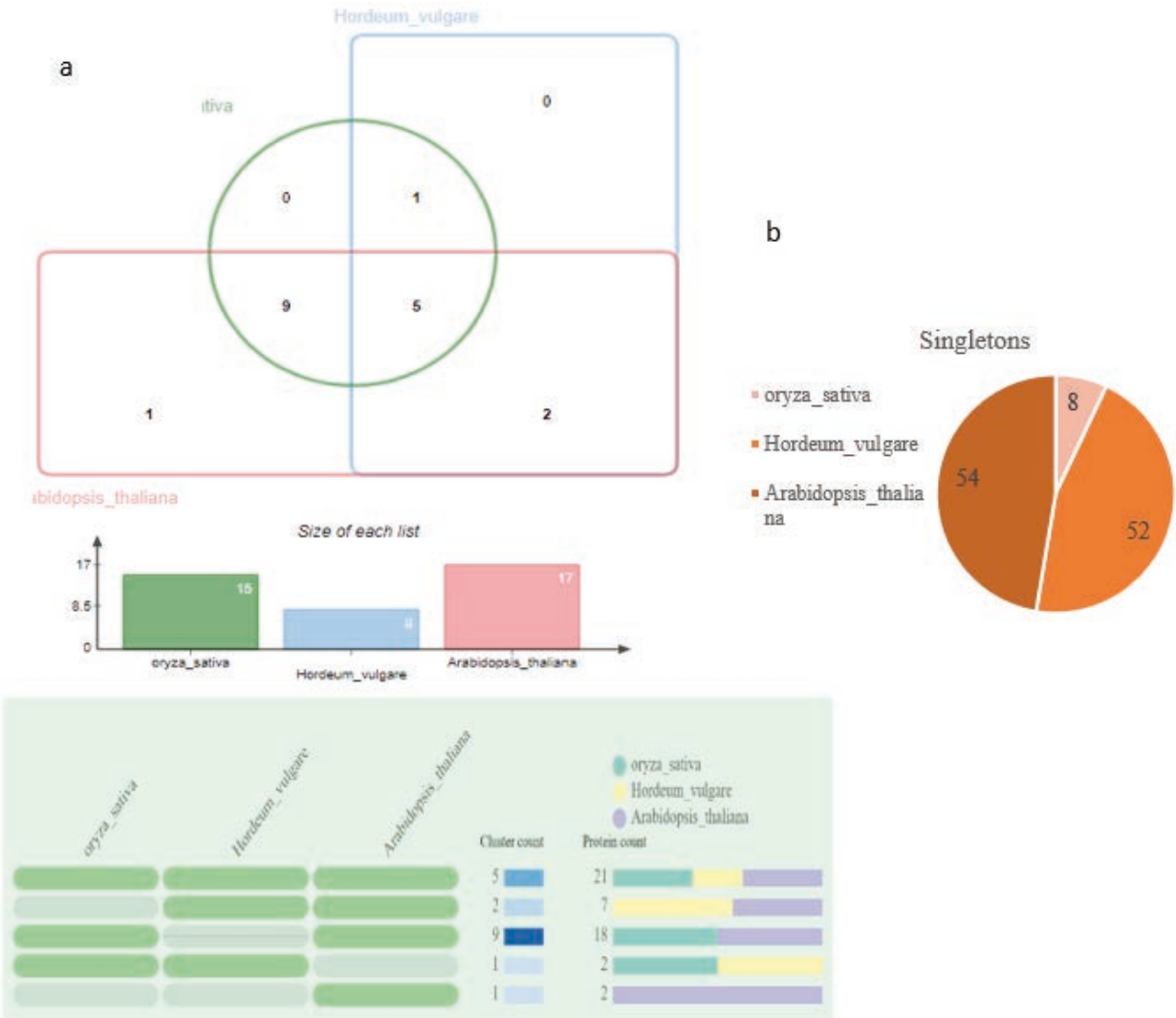

Figure 4 a: Orthologous gene clustering analysis. The orthologous gene clusters among the AGC kinase gene families in *A. thaliana, O. sativa,* and *H. vulgare* were identified and visualized using the OrthoVenn2 web platform. The e-value cut-off 1e-10 was used for the analysis. **b.** Number of singletons identified in *A. thaliana, O. sativa,* and *H. vulgare*

3.5 EXPRESSION PROFILES ANALYSIS OF AGC KIANASE GENES IN SPECIFIC TISSUES UNDER COLD AND HEAT STRESS CONDITIONS

In Azaran cultivar, most of AGC kinase genes showed a wide range of expression levels under heat and cold stress conditions. The expression profile of 5 AGC kinase genes under heat and cold stress conditions were performed using reverse transcription-PCR (qRT-PCR) quantitative analysis in three different tissues: root, stem, and leaf. Most of genes showed increased expression under heat stress as compared to cold stress and were expressed in the leaves showed increased expression, indicating that the AGC kinase genes were involved in leaf development. AGC kinase genes were decreased in cold stress but were increased in response to heat stress. The HvNDR6.2 gene showed high expression in root, shoot, and leaf tissues under heat stress, while it was down-regulated in response to cold (Figure 5a).

In Jolgeh cultivar, AGC kinase genes showed different expressions in response to cold and heat stresses. The HvNDFR4 gene was up-regulated in roots under heat stress but was down-regulated under cold stress condition. In Jolgeh cultivar, most genes were down-regulated in response to heat and cold stresses. Only the HvNDR6.2 gene was up-regulated in leaves and stems under heat stress. The HvNDR6.2 gene showed up-regulated expression in roots, stems, and leaves under cold stress. Most genes showed high levels of relative expression under normal conditions in leaves. But AGC kinase genes was down-regulated under cold stress. The HvNDR4 gene had high expression in roots under heat condition. These results showed that the HvNDR4 gene can be used as a molecular marker in barley root improvement under heat and cold stresses. Also, the HvNDR6.2 gene can be utilized as a molecular marker under cold stress in all three tissues (Figure 5b). In our analysis, the expression profiles of AGC kinase genes under heat and cold stresses revealed differential and overlapping expression patterns. Various expression patterns of AGC kinase genes may suggest various roles in response to heat and cold stress conditions. Based on the synthetic analysis of the AGC kinase genes, the HvNDR4 and HvNDR6 genes were orthologous, indicating that the orthologous genes had similar expression. As a result, the HvNDR4 and HvNDR6 genes can respond almost identically under heat and cold stress conditions.

3.6 GENE ONTOLOGY ANALYSIS

In barley, the largest percentage of detected proteins was involved in protein metabolism (90.91 %) followed by hormonal metabolism (6.06 %) (Figure 6). Most of the AGC kinase genes in the barley were involved in protein metabolism (post-translational modification and protein synthesis) and signaling, where many of the AGC2 kinases were involved in light signaling. Plants possess the same basic AGC kinase subfamilies (PDK1, S6K, and NDR) as other eukaryotes but they do not encode for the AGC kinases, such as PKA and PKC, implicated in the control of cell expansion, proliferation, and auxin polarity in fungi and animals (Zhang and Friml, 2020). One NDR protein, TaAGC1, has been indicated to have bio-

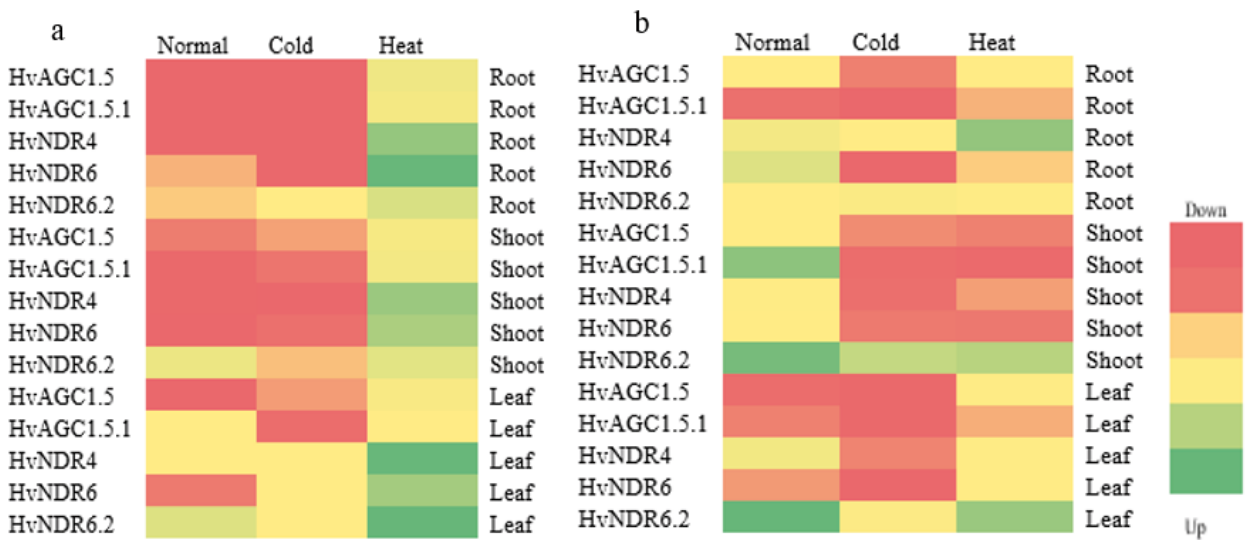

Figure 5: Differential gene expression of Azaran (a) and Jolgeh (b) cultivars under heat and cold stress conditions. Green and red indicate up and down-regulated genes, respectively. Also, yellow color indicates low level expression under both stresses. Gene expression patterns in the three tissues (stem, root, leaf)

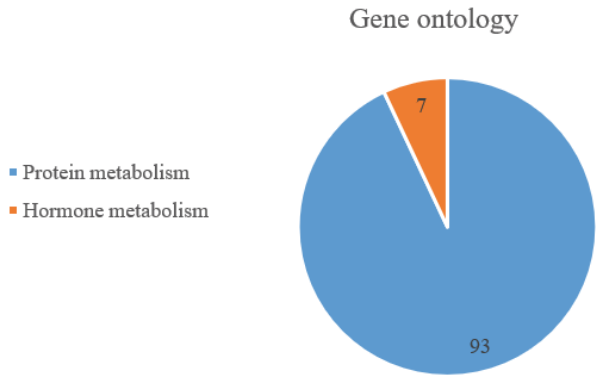

Figure 6: GO classification of the DEGs in barley. Results of significantly enriched pathways involving the AGC kinases genes in barley

logical function, implicated in response to *Rhizoctonia cerealis* E.P. Hoeven used for controlling the induction of ROS-related and defense-related genes in wheat (Zhang and Friml, 2020). Plant responses to external or internal stimuli include rapid protein changes that ultimately lead to the activation of transcriptional processes. Phosphorylation of proteins is commonly used in cellular signaling (protein kinase), where a phosphate group is added to the amino acid side chain. PID and phototropins are main factors in triggering and regulating growth by controlling auxin transport such as PIN. Phototropins have a key role in plant growth and were used directly in polar auxin transport (Galván-Ampudia and Offringa, 2007). The AGC kinase protein kinase family is involved in various signaling pathways, containing light blue and auxin signaling (Christensen et al., 2000; Robert and Offringa, 2008).

4 CONCLUSION

In this study, 28 AGC kinase genes were detected in barley. We studied their phylogenetic relationships, gene structures, chromosomal locations, genes duplication, gene ontology, and clustering of orthologous genes. Further, genome-wide identification of AGC kinase genes were performed. According to structure analysis results, various genes of the same subfamily had similar gene structure, proposing that they have the same evolutionary origin and probably the same functions. These results can provide insights into the functional differences, evolutionary relationships, and comparative genomics analysis of AGC kinases in barley. The HvNDR4 and HvNDR6 genes were both orthologous and were found to serve as molecular signaling in different stresses. AGC kinase genes showed to have different functions, indicating the presence of various conserved domains in these genes. Using this characteristic, candidate genes can be used to genetically improve plants in response to abiotic stresses. Based on the synthetic analysis of the AGC kinase genes, the HvNDR4 and HvNDR6 genes were orthologous, indicating that the orthologous genes show similar expression.

5 CONFLICTS OF INTERESTS

The authors declare that they have no competing interests.

6 REFERENCES

Altenhoff, A.M. & Dessimoz, C. (2009). Phylogenetic and functional assessment of orthologs inference projects and methods. *PLoS Computational Biology*, *5*(1), p.e1000262. https://doi.org/10.1371/journal.pcbi.1000262

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bradley D. & Beltrao, P. (2019). Evolution of protein kinase substrate recognition at the active site. *PLoS Biology*, *17*(6), p.e3000341. https://doi.org/10.1371/journal.pbio.3000341

Cheung, J., Estivill, X., Khaja, R., MacDonald, J.R., Lau, K., Tsui, L.C. & Scherer, S.W. (2003). Genome-wide detection of segmental duplications and potential assembly errors in the human genome sequence. *Genome Biology*, *4*(4), 1-10. https://doi.org/10.1186/gb-2003-4-4-r25

Christensen, S.K., Dagenais, N., Chory, J. & Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. *Cell*, *100*(4), 469-478. https://doi.org/10.1016/S0092-8674(00)80682-0

Dhonukshe, P., Huang, F., Galvan-Ampudia, C.S., Mähönen, A.P., Kleine-Vehn, J., Xu, J., Quint, A., Prasad, K., Friml, J., Scheres, B. & Offringa, R. (2010). Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS (N/S) motifs to direct apical PIN recycling. *Development*, *137*(19), 3245-3255. https://doi.org/10.1242/dev.052456

Galván-Ampudia, C.S. & Offringa, R. (2007) Plant evolution: AGC kinases tell the auxin tale. *Trends in Plant Science*, *12*(12), 541-547. https://doi.org/10.1016/j.tplants.2007.10.004

Hergovich, A. (2016). The roles of NDR protein kinases in hippo signalling. *Genes*, *7*(5), 21. https://doi.org/10.3390/genes7050021

Huang, F., Kemel Zago, M., Abas, L., van Marion, A., Galván-Ampudia, C.S. & Offringa, R. (2010). Phosphorylation of conserved PIN motifs directs *Arabidopsis* PIN1 polarity and auxin transport. *The Plant Cell*, *22*(4), 1129-1142. https://doi.org/10.1105/tpc.109.072678

Kong, W., Tan, S., Zhao, Q., Lin, D.L., Xu, Z.H., Friml, J. & Xue, H.W. (2021). mRNA surveillance complex PELOTA–HBS1 regulates phosphoinositide-dependent protein kinase1 and

plant growth. *Plant Physiology*, *186*(4), 2003-2020. https://doi.org/10.1093/plphys/kiab199

Krupnick, J.G. & Benovic, J.L. (1998). The role of receptor kinases and arrestins in G protein–coupled receptor regulation. *Annual Review of Pharmacology and Toxicology*, *38*(1), 289-319. https://doi.org/10.1146/annurev.pharmtox.38.1.289

Kyoko, K., Ohno, S., Serji, M., Ichiro, Y. & Koichi, S. (1989) A novel yeast gene coding for a putative protein kinase. *Gene*, *76*(1), 177-180. https://doi.org/10.1016/0378-1119(89)90021-8

Oyama, T., Shimura, Y. & Okada, K. (2002). The IRE gene encodes a protein kinase homologue and modulates root hair growth in *Arabidopsis*. *The Plant Journal*, *30*(3), 289-299. https://doi.org/10.1046/j.1365-313X.2002.01290.x

Petersen, L.N., Ingle, R.A., Knight, M.R. & Denby, K.J. (2009). OXI1 protein kinase is required for plant immunity against *Pseudomonas syringae* in *Arabidopsis*. *Journal of Experimental Botany*, *60*(13), 3727-3735. https://doi.org/10.1093/jxb/erp219

Pislariu, C.I. & Dickstein, R. (2007). An IRE-like AGC kinase gene, MtIRE, has unique expression in the invasion zone of developing root nodules in *Medicago truncatula*. *Plant Physiology*, *144*(2), 682-694. https://doi.org/10.1104/pp.106.092494

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. & Knight, M.R. (2004). OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. *Nature 427*(6977), 858-861. https://doi.org/10.1038/nature02353

Robert, H.S. & Offringa, R. (2008). Regulation of auxin transport polarity by AGC kinases. *Current Opinion in Plant Biology*, *11*(5),495-502. https://doi.org/10.1016/j.pbi.2008.06.004

Saidi, A. & Hajibarat, Z. (2020a). Computational study of environmental stress-related transcription factor binding sites in the promoter regions of maize auxin response factor (ARF) gene family. *Notulae Scientia Biologicae*, *12*(3), 646-657. https://doi.org/10.15835/nsb12310823

Saidi, A., Hajibarat, Z. & Hajibarat, Z. (2020b). Identification of responsive genes and analysis of genes with bacterial-inducible cis-regulatory elements in the promoter regions in *Oryza sativa* L.. *Acta agriculturae Slovenica*, *116*(1), 115-123. https://doi.org/10.14720/aas.2020.116.1.1035

Saidi, A., Hajibarat, Z. & Hajibarat, Z. (2021a). Phylogeny, gene structure and GATA genes expression in different tissues of Solanaceae species. *Biocatalysis and Agricultural Biotechnology*,102015. https://doi.org/10.1016/j.bcab.2021.102015

Saidi, A., Hajibarat, Z. & Ahmadikhah, A. (2021b). Computational analysis of responsive transcription factors involved in drought and salt stress in rice. *Journal of Applied Biotechnology Reports*, *8*(4), 406-413. doi:10.30491/jabr.2020.243913.1272

Thimm, O., Bläsing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L.A., Rhee, S.Y. & Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal*, *37*(6), 914-939. https://doi.org/10.1111/j.1365-313X.2004.02016.x

Toda, T., Cameron, S., Sass, P. & Wigler, M. (1988). SCH9, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes & Development*, *2*(5), 517-527. https://doi.org/10.1101/gad.2.5.517

Voorrips, R.E. (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. *Journal of Heredity*, *93*(1), 77-78. https://doi.org/10.1093/jhered/93.1.77

Wang, L., Yin, H., Qian, Q., Yang, J., Huang, C., Hu, X. & Luo, D. (2009). NECK LEAF 1, a GATA type transcription factor, modulates organogenesis by regulating the expression of multiple regulatory genes during reproductive development in rice. *Cell Research*, *19*(5), 598-611. https://doi.org/10.1038/cr.2009.36

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de Beer, T.A.P., Rempfer, C., Bordoli, L. & Lepore, R. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research*, *46*(W1), W296-W303. https://doi.org/10.1093/nar/gky427

Xue, Y.J., Tao, L. & Yang, Z.M. (2008). Aluminum-induced cell wall peroxidase activity and lignin synthesis are differentially regulated by jasmonate and nitric oxide. *Journal of Agricultural and Food Chemistry*, *56*(20), 9676-9684. https://doi.org/10.1021/jf802001v

Yang, S., Zhang, X., Yue, J.X., Tian, D. & Chen, J.Q. (2008). Recent duplications dominate NBS-encoding gene expansion in two woody species. *Molecular Genetics and Genomics*, *280*(3), 187-198. https://doi.org/10.1007/s00438-008-0355-0

Zhang, Y. & Friml, J. (2020). Auxin guides roots to avoid obstacles during gravitropic growth. *The New Phytologist*, *225*(3), 1049. https://doi.org/10.1111/nph.16203

Zhou, P.M., Liang, Y., Mei, J., Liao, H.Z., Wang, P., Hu, K., Chen, L.Q., Zhang, X.Q. & Ye, D. (2021). The *Arabidopsis* AGC kinases NDR2/4/5 interact with MOB1A/1B and play important roles in pollen development and germination. *The Plant Journal*, *105*(4), 1035-1052. https://doi.org/10.1111/tpj.15085

Zhu, X., Yang, K., Wei, X., Zhang, Q., Rong, W., Du, L., Ye, X., Qi, L. & Zhang, Z. (2015). The wheat AGC kinase TaAGC1 is a positive contributor to host resistance to the necrotrophic pathogen *Rhizoctonia cerealis*. *Journal of Experimental Botany*, *66*(21), 6591-6603. https://doi.org/10.1093/jxb/erv367