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ORIGINAL ARTICLE

Identification and expression of the *AREB/ABF/ABI5* subfamily genes in chickpea and lentil reveal major players involved in ABA-mediated defense response to drought stress

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Abstract

Main conclusion **This study identified and evaluated the expression of the *AREB/ABF/ABI5* subfamily genes in chickpea and lentil, and revealed the major players involved in defense response to PEG-induced drought stress.**

Abstract Abscisic acid (ABA)-responsive element-binding protein/ABRE-binding factor/ABA-INSENSITIVE 5 (AREB/ABF/ABI5) subfamily proteins are major players in the ABA-mediated signaling pathway triggered by multiple stresses. AREB/ABF/ABI5 subfamily proteins belong to the basic-leucine zipper transcription factors that regulate the expression of several downstream defense genes to abiotic and biotic stresses. This protein set is highly targeted when trying to understand plant defense against abiotic stress or to improve plant tolerance to drought, cold, and salinity stresses. However, there is still very little information available about the genes of the *AREB/ABF/ABI5* subfamily in chickpea and lentil. Herein, 8 chickpea and 9 lentil genes of the AREB/ABF/ABI5 subfamily were identified based on sequence analysis, and their expression levels were tested in a polyethylene glycol-induced drought experiment (20% PEG in Hoagland solution) using real-time RT-PCR and metadata analysis. Sequence analysis showed that members of this subfamily are highly conserved among themselves and with their orthologous genes in other closely related plant species. Overall, sequence data suggested that these genes may possess close or overlapping biological roles in regulating the transcription of abiotic stress-related defense genes. The meta-analysis from RNA-Seq datasets of unstressed plants showed that some members of this gene subfamily have a tissue-specific expression in both chickpea and lentil. Drought-contrasting chickpea and lentil cultivars showed that most *AREB/ABF/ABI5* genes are modulated by PEG-induced drought. Furthermore, *AREB/ABF/ABI5* genes had also a tendency for higher expression as cultivar tolerance increases. Therefore, this study identified the *AREB/ABF/ABI5* subfamily genes in chickpea and lentil, and provides a comprehensive characterization of these members to support further focused research.

Keywords Abiotic stress · ABRE *cis*-elements · Abscisic acid · bZIP transcription factor · DNA binding · Legumes

Abbreviations

ABF	ABRE-binding factor
ABRE	ABA-responsive element
AREB	ABA-responsive element-binding protein
PP2C	Protein phosphatase 2C
SnRK	Sucrose non-fermenting 1-related protein kinase

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Introduction

Chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medik) are important legumes used for human food, nutritionally rich in high-quality proteins, and with reduced antinutritional factors (Landi et al. 2021). They are widely cultivated in Europe and Asia and have recently gained prominence in South and North America (Karalija et al. 2022). Chickpea is a self-pollinated diploid, dicotyledonous, originating from the Middle East, with a 738 Mb size genome organized into eight chromosomes (2n = 2x = 16) and more than 28,200 annotated genes (Varshney et al. 2013). In turn, lentil is a self-pollinated diploid, dicotyledonous, originating from Southwest Asia, with a 3.69 Gb size genome organized in seven chromosomes (2n = 2x = 14) and more than 58,243 annotated genes (Ramsay et al. 2021). Several chickpea and lentil germplasm banks are distributed worldwide with numerous accessions, lines, genotypes, and cultivars with enormous genetic and phenotypic variability and contrasting traits to abiotic stress (Singh et al. 2019b; Silva-Perez et al. 2022; Basso et al. 2023a). Chickpea and lentil are considered well adapted to low-water environments but are potentially sensitive to drought stress during the booting stage, flowering, and grain filling (Korbu et al. 2022). Despite the high phenotypic variability, several commercial cultivars are sensitive to drought stress (Rani et al. 2020). Climate change has increasingly become intense worldwide and drought stress has become a major limiting factor for chickpea and lentil production (Basso et al. 2024a). Therefore, studies to identify genetic elements related to increased plant tolerance to drought are promising alternatives for use in the breeding and genetic engineering of chickpea and lentil (Basso et al. 2019, 2020, 2024b; Karalija et al. 2022). Furthermore, understanding the molecular mechanisms that contribute to increased plant tolerance or resilience to drought stress in these crops can contribute to the development of superior cultivars (Rani et al. 2020).

Abscisic acid (ABA) is involved in signaling for the regulation of numerous defense processes against abiotic and biotic stresses, as well as plant development (Sah et al. 2016). The ABA signaling pathway contains several important components, such as PYR/PYL receptors, protein phosphatase 2C (PP2Cs), sucrose non-fermenting 1-related protein kinase 2 (SnRKs), and ABRE-binding factor (ABFs) proteins (Valdés et al. 2012; Fujita et al. 2013; Basso et al. 2021). In addition, several players act downstream in the ABRE-dependent transcriptional activation of stress-related defense genes, such as the basic-leucine zipper (bZIP) transcription factors (Dröge-Laser et al. 2018). The bZIP family is organized into 13 major groups, with the A group formed mostly by the ABA-responsive element-binding protein, ABRE-binding factor, and ABA-INSENSITIVE 5 (AREB/ABF/ABI5) subfamily members (Collin et al. 2021). These ABA-responsive defense genes have conserved coupling elements and ABRE (PyACGTGG/TC) *cis*-acting regulatory elements in promoter sequences, where AREB/ABF/ABI5 subfamily members bind to promote their transcriptional activation (Fujita et al. 2013). In the *Arabidopsis thaliana*, 9 AREB/ABF/ABI5 subfamily proteins were identified acting downstream of the SnRK1 to SnRK3 (Finkelstein 1994; Lopez-Molina et al. 2001; Fujita et al. 2013). The AtABF2/AREB1, AtABF4/AREB2, AtDPBF5/ABF3, and AtABF1 are activated by AtSnRK2 which subsequently activate the transcription of several ABA-dependent defense genes (Fujita et al. 2005, 2013; Yoshida et al. 2015). The AtDPBF3/AREB3 is activated by AtSnRK2 and has a remarkable role in signaling and floral transition, seed development, maturation, and germination (Martignago et al. 2023). In turn, the AtDPBF1/ABI5 is activated by AtSnRK1 to AtSnRK3 and acts as an important player in the ABA-dependent post-germinative growth arrest checkpoint (Lopez-Molina et al. 2001; Zhou et al. 2015). AtDPBF4/bZIP12 and AtDPBF2/bZIP67 are activated by AtSnRK1 and play significant roles in seed maturation and germination (Mendes et al. 2013; Fàbregas and Fernie 2021). Finally, the AtEEL/bZIP15 is highly overaccumulated in flower tissues and pods and was speculated to play an important role in seed formation and maturation (Wang et al. 2021). Overall, *AtAREB/ABF/ABI5* subfamily genes are modulated by different abiotic stress conditions and demonstrated a tendency for greater expression with increased tolerance of *A. thaliana*.

AREB/ABF/ABI5 subfamily members have already been characterized in several plant species but still remain to be performed in chickpea and lentil (Liu et al. 2019; Li et al. 2020; Yong et al. 2021; Fiallos-Salguero et al. 2023; Pan et al. 2023). Therefore, considering the importance of this subfamily, our work aimed to identify and characterize these genes in chickpea and lentil improving the knowledge of their role in drought response. Herein, 8 and 9 *AREB/ABF/ABI5* subfamily genes were identified and characterized in chickpea and lentil, respectively. A systematic analysis of nucleotide, protein, and promoter sequences, phylogenetic relationship, gene structure, protein–protein network, and protein structural similarities was performed. Moreover, a meta-analysis showed a tissue-specific gene expression in unstressed and stressed plants kept under different abiotic stresses. Subsequently, the expression profile of *AREB/ABF/ABI5* subfamily genes in contrasting (drought-tolerant and sensitive) chickpea and lentil cultivars kept under polyethylene glycol (PEG 6000)-induced drought stress was performed by real-time RT-PCR. Furthermore, the importance

of this gene subfamily for chickpea and lentil in ABA-mediated defense against drought stress was discussed. Therefore, this study provides a consistent basis for further focused functional characterization of each AREB/ABF/ABI5 subfamily member and substantial support for the biotechnological improvement of these crops.

Materials and methods

AREB/ABF/ABI5 sequences from chickpea and lentil

Gene and protein sequences of chickpea and lentil were retrieved from the CGIAR v1.0 assembly (*Cicer arietinum* v1.0 annotation dataset, chickpea CDC Frontier variety; Varshney et al. 2013) and *Lens culinaris* CDC Redberry genome v2.0 annotation dataset (Ramsay et al. 2021) from the Pulse Crop Database (Humann et al. 2019) and *Lens culinaris* v1 dataset from Phytozome v.13 (Goodstein et al. 2012). The gene and protein sequences of *A. thaliana* were retrieved from the TAIR10 dataset (Cheng et al. 2017).

AREB/ABF/ABI5 sequence analysis

Conserved domains were identified using the CDD database (Marchler-Bauer et al. 2015), PFAM database (El-Gebali et al. 2019), and InterPro Scan (Blum et al. 2021). Protein localization was predicted using LOCALIZER (Sperschneider et al. 2017). Transmembrane domains were predicted using TMHMM Server v. 2.0 (Krogh et al. 2001). Evolutionary analyses were performed by the Phylogeny.fr web service using the MLE method by PhyML with aLRT SH-like branch support and GTR or WAG substitution models (Dereeper et al. 2008). The structure of *AREB/ABF/ABI5* genes was constructed with Gene Structure Display Server (Hu et al. 2014), while an unrooted evolutionary tree was inferred using the NJ method with 5000 bootstrap replicates by MEGA11 (Tamura et al. 2021). Chromosomal locations of *AREB/ABF/ABI5* genes were generated with the MapGene2Chrom v2 (Jiangtao et al. 2015). *Cis*-acting regulatory elements in promoter sequences were predicted from 2 kb genomic sequences upstream of the ATG by the PlantCARE database (Lescot et al. 2002). Protein motifs were identified with MEME (Bailey et al. 2015). The interaction network among AREB/ABF/ABI5 with partner proteins was provided by the STRING database using as a reference the *Cicer arietinum* NCBI:txid3827 (Szklarczyk et al. 2020) and STRG0A15LLR datasets (standard datasets to run analysis in the STRING) for chickpea and lentil, respectively. Conserved motifs and target sites in the AREB/ABF/ABI5 subfamily proteins of chickpea and lentil for SnRK-mediated activation were identified based on the previous information generated from *A. thaliana* AREB/ABF/ABI5 proteins (Fujita et al. 2005, 2006).

AREB/ABF/ABI5 protein three-dimensional (3D) structure

AREB/ABF/ABI5 protein sequences were processed by the ChimeraX (Pettersen et al. 2021) using the ColabFold, an open-source, optimized version of Alphafold 2 (Mirdita et al. 2022). Displayed results refer to the "best model fit", which represents the 3D structures according to the predicted local distance difference test (plDDT) with a scale going from 0 to 100%. This parameter indicates an estimative of how the prediction agrees with an experimental structure (Tunyasuvunakool et al. 2021). Pair-to-pair comparison of the 3D protein structures was processed with homology modeling by the MODELLER v10.4 (Webb and Sali 2014).

Meta-analysis from RNA-Seq datasets generated under abiotic and biotic stresses

For tissue-specific expression in chickpea, RNA-Seq datasets generated by Jain et al. (2022) from cv ICC 4958 grown under growth room and field conditions were used. The expression level was generated from 32 different tissue samples with at least three biological replicates and normalized by FPKM (Fragments Per Kilobase per Million mapped fragments). For lentil, RNA-Seq datasets generated by Sudheesh et al. (2016) from the cv Cassab grown in a glasshouse at 22 °C were used. The expression level was generated from different tissues using three biological replicates and normalized by log-transformed counts using the 75th percentile method. For chickpea data mining related to abiotic stress were used RNA-Seq datasets generated from plants kept under drought, salinity, and heat stresses (Garg et al. 2016; Mashaki et al. 2018; Kumar et al. 2019, 2021b; Kaashyap et al. 2022; Kudapa et al. 2023). Similarly, for lentil data mining were used RNA-Seq datasets generated from plants kept under drought, salinity, heat, alkalinity, and biotic stresses (Khorramdelazad et al. 2018; Morgil et al. 2019; Singh et al. 2019a, 2021, 2022a, b; Kumar et al. 2021a; Mishra et al. 2021; Sohrabi et al. 2022). Heatmaps were generated by SRplot (https://www.bioinformatics.com.cn/srplot) using log2(fold change) values contrasting "stress treatment" *versus* "control treatment".

Plant material and PEG-induced drought stress

Chickpea cvs Blanco lechoso (PEG-tolerant) and Pedrosillano (PEG-sensitive), and lentil cvs Castellana (PEG-tolerant) and CDC Redberry (PEG-sensitive) were previously determined as contrasting to PEG-induced drought stress (Basso et al. 2023b). Basso et al. (2023b) performed

a screening with several chickpea and lentil cultivars to identify the most contrasting ones (PEG-tolerant and PEG-sensitive), when the Blanco lechoso and Castellana cultivars were considered PEG-tolerant, since they showed a lower reduction in the accumulation of vegetative biomass and a higher growth rate compared to the Pedrosillano and CDC Redberry cultivars when kept under PEG-induced drought stress. Here, seeds were sterilized and germinated as described by Basso et al. (2023a). Germinated seeds with 1–2 cm radicles were transferred to a hydroponic system with Hoagland solution (Hoagland and Arnon 1938) and kept for 18 days under greenhouse conditions. Uniform plants were chosen to proceed with the PEG-induced drought treatment with Hoagland + 20% (w/v) PEG 6000 (Duchefa Biochemie, Haarlem, The Netherlands) solution (water potential: −0.75 to −0.85 MPa; Zhenyi et al. 2019) compared to control unstressed plants kept only in Hoagland solution (−0.03 MPa) for 8 days. Three biological replicates composed of three plants for each genotype ($n = 3 \times 3$) were collected and evaluated for gene expression, as well as for further biochemical analysis.

ABA content in contrasting cultivars of chickpea and lentil

For ABA determination, approximately 250 mg of plant materials (fresh weight) were used from three to four biological replicates per treatment. Entire leaf samples of well-watered plants were collected at the onset of the PEG-induced drought treatment (named Hoagland 0 days) and 8 days after (Hoagland 8 days). Meanwhile, plants kept under PEG-induced drought stress were collected 1, 3, and 8 days after the onset of the 20% PEG 6000 treatment through a Hoagland solution. Deuterium-labeled ABA (ABA-d6; National Research Council of Canada, Ottawa, Ontario, Canada) was used as an internal standard. Finely, ground leaf samples were suspended in 1 mL of extraction buffer (80% acetone and 1% acetic acid) and mixed with 100 ng internal standard, followed by a sonication lysis step of three cycles for 3 min each. The samples were centrifuged at 10,000 *g* for 2 min, and the pellets were resuspended in 1 mL extraction buffer and sonicated again. The collected supernatants were evaporated under a nitrogen flux. To remove chlorophylls, 1 mL hexane was added to the samples, followed by centrifugation at 10,000 *g* for 2 min. The ABA content was determined from the aqueous phase using a high-performance liquid chromatograph HP-1260 infinity II interfaced with a mass spectrometer 6530 LC/Q-TOF (HPLC-MS/Q-TOF) equipped with an ESI Jet Stream source (Agilent, Santa Clara, CA, USA). The eluting phases consisted of H_2O supplemented with 0.1% formic acid (solvent A) and acetonitrile:methanol 85:15 (v/v) supplemented with 0.1% formic acid (solvent B). The analysis was performed in MS/MS negative-ion mode, using a 2 × 150 mm Polaris 3C18-A column (Agilent Technologies) and eluted with a 12-min run from 90% solvent A to 90% solvent B at a flow rate of 250 µL min^{-1}. Q-TOF instrument parameters were: nitrogen gas, temperature 325 °C, gas flow 15 L/min^{-1}, nebulizer 15 psi, capillary voltage 3.5 kV, extended dynamic range 2 GHz, MS1 and MS2 acquisition speed 4 spectra/s, MS1 and MS2 mass range 90 to 1100 m/z, fragmentor 170 eV, and collision energy 15 eV. ABA quantification was performed by HPLC-Q/TOF using a 10-point regression line built with ABA standard (Sigma-Aldrich, St. Louis, MO, USA). The ABA concentrations used for calibration of the quantification method were 1, 2, 5, 10, 50, 100, 200, 500, 1000, and 1500 ng. The peak area ratios of the accurate mass chromatograms for the 153.09 and 159.13 product ions of ABA and ABA-d6, respectively, were adopted for the calibration line. Lines with an $r^2 > 0.996$ were considered with a LOQ of 1.5 ng. The data were elaborated using the MassHunter v.10.1 (Agilent). A Tukey's test was performed to analyze the statistical differences in ABA contents within each cultivar at a 95% significance level.

RNA isolation and *AREB/ABF/ABI5* gene expression in contrasting cultivars of chickpea and lentil

Leaves were collected from plants kept in Hoagland + PEG solution at 1, 3, and 8 days after PEG treatment and from plants kept only in Hoagland solution. RNA was purified with GenUP™ Total RNA Kit (Biotechrabbit, Berlin, Germany) and integrity was checked in agarose gel electrophoresis. RNA samples were treated with RNase-free RQ1 DNase I (Promega, Madison, WI, USA) and used for cDNA synthesis using oligo-dT primer and SuperScript III RT mix (Life Technologies, Carlsbad, CA, USA). The cDNA samples were diluted 1:10 (v:v) and real-time RT-PCR assays were performed in QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using 3 µL cDNA, 0.1 µM gene-specific primers (Table S1), and 2X SYBR Green PCR Master Mix (Applied Biosystems). The cycling program used for real-time RT-PCR was 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C, followed by melting curve analysis using default parameters. Relative expression was calculated using the 2^-ΔCt formula (Schmittgen and Livak 2008) with *CaCAC* (Reddy et al. 2016) and *LcTUB* (Sinha et al. 2019) as endogenous reference genes (Table S1). *CaG6PD*, *CaTIP41*, *LcRPL2*, and *LcRBC1* reference genes were also tested, but *CaCAC* and *LcTUB* were more stable in our samples. The *CaGOLS2* and *LcGOLS2* genes were used as molecular markers of plant defense activation against drought stress (Duarte et al. 2022; Martins et al. 2022; Molinari et al. 2023) in chickpea and lentil (Table S1). All cDNA samples were carried out in technical triplicates. Target-specific

amplification was confirmed by the occurrence of a single peak in the melting curve analysis. Data were evaluated for statistical significance using the SASM-Agri (Canteri et al. 2001).

Results

Identification of *AREB/ABF/ABI5* subfamily genes in chickpea and lentil

Herein, 8 and 9 *AREB/ABF/ABI5* subfamily genes were identified in chickpea and lentil, respectively, using as reference the *AREB/ABF/ABI5* subfamily genes previously identified in *A. thaliana* (Finkelstein 1994; Lopez-Molina et al. 2001; Fujita et al. 2013) (Fig. 1; Tables 1 and 2). In silico analysis revealed that most of the proteins share several important features, such as nuclear localization, conserved domains such as bZIP, NLS, PF00170, cd14707, cl21462, IPR004827, IPR043452, IPR046347, PTHR22952, and K14432, and absence of transmembrane domain (Tables 1 and 2). In particular, Lcu.2RBY.4g037060.1 protein has the bZIP domain truncated and lacks of nuclear localization signal, indicating that this protein may not act as a transcription factor (Table 2). The interfamily phylogenetic relationship using coding nucleotide (CDS) sequences revealed the formation of three main subgroups (A, B1, and B2) with high bootstrap support, where *AREB/ABF/ABI5* members of chickpea and lentil showed close evolutionary relationships with their *A. thaliana* orthologous genes (Fig. 1). These three subgroups are supported by the clustering of *A. thaliana AREB/ABF/ABI5* genes into two main subgroups (A and B) as previously shown by Fujita et al. (2013) and Liu et al. (2019). Phylogenetic trees constructed using entire amino acid sequences or only conserved motifs in proteins revealed that some of these genes or proteins are highly conserved between chickpea and lentil, while others are less conserved (Fig. S1). These collective data indicate that some of these proteins may develop similar activities to each other or be conserved in different plant species, while other proteins may have additional or non-overlapping functions. The analysis of conserved domains and target sites of protein kinases in AREB/ABI/ABF subfamily proteins of chickpea and lentil revealed that several of these proteins show high conservation for almost all these criteria, while others have some of them poorly conserved or absent (*e.g.*, Lcu.2RBY.4g061510.1 and Lcu.2RBY.1g044680.1) (Fig. 2). These data suggest that these proteins may have functional activities regulated differentially or specifically when compared to each other, but these features provide support for characterizing them as typical AREB/ABF/ABI5 proteins. Detailed information concerning the chickpea and lentil genes, such as gene name, gene identifier, chromosomal location, CDS, amino acid sequence length, protein annotation, molecular weight, isoelectric point, subcellular localization, and the presence or absence of conserved domains, are listed in Tables 1 and 2. Therefore, sequence analyses allowed the identification of *AREB/ABF/ABI5* subfamily genes in chickpea ($n = 8$ genes) and lentil ($n = 9$ genes).

AREB/ABF/ABI5 gene structure and chromosomal localization

Variations in the number and size of exons and intron sequences, 5'-UTR, and 3'-UTR sequences even between homologous *AREB/ABF/ABI5* genes of chickpea and lentil were observed (Fig. S2). Similarly, as already shown by the variation in the size of CDS and protein between these genes (Tables 1 and 2), the number of exons ranged from 3 to 4. The chromosomal localization showed that *AREB/ABF/ABI5* genes are widely distributed in all chromosomes, except for the absence in chromosomes 4 and 8 in chickpea, and 5 in lentil (Fig. S3a and b). Therefore, these collective data revealed the organization and chromosomal localization of the *AREB/ABF/ABI5* subfamily genes in chickpea and lentil.

Cis-acting regulatory elements in AREB/ABF/ABI5 promoter sequences

The *cis*-acting regulatory elements were identified and the top 14 were quantitatively analyzed among different promoters (Fig. 3). The six most prevalent in all AREB/ABF/ABI5 promoters were ABA-responsive, light-responsive, ethylene-responsive, MYB-related, MYC-related, and defense and stress-responsive. Although it has similar features to the other AREB/ABF/ABI5 members, the promoter of the *Lcu.2RBY.1g044680.1* gene did not show typical ABA-responsive elements. In addition, other *cis*-acting regulatory elements associated with tissue-specific (such as seed, xylem, and meristem) expression and responsive to other hormones (such as gibberellin, auxin, and salicylic acid) were less represented but also present in different promoters. The top three with the higher number of *cis*-acting regulatory elements associated with defense responses to abiotic stress were promoters of the *Lcu.2RBY.2g084200.1* and *Lcu.2RBY.3g062010.1* genes. Therefore, these data indicated that these *AREB/ABF/ABI5* subfamily genes may have their expression modulated by different hormones, abiotic stress, and in a tissue-specific manner, potentially developing an important role in the defense response of chickpea and lentil.

Conserved motifs in AREB/ABF/ABI5 proteins and protein–protein network

Several conserved motifs were identified in all AREB/ABF/ABI5 proteins, while some motifs were specific for

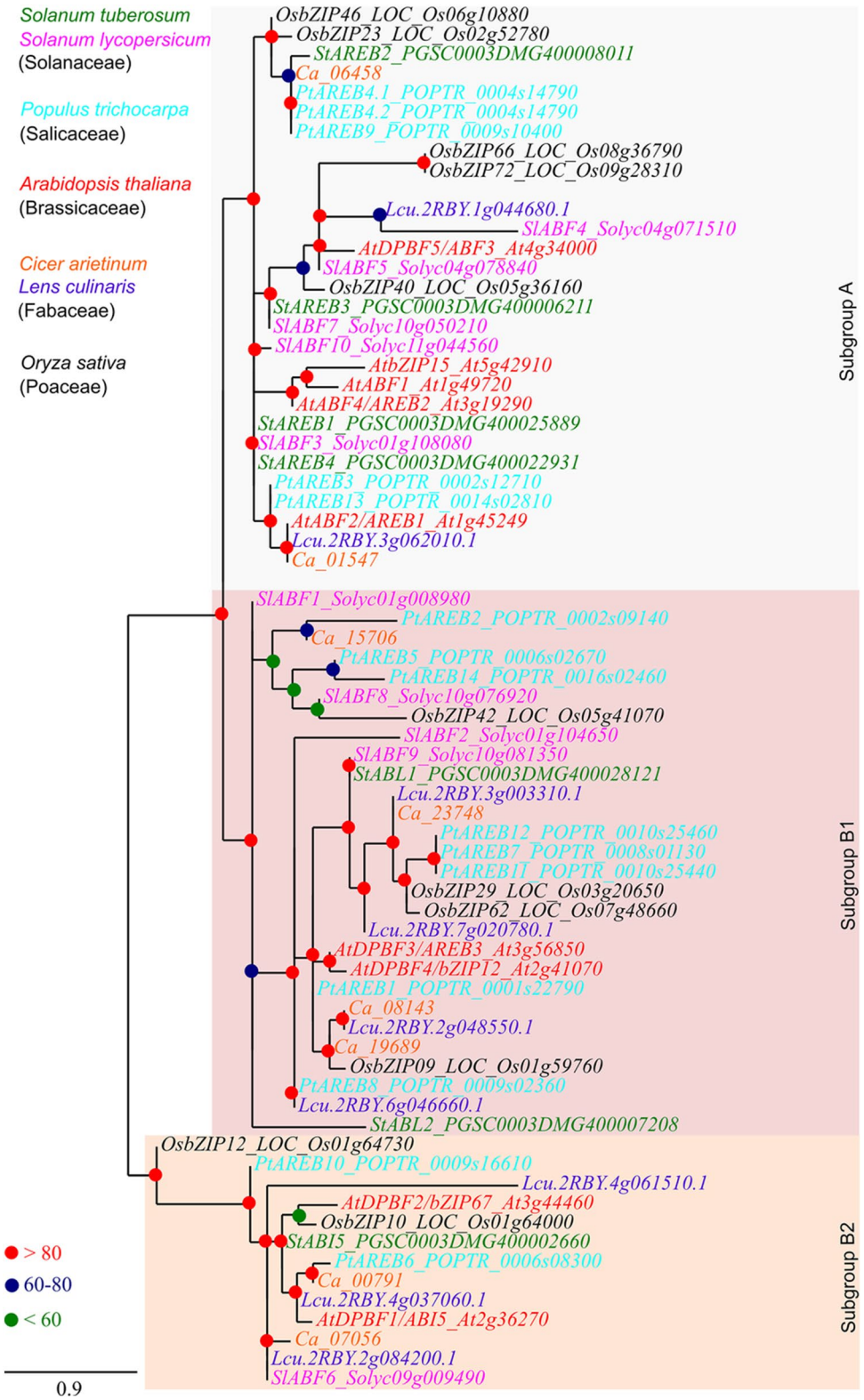

Fig. 1 *AREB/ABF/ABI5* subfamily genes in chickpea and lentil. Interfamily evolutionary tree using coding nucleotide sequences of the *AREB/ABF/ABI5* orthologous genes identified in chickpea and lentil (Tables 1 and 2) using *AREB/ABF/ABI5* subfamily genes of *Arabidopsis thaliana* as a reference (Fujita et al. 2005, 2013; Furihata et al. 2006; Yoshida et al. 2015), in addition to *AREB/ABF/ABI5* subfamily genes of *Solanum lycopersicum* (Pan et al. 2023), *Oryza sativa* (Lu et al. 2009), *Solanum tuberosum* (Liu et al. 2019), and *Populus trichocarpa* (Ji et al. 2013)

certain proteins, such as motif 10 in Lcu.2RBY.1g044680.1 protein (Fig. S4). The number and organization of these motifs were also conserved among some proteins of the same plant species or even between chickpea and lentil, for example, Ca_23748 *versus* Lcu.2RBY.3g003310.1, and Lcu.2RBY.6g046660.1 *versus* Lcu.2RBY.2g048550.1 proteins. Meanwhile, other homologous proteins showed a different number and organization of conserved motifs,

Table 1 *AREB/ABF/ABI5* subfamily genes and proteins identified in chickpea

Gene identifier	Evolutionary relationship (Arabidopsis)	Orthologs in lentil	Temporary name	Annotation from database	Subcellular localization	Pfam domain	CD domain	InterPro	TMH	bZIP	NLS	PANTHER	KEGG	Chr	CDS (nt)	Amino acid	Mw kDa	pI
*Ca_01547**	*At1g45249* (nt) *At1g49720* (protein) *At5g42910* (domains)	*Lcu.2RBY.3g062010.1*	undefined	CAMP-response element-binding protein-related abscisic acid-insensitive 5-like	Nucleus	PF00170	cd14707	IPR043452 IPR046347 IPR004827	no	yes	yes	PTHR22952 PTHR22952:SF49	K14432	5	1290	429	47.04	9.45
Ca_19689	*At3g56850* (nt and domains)	*Lcu.2RBY.2g048550.1*	*CaAREB3*	CAMP-response element-binding protein-related	Nucleus	PF00170	cl21462	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF192	K14432	2	912	304	33.9	6.81
Ca_23748	undefined	*Lcu.2RBY.3g003310.1*	undefined	CAMP-response element-binding protein-related abscisic acid-insensitive 5-like protein 3	Nucleus	PF00170	cd14707 cl41729	IPR004827 IPR029053 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF205	K14432	5	969	323	35.7	7.04
Ca_06458	*At1g45249* (nt, protein, and domains)	undefined	*CaAREB1*	CAMP-response element-binding protein-related abscisic acid-insensitive 5-like protein 6	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF158	K14432	6	1158	386	42.5	9.17
Ca_00791	*At2g36270* (nt, protein, and domains)	*Lcu.2RBY.4g061510.1*	*CaABI5*	Protein abscisic acid-insensitive 5	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF217	K14432	3	1284	428	46.3	8.88
Ca_08143	*At2g41070* (nt and domains) *At3g56850* (protein)	*Lcu.2RBY.6g046660.1*	*CabZIP12*	CAMP-response element-binding protein-related	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF192	K14432	3	909	303	33.5	6.40
Ca_15706	undefined	*Lcu.2RBY.7g020780.1*	undefined	CAMP-response element-binding protein-related abscisic acid-insensitive 5-like protein 3	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF205	K14432	7	969	323	35.8	6.27

Table 1 (continued)

Gene identifier	Evolutionary relationship (Arabidopsis)	Orthologs in lentil	Temporary name	Annotation from database	Subcellular localization	Pfam domain	CD domain	InterPro	TMH	bZIP	NLS	PANTHER	KEGG	Chr	CDS (nt)	Amino acid	Mw kDa	pI
Ca_07056	*At3g44460* (nt, protein, and domains)	*Lcu.2RBY.2g084200.1*	*CabZIP67*	Abscisic acid-insensitive 5-like protein 1	Nucleus	PF00170	cl21462 cl05445	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF119	K14432	1	885	295	33.3	8.10

*The *Ca_01547* gene named here in this study comprises the sequence of both *Ca_01547* and *Ca_01548* genes. The *Ca_01547* and *Ca_01548* were annotated erroneously as two independent genes in the CGIAR v1.0 assembly (*Cicer arietinum* v1.0 annotation dataset; Varshney et al. 2013)). Other sequence data about these two genes confirm this information that *Ca_01547* plus *Ca_01548* make a single gene (XP_004501562.1 and rna-XM_004501505.3)

nt nucleotide, *TMH* transmembrane helix, *bZIP* Basic Leucine Zipper Domain, *NLS* nuclear localization signal, *Chr* chromosome, *Mw* molecular weight, *pI* isoelectric point, *THM* prediction of transmembrane helices in proteins, *PTHR22952* abscisic acid-insensitive 5-like protein 8-related, *PTHR22952:SF49* CAMP-response element-binding protein-related, *K14432* ABF, *IPR043452* plant bZIP transcription factors; PF00170: bZIP_1; PTHR22952:SF194: subfamily not named, *IPR004827* Basic-leucine zipper domain, *cd14707* bZIP_plant_BZIP46, *IPR046347* basic-leucine zipper domain superfamily, *cl21462* bZIP super family, *PTHR22952:SF192* subfamily not named, *cl41729* KLF1_2_4_N super family, *PTHR22952:SF205* abscisic acid-insensitive 5-like protein 3, *IPR029053* viral coat protein subunit, *PTHR22952:SF158* abscisic acid-insensitive 5-like protein 6, *PTHR22952:SF217* protein abscisic acid-insensitive 5, *cl05445* Mt_ATP-synt_D super family, *PTHR22952:SF119* abscisic acid-insensitive 5-like protein 1

for example, Ca_00791 *versus* Lcu.2RBY.4g037060.1, Ca_07056 *versus* Lcu.2RBY.2g084200.1. These data showed that chickpea and lentil proteins share several motifs and also present particular motifs suggesting that they have functions ranging from conserved to additional ones in the ABA-signaling pathway. The meta-analysis of the protein–protein interaction and gene co-expression network predicted by the STRING database version 12.0 using the STRG0A15LLR dataset showed that chickpea and lentil AREB/ABF/ABI5 proteins are highly interconnected in a group of at least 13 and 15 proteins, respectively (Fig. 4a and b). In addition, several chickpea and lentil serine/threonine kinase proteins that can be involved in MAPK signal transduction in the ABA signaling pathway, based on the previous information on orthologous proteins (Danquah et al. 2014), were predicted to be hub proteins together with AREB/ABF/ABI5 proteins. Therefore, these collective data revealed that chickpea and lentil AREB/ABF/ABI5 proteins can play from quite conserved to particular functions and showed protein–protein interaction networks with important hub proteins potentially involved in the ABA signaling pathway.

3D structure of AREB/ABF/ABI5 proteins

The 3D structures of AREB/ABF/ABI5 proteins identified in chickpea and lentil were successfully modeled (Fig. S5a–q) and compared among them to verify potential structural similarity (Table S2). In accordance with the previous sequence data observed with AREB/ABF/ABI5 proteins identified in other plant species, this in silico analysis indicates that these AREB/ABF/ABI5 subfamily proteins are structurally conserved also in both chickpea and lentil, and when compared to AREB/ABF/ABI5 proteins of *A. thaliana*. A detailed analysis in Tables S2 and S3 shows that the alignment between the conserved regions of the proteins of the AREB families is high and always above 80%. A graphic example of alignment is shown in Fig. S6, where Lcu.2RBY.3g062010.1 and Ca_01547 are compared.

AREB/ABF/ABI5 gene expression in different organs and under different stress conditions

A meta-analysis from RNA-Seq datasets retrieved from literature for chickpea and lentil was performed to investigate the expression profile of the *AREB/ABF/ABI5* subfamily genes that we identified in this study. *AREB/ABF/ABI5* genes show a higher number of normalized transcripts in a tissue- or stage-specific manner in unstressed chickpea and lentil plants (Fig. 5a and b). Particularly, *Ca_23748*, *Ca_07056*, *Ca_15706*, and *Ca_00791* showed higher expression levels in seed, embryo, and endosperm even in the absence of severe stress conditions (Fig. 5a).

Table 2 *AREB/ABF/ABI5* subfamily genes and proteins identified in lentil

Gene identifier	Evolutionary relationship (Arabidopsis)	Orthologs in chick-pea	Tempo-rary name	Annota-tion from database	Subcel-lular localiza-tion	Pfam domain	CD domain	InterPro	TMH	bZIP	NLS	PANTHER	KEGG	Chr	CDS (nt)	Amino acid	Mw kDa	pI
Lcu.2RBY.3g062010.1	*At1g45249* (nt) *At1g49720* (protein) *At5g42910* (domains)	*Ca_01547*	undefined	CAMP-response element-binding protein-related	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF194	K14432	3	1317	439	48.1	9.36
Lcu.2RBY.4g061510.1	*At3g44460* (nt) *At2g36270* (protein)	*Ca_00791*	undefined	CAMP-response element-binding protein-related	Nucleus	PF00170	cd14707	IPR004827 IPR001630 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF175	K14432	4	816	272	30.7	6.13
*Lcu.2RBY.1g044680.1**	*At4g34000* (nt)	undefined	*LcABF3*	cAMP response element-binding (CREB) protein basic-leucine zipper domain	Nucleus	PF00170	cd14707	IPR004827 IPR001630 IPR046347 IPR043452	no	yes	yes	PTHR22952 PTHR22952:SF184	K14432	1	738	246	27.5	5.53
Lcu.2RBY.3g003310.1	undefined	*Ca_23748*	undefined	abscisic acid-insensi-tive 5-like protein 3	Nucleus	PF00170	cd14707 cl25753	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF205	K14432	3	1023	341	36.1	6.68
Lcu.2RBY.2g048550.1	*At3g56850* (nt) *At2g36270* (protein)	*Ca_19689*	undefined	CAMP-response element-binding protein-related	Nucleus	PF00170	cl21462	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF192	K14432	2	969	323	36.2	8.59
Lcu.2RBY.4g037060.1	*At2g36270* (nt)	Undefined	*LcABI5*	Protein abscisic acid-insensi-tive 5	unde-fined	no	no	IPR043452	no	trun-cated	no	PTHR22952 PTHR22952:SF217	K14432	4	846	281	32.0	6.27
Lcu.2RBY.6g046660.1	*At3g56850* (protein) *At2g41070* (nt, domains)	*Ca_08143*	undefined	CAMP-response element-binding protein-related	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF192	K14432	6	963	321	35.7	7.24

Table 2 (continued)

Gene identifier	Evolutionary relationship (Arabidopsis)	Orthologs in chickpea	Temporary name	Annotation from database	Subcellular localization	Pfam domain	CD domain	InterPro	TMH	bZIP	NLS	PANTHER	KEGG	Chr	CDS (nt)	Amino acid	Mw kDa	pI
Lcu.2RBY.7g020780.1	undefined	*Ca_15706*	undefined	abscisic acid-insensitive 5-like protein 2	Nucleus	PF00170	cd14707	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF137	K14432	7	981	327	36.1	6.48
Lcu.2RBY.2g084200.1	*At3g44460* (protein and domains)	*Ca_07056*	*LcbZIP67*	Protein abscisic acid-insensitive 5	Nucleus	PF00170	cl21462 cl05445	IPR004827 IPR043452 IPR046347	no	yes	yes	PTHR22952 PTHR22952:SF217	K14432	2	969	323	36.3	8.56

*The *Lcu.2RBY.L013250.1* gene is located in chromosome 1 and it has a very similar gene (*Lcu.2RBY.L013250.1*), but this second gene located in the unitig2983 contig from the *Lens culinaris* CDC Redberry genome v2.0 annotation dataset (Ramsay et al. 2021) is truncated or incompletely assembled and was disregarded in this study

nt nucleotide, *TMH* transmembrane helix, *bZIP* Basic Leucine Zipper Domain, *NLS* nuclear localization signal, *Chr* chromosome, *Mw* molecular weight, *pI* isoelectric point, *THM* prediction of transmembrane helices in proteins, *cl25753* PHA03369 superfamily, *PTHR22952:SF137* abscisic acid-insensitive 5-like protein 2, *PTHR22952:SF175* subfamily not named, *IPR001630* cAMP response element-binding (CREB) protein, *PTHR22952:SF184* basic-leucine zipper transcription factor-related

Similarly, *Lcu.2RBY.3g003310.1*, *Lcu.2RBY.2g084200.1*, *Lcu.2RBY.7g020780.1*, and *Lcu.2RBY.4g037060.1* also showed higher expression levels in immature seeds and pods (Fig. 5b). The *Lcu.2RBY.1g044680.1* gene showed higher expression in leaves while the *Lcu.2RBY.3g062010.1* gene in stems. These collective data suggested that these *AREB/ABF/ABI5* subfamily genes could have dynamic expression and can modulate additional biological roles in addition to defense response to a given abiotic stress. The meta-analysis also addressed the expression profile of the *AREB/ABF/ABI5* genes in different chickpea and lentil tissues in response to drought, salinity, heat, and biotic stress conditions (Fig. S7a–f; Fig. S8a–b). For chickpea, *AREB/ABF/ABI5* genes were up-regulated by all stress conditions in at least 1 evaluated tissue, with greater expression levels in leaves when under drought stress, flower, and shoot when under salinity stress, and before-flowering leaf when under heat stress (Fig. S7a–c). Similarly, all *AREB/ABF/ABI5* genes of lentil were up-regulated by all three stress conditions in the different tissues evaluated, mainly in roots and leaves of stress-tolerant cultivars (Fig. S7d–f). Particularly, *Lcu.2RBY.7g020780.1* and *Lcu.2RBY.2g084200.1* genes showed higher expression in roots of sensitive plants compared to tolerant plants kept under alkalinity stress, while the expression profiles of the other genes were all correlated with plant tolerance (Fig. S8a). The *AREB/ABF/ABI5* genes also showed a tendency for greater expression profile with increased tolerance of cultivars, except for the *Lcu.2RBY.1g044680.1* and *Lcu.2RBY.4g037060.1* genes in lentil plants under biotic stress caused by fungal disease (Fig. S8b). Therefore, these collective data showed that both *AREB/ABF/ABI5* subfamily genes were up-regulated by different abiotic and biotic stress conditions, with dynamic expression in different tissues, and tended to have greater expression as cultivar tolerance increased. Furthermore, the *AREB/ABF/ABI5* genes of chickpea and lentil were suggested to be important in the ABA-dependent signaling pathway for both abiotic and biotic stresses.

PEG-induced drought stress, ABA content, and expression of drought marker genes

A PEG-induced drought stress experiment in a hydroponic system was carried out using PEG 6000 as an osmotic agent. PEG is a non-ionic and inert polymer for plant cells widely used to artificially cause osmotic tension or simulate drought stress in plants under hydroponic system changing the water potential of solutions, which maintains the same water potential throughout the trials (Michel and Kaufmann 1973). For this, contrasting drought-tolerant and drought-sensitive chickpea and lentil cultivars (Basso et al. 2023b) were successfully subjected to PEG-induced drought stress and

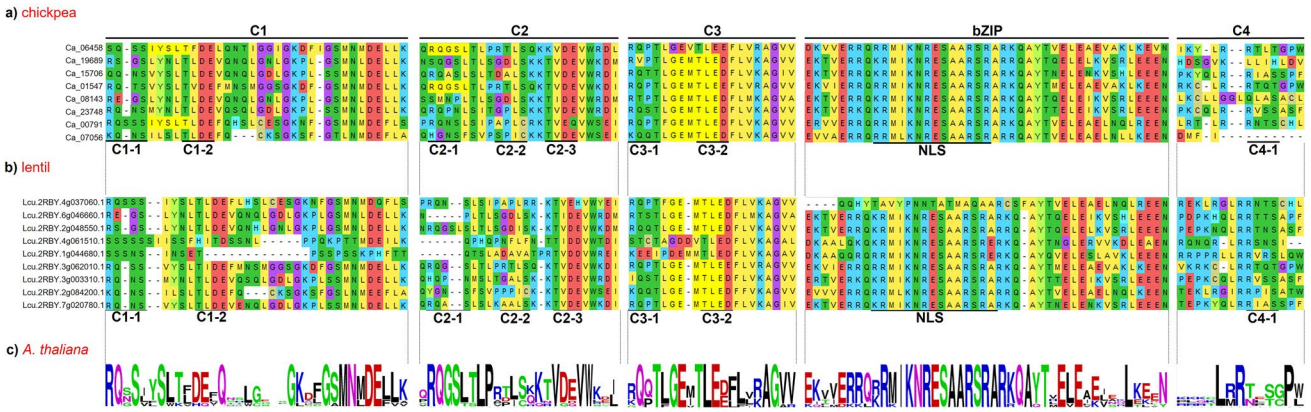

Fig. 2 Conserved motifs and target sites of kinases in the AREB/ABF/ABI5 subfamily proteins in chickpea and lentil. Chickpea (**a**) and lentil (**b**) protein sequences, highlighting in the sequence alignment only the motifs and conserved regions. C1 to C4 motifs and bZIP conserved domains and nuclear localization signal (NLS) proposed by Fujita et al. (2005) and putative target sites of protein kinases proposed by Furihata et al. (2006) within conserved regions of AREB/ABI/ABF subfamily proteins of *Arabidopsis thaliana* and identified in this study in chickpea and lentil proteins. **c** Sequence logo of C1 to C4 and bZIP conserved domains of AREB/ABI/ABF subfamily proteins of *Arabidopsis thaliana*

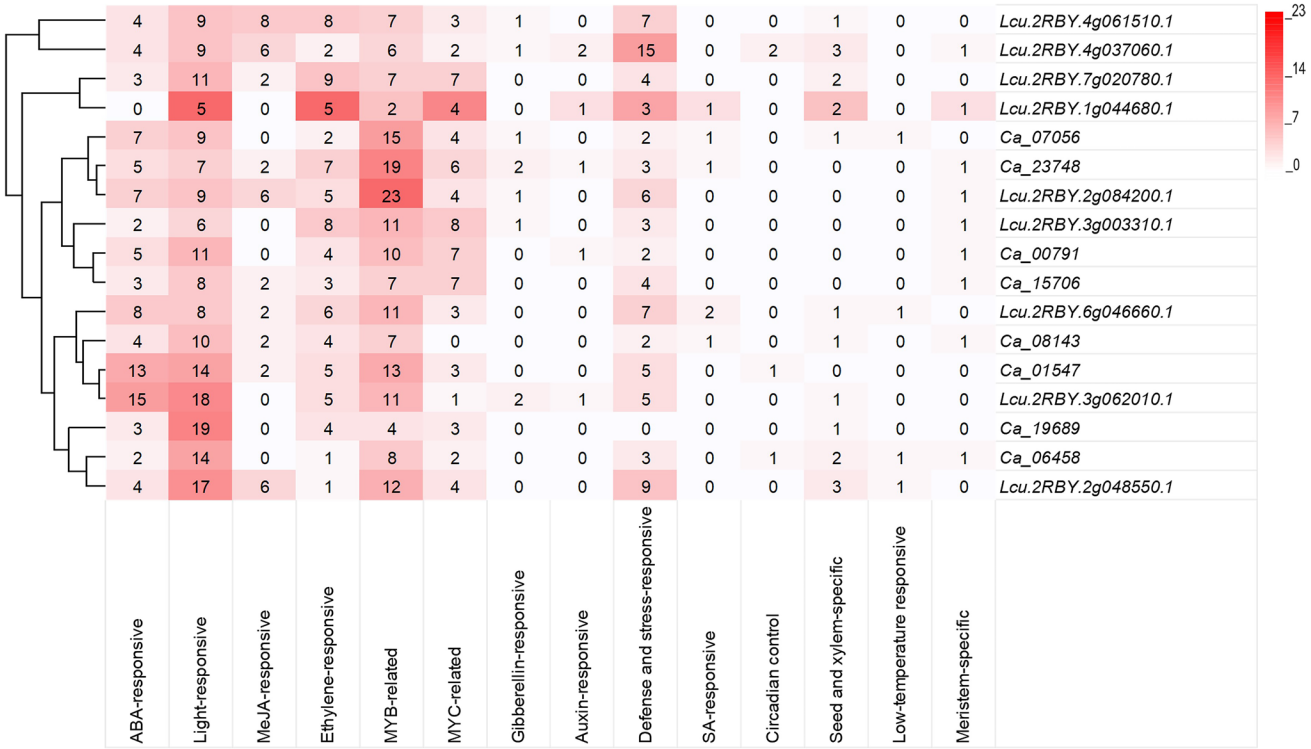

Fig. 3 *Cis*-acting regulatory elements in 2 kb-promoter regions of the *AREB/ABF/ABI5* subfamily genes identified in chickpea and lentil. The number of *cis*-acting regulatory elements in promoter sequences of each gene is shown inside the heatmap. *ABA* abscisic acid, *MeJA* methyl jasmonate, *MYB* MYB transcription factor binding domain, *MYC* MYC transcription factor binding domain, *SA* salicylic acid

evaluated at 0, 1, 3, and 8 days after onset 20% PEG and unstressed control treatments. The determination of ABA content and expression profile of drought stress marker genes *CaGOLS2* and *LcGOLS2* (Duarte et al. 2022; Martins et al. 2022; Molinari et al. 2023) were analyzed to monitor the intensity of the imposed stress. The hormonal analysis revealed that PEG treatment significantly affected the ABA content in chickpea and lentil cultivars compared to unstressed control plants (Fig. 6a). In both cultivars, ABA content tended to increase with the

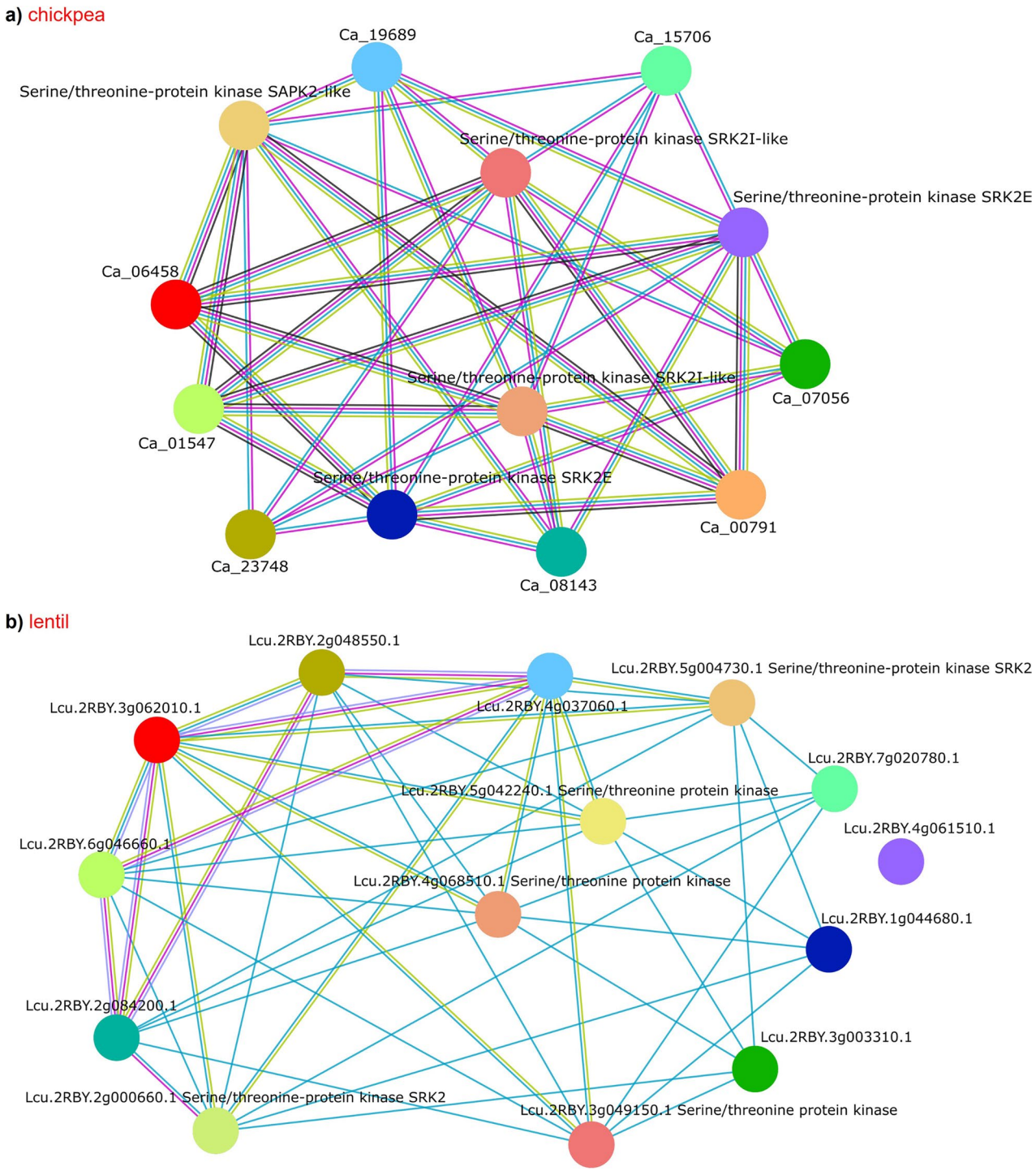

Fig. 4 Protein–protein interaction and gene co-expression network among AREB/ABF/ABI5 and partner proteins from chickpea (**a**) and lentil (**b**) predicted using the STRING database. In addition to the AREB/ABF/ABI5 proteins used as input, more nodes were added to current networks. The protein–protein interaction network represents the known interactions which are shown in light blue lines when from curated databases, and pink lines when experimentally determined; predicted interactions are shown in dark green lines when gene neighborhood, red lines when gene fusions, and dark yellow lines when gene co-occurrence; while other protein–protein associations are shown in light green lines when text-mining, black lines when co-expression, and light blue lines when by protein homology. Colored nodes indicate query proteins and the first shell of interactors

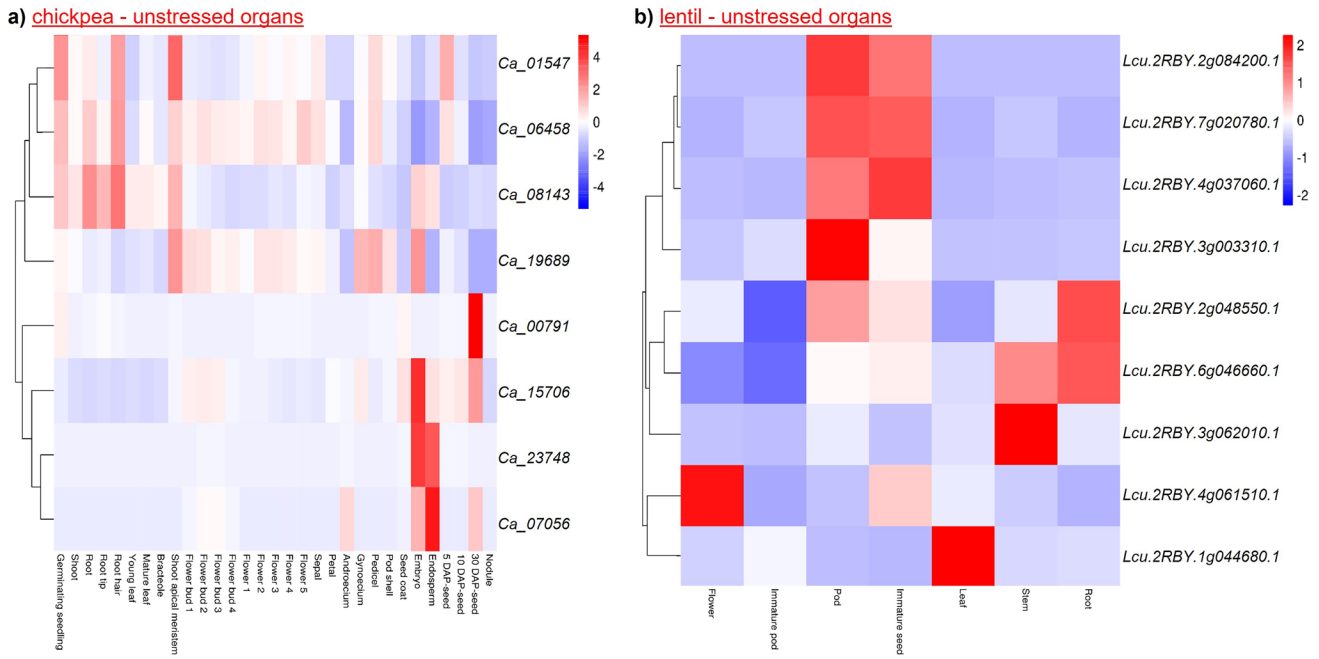

Fig. 5 Expression profile of the *AREB/ABF/ABI5* subfamily genes in different chickpea and lentil organs using meta-analysis of RNA-Seq datasets. Expression of *AREB/ABF/ABI5* subfamily genes in different organs of chickpea (**a**) and lentil (**b**). The same tissues but different numbers represent data from different articles used in the meta-analysis, for example, Flower bud 1 to 4. Expression values in different chickpea organs correspond to FPKM-normalized counts for each gene, while expression values in different lentil organs correspond to log-transformed counts using the 75th percentile method. The color scale bars indicate the range of expression profiles. *DAP* days after pollination

duration of PEG-induced drought stress. After 1 day of PEG-induced drought stress, the chickpea cvs Blanco lechoso (PEG-tolerant) and Pedrosillano (PEG-sensitive) have ABA levels ranging from 1.381 (± 0.420) and 4.770 (± 0.972) nmol g^{-1} fresh weight, respectively. At the same time point, the ABA content was increased from about fourfold in lentil cv Castellana (PEG-tolerant) to sevenfold in the cv CDC Redberry (PEG-sensitive) having values equal ranging from 0.522 (± 0.167) and to 5.108 (± 3.331) nmol g^{-1} fresh weight, respectively. After 3 days of PEG-induced drought stress, there was a further ABA accumulation in all the stressed cultivars except for the chickpea cv Blanco lechoso, 1.366 (± 0.359) nmol g^{-1} fresh weight. Notably, the chickpea cv Pedrosillano had an upper fold increase equal to about a fourth displaying 11.384 (± 4.823) nmol g^{-1} fresh weight of ABA. In contrast, in lentil cv Castellana. it increased approximately ten times compared to the previous time point, staying at 9.921 (± 1.330) nmol g^{-1} fresh weight, while in cv CDC Redberry, there was a small increase, staying at 6.276 (± 1.693) nmol g^{-1} fresh weight. After 8 days of PEG-induced drought stress, the tolerant chickpea and lentil cultivars maintained an increase in ABA content, while sensitive cultivars remained at a similar level compared to the previous time point (Fig. 6a). Meanwhile, the up-regulation of the drought marker genes, *CaGOLS2* and *LcGOLS2*, confirmed the stress levels imposed in our experiments and suggested modulation of the expression of other drought-responsive genes, such as *AREB/ABF/ABI5* subfamily genes (Fig. 6b and c). This expression data indicated that the activation of stress marker genes in tolerant cultivars is lower or similar after 1 day of PEG-induced drought stress, complying with ABA levels, but the expression profile of these genes is higher or equal after 3 days of PEG-induced drought stress compared to sensitive cultivars.

AREB/ABF/ABI5 gene expressions in contrasting cultivars under PEG-induced drought stress

The relative expression of 8 and 9 *AREB/ABF/ABI5* subfamily genes identified in chickpea and lentil was determined during the PEG-induced drought experiment. Real-time RT-PCR results were similar to those observed in the meta-analysis with different stress conditions, in which most *AREB/ABF/ABI5* genes of chickpea and lentil showed a tendency for greater expression as the tolerance of the cultivars increased (Fig. 7a and b). The only exception compared to other genes, the *Ca_07056* gene was more up-regulated by PEG-induced drought stress in the sensitive chickpea

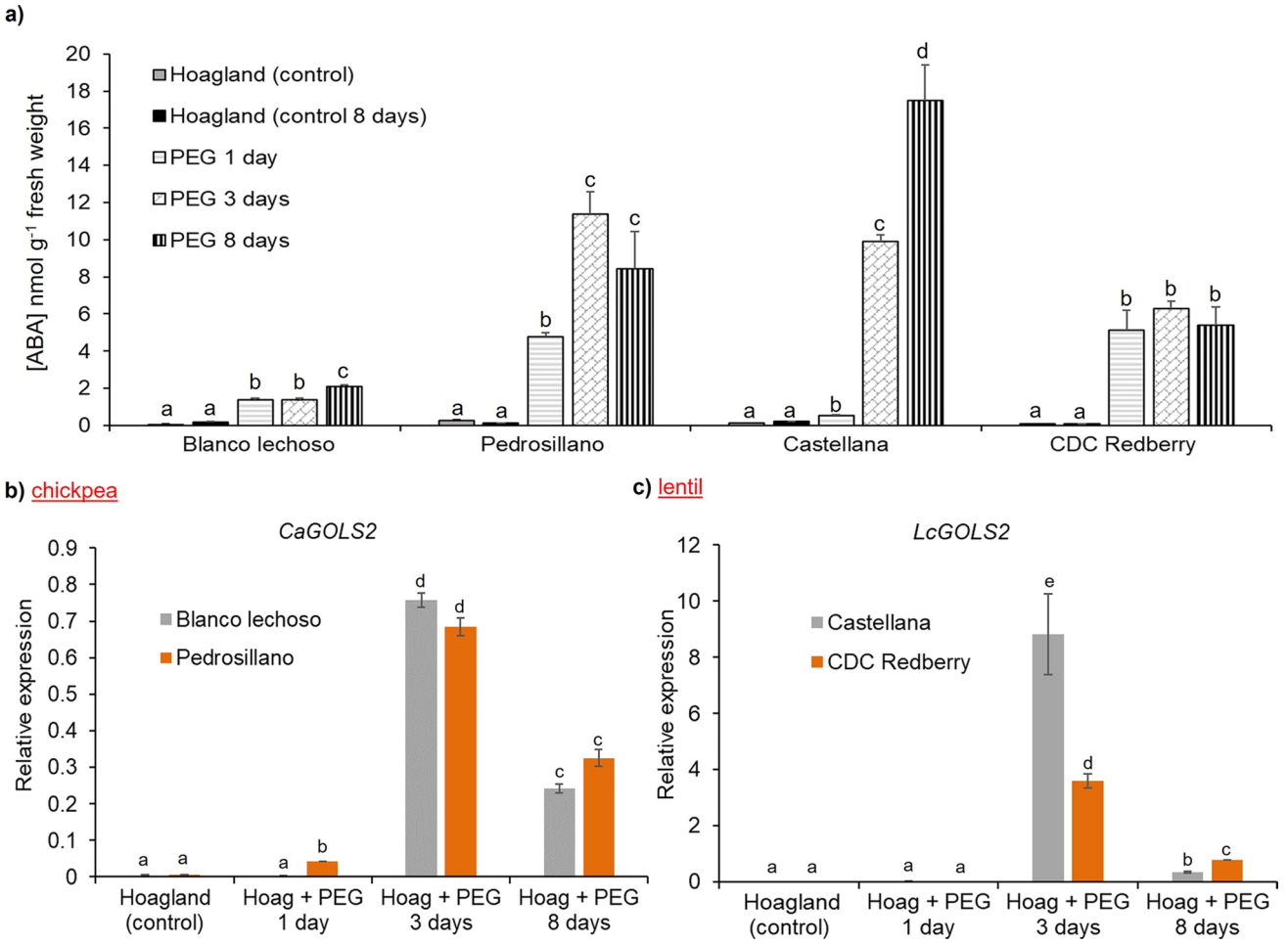

Fig. 6 Abscisic acid (ABA) content and expression profile of the *GOLS2* (galactinol synthase 2, drought stress markers) genes in chickpea cvs Blanco lechoso (PEG-tolerant) and Pedrosillano (PEG-sensitive) and lentil cvs Castellana (PEG-tolerant) and CDC Redberry (PEG-sensitive) kept under drought stress conditioned by 20% polyethylene glycol (PEG 6000) treatment compared to unstressed control condition for 1, 3, and 8 days. **a** ABA content in vegetative tissues (entire leaves) of chickpea and lentil plants kept under PEG treatment compared to unstressed control conditions. Error bars represent the standard error of means ($n=3$ to 4 biological replicates per treatment). Different letters on the bars indicate significant statistical differences within each cultivar according to Tukey's test at a 95% significance level. **b**, **c** Expression profile of *CaGOLS2* (**b**) and *LcGOLS2* (**c**) genes measured by real-time RT-PCR in the same plants kept under PEG treatment compared to unstressed control conditions. Relative expression was calculated using the 2^-ΔCt formula and normalized with *CaCAC* and *LcTUB* as endogenous reference genes (Table S1). Error bars represent confidence intervals corresponding to three biological replicates consisting of three plants each ($n=3\times3$). Different letters on the bars indicate significant statistical differences between contrasting cultivars according to Tukey's test at a 95% significance level. PEG 1 day, 3 days, and 8 days indicate the number of days after the onset of drought stress

cultivar. For this gene, additional conditions to PEG-induced drought stress and tissue-specific expression may be associated with their unusual expression profile. Several *AREB/ABF/ABI5* genes had their expression profile higher in tolerant cultivars compared to sensitive cultivars when under unstressed conditions (Fig. S9a–q). The *Ca_06458*, *Ca_00791*, *Lcu.2RBY.4g037060.1*, *Lcu.2RBY.6g046660.1*, and *Lcu.2RBY.2g084200.1* genes were consistently up-regulated along the PEG-induced drought stress in chickpea and lentil (Fig. S9d, S8e, n, o, and q). Meanwhile, the other genes had specific modulations at a given time of exposure to PEG-induced drought stress or did not have their expression significantly modulated. In the expression profile of homologous genes between chickpea and lentil, it was observed that mainly *Ca_01547* and *Lcu.2RBY.3g062010.1*, *Ca_08143* and *Lcu.2RBY.6g046660.1*, and *Ca_07056* and *Lcu.2RBY.2g084200.1* genes show slightly different profiles. Therefore, these gene expression data support the results obtained with sequence analysis (gene, protein, and promoter) and meta-analysis using RNA-Seq datasets, confirming that *AREB/ABF/ABI5* subfamily genes are important in the ABA-mediated defense response, mainly to drought, salinity, and heat stress. These data also showed that some of these genes have a similar expression profile

while others have a very particular behavior, highlighting the potential of the *Ca_06458*, *Lcu.2RBY.1g044680.1*, *Lcu.2RBY.6g046660.1*, and *Lcu.2RBY.7g020780.1* genes as key players involved in the defense response to PEG-induced drought stress in chickpea and lentil.

Discussion

Chickpea and lentil crops are exposed and susceptible to abiotic stresses (Singh et al. 2019b; Silva-Perez et al. 2022; Basso et al. 2023b). Among them, drought stress is the most relevant as a consequence of global climate changes that lead to a lack or excess of rainfall, severely compromising agricultural production (Basso et al. 2024a). Most commercial cultivars of chickpea and lentil are susceptible or have a low tolerance to the levels of water deficit currently observed in field conditions (Basso et al. 2023b). The ABA-dependent and ABA-independent signaling are the major plant defense pathways against abiotic stresses, such as drought and high salinity (Yoshida et al. 2014). In the ABA-dependent pathway, the primary players are PYR/PYL/RCAR receptors, PP2C phosphatases, SnRK2 kinases, and ABA-responsive transcription factors (Fujita et al. 2013; Danquah et al. 2014). In the absence or reduced levels of ABA, these receptor-encoding genes are up-regulated to improve ABA sensitivity, but these receptors do not bind to and do not inactivate the PP2Cs, whereas PP2Cs bind and inactivate SnRKs by dephosphorylation mediated by CK2 kinase repressing the ABA signaling pathway (Ma et al. 2009; Danquah et al. 2014; Sah et al. 2016). Meanwhile, abiotic stress-related defense genes remain only at

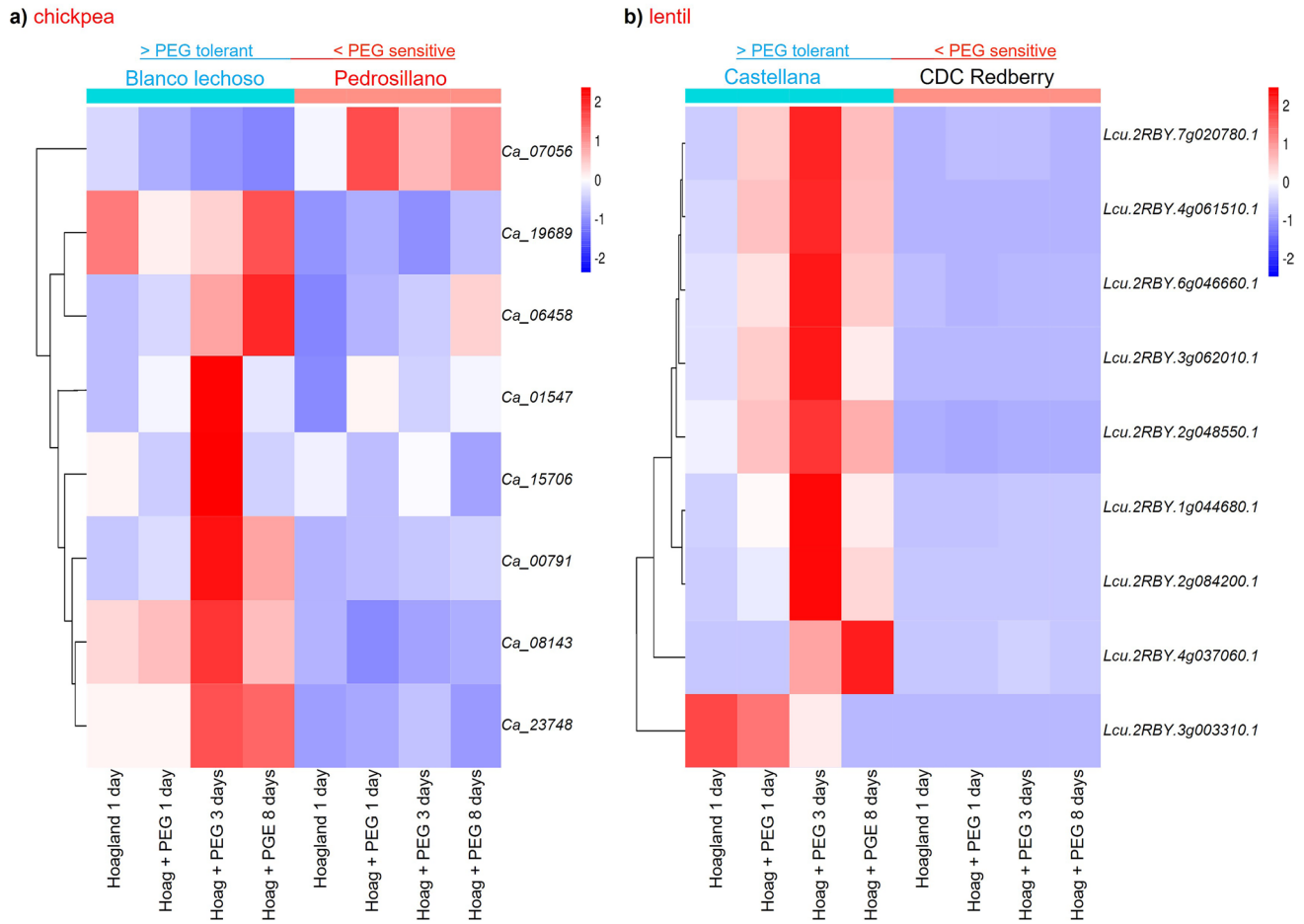

Fig. 7 Expression patterns of the *AREB/ABF/ABI5* subfamily genes in **a** chickpea cvs Blanco lechoso (PEG-tolerant) and Pedrosillano (PEG-sensitive) and **b** lentil cvs Castellana (PEG-tolerant) and CDC Redberry (PEG-sensitive) kept under drought stress conditioned by 20% polyethylene glycol (PEG 6000) compared to unstressed control plants. Gene expression was measured by real-time RT-PCR in plants 1, 3, and 8 days after the onset of drought stress. Data are presented as normalized gene expression. Relative expression was calculated using the 2^-ΔCt formula and normalized with *CaCAC* and *LcTUB* as endogenous reference genes (Table S1). Error bars represent confidence intervals corresponding to three biological replicates consisting of three plants each ($n = 3 \times 3$). The color scale bar indicates the relative expression level. PEG 1 day, 3 days, and 8 days indicate the number of days after the onset of drought stress. The statistical support for the expression level of each gene is shown in Fig. S9

the basal or not expressed (Sah et al. 2016). In the presence of ABA, ABA receptor-encoding genes are downregulated, ABA receptor proteins coupled to ABA bind and inactivate PP2Cs, releasing the PP2Cs–SnRKs complex, and free SnRK2s are phosphorylated by B3-MAPKKK and activate the ABA signaling pathway by phosphorylation of several ABA-responsive transcription factors (Fujita et al. 2005; Danquah et al. 2014; Sah et al. 2016). The AREB/ABF/ABI5 proteins are some of the transcription factors involved in the ABA signaling pathway, which are activated by SnRKs and control the downstream expression of numerous abiotic stress-related defense genes (Furihata et al. 2006; Fujita et al. 2013; Yoshida et al. 2015). Downstream genes modulated by AREB/ABF/ABI5 transcription factors are associated with seed germination (*e.g.*, *CAT1* and *PHO1*, and *PGIP1* and *PGIP2*, which are responsible for reactive oxygen species scavenging and inhibit germination, respectively) and associated with stress adaptation in vegetative tissues (*e.g.*, *SGR1*, *NYC1*, *SAG29*, *SOC1*, *GLDT1*, *LEA*, *RBA18*, and *RD29*, among others) (Fujita et al. 2005; Collin et al. 2021). Therefore, these transcription factors are powerful tools for the genetic engineering of crops, since each one is involved in the modulation of numerous downstream genes associated with plant tolerance to abiotic stresses (Basso et al. 2020).

To date, *AREB/ABF/ABI5* subfamily genes of *A. thaliana* have been characterized in terms of functional and activation domains (Fujita et al. 2005; Furihata et al. 2006) and organized into two functional groups in *A. thaliana* (Fujita et al. 2013; Yoshida et al. 2015; Liu et al. 2019). The first group is formed by *AtDPBF3/AREB3*, *AtDPBF1/ABI5*, *AtDPBF4/bZIP12*, *AtEEL/bZIP15*, and *AtDPBF2/bZIP67* genes which are more expressed in seeds and involved in seed development, maturation and germination, and ABA-dependent signaling pathway in seeds (Finkelstein 1994; Fujita et al. 2013; Yoshida et al. 2015). The second group is formed by *AtABF1*, *AtABF2/AREB1*, *AtABF4/AREB2*, and *AtDPBF5/ABF3* genes which are more highly expressed in vegetative tissues in response to different abiotic stress conditions (Lopez-Molina et al. 2001; Fujita et al. 2013). Transgenic overexpression or transcriptional up-regulation mediated by genome editing using deactivated Cas9 (dCas9) of the *AREB/ABF/ABI5* subfamily genes can improve plant tolerance to abiotic stresses (Fujita et al. 2005; Li et al. 2020). However, information about *AREB/ABF/ABI5* subfamily genes is still very scarce for chickpea and lentil. In this study, a comprehensive characterization of these *AREB/ABF/ABI5* genes in chickpea and lentil was performed at the sequence and gene expression level in response to different abiotic stresses. A total of 8 and 9 *AREB/ABF/ABI5* genes were identified in chickpea and lentil, respectively, and considered homologous to the *AREB/ABF/ABI5* genes of *A. thaliana*. Furthermore, these proteins identified in chickpea and lentil showed the C1–C4 motifs, the bZIP and NLS domains, and putative target sites of protein kinases to be highly conserved in most of them (Fujita et al. 2005; Furihata et al. 2006). Some of these components were absent or less conserved in some of these proteins, indicating that they may be activated or act in a particular way compared to the other AREB/ABF/ABI5 proteins. Sequence features, evolutionary relationships, gene structures, chromosomal localization, *cis*-acting regulatory elements, conserved motifs, protein–protein interaction networks, protein 3D structures, and gene expression profiles were provided in this study. It is summarized that these chickpea and lentil genes showed sequence features, subcellular localization, domains and motifs in protein sequences, and *cis*-acting regulatory elements that are highly conserved and are closely linked with defense response to abiotic stresses and seed dehydration. In addition, some of these *AREB/ABF/ABI5* genes presented the same functional domains or motifs, suggesting that these can have similar or redundant biological functions in chickpea and lentil. These features were conserved not only when compared with *AREB/ABF/ABI5* subfamily genes of *A. thaliana* (Yoshida et al. 2010, 2015; Fujita et al. 2013), but also when compared with *Ipomoea batatas* (Mathura et al. 2023), *Solanum tuberosum* (Liu et al. 2019), *Corchorus olitorius* (Fiallos-Salguero et al. 2023), *Oryza sativa* (Lu et al. 2009), *Populus trichocarpa* (Ji et al. 2013), *Solanum lycopersicum* (Pan et al. 2023), *Prunus mume* (Yong et al. 2021), *Santalum album* (Liu et al. 2023), and *Dendrobium catenatum*, *Apostasia shenzhenica*, and *Phalaenopsis equestris* (Xie et al. 2024), which ranged from 3 to 14 *AREB/ABF/ABI5* genes per plant species. Particular features were also observed in certain subgroups within this gene subfamily, which may explain the modulation of gene expression in defense response to different stress conditions, seed- or vegetative-specific activity, broad protein–protein interaction networks, specific biological functions, and protein dimerization (Dröge-Laser et al. 2018). It is important to mention that *AREB/ABF/ABI5* transcription factors also participate in broad hormonal responses, seed and embryo integrity, and plant growth and development (Sah et al. 2016). Based on these previous sequence data, the *AREB/ABF/ABI5* subfamily genes identified here in the chickpea and lentil were suggested to participate in the ABA-dependent signaling pathway and are responsible for the transcriptional activation of abiotic stress-related defense genes.

The *cis*-acting regulatory elements present in promoter sequences of these *AREB/ABF/ABI5* genes illustrated the potential of these genes to be dynamically modulated by different abiotic stresses, multiple hormones, and in a tissue-specific manner. These features confirm the functional complexity of these transcription factors and their importance in the different biological functions previously mentioned. A similar profile of *cis*-acting regulatory elements was also

observed in promoter sequences of *AREB/ABF/ABI5* genes identified in *Prunus mume* and *Corchorus olitorius* plants, indicating interspecies conservation of the transcriptional modulation profile of these genes (Yong et al. 2021; Fiallos-Salguero et al. 2023). Protein–protein interaction networks based on known, validated or predicted interaction, gene co-occurrence, gene co-expression, gene neighborhood, and protein homology showed that these chickpea and lentil AREB/ABF/ABI5 subfamily proteins are interconnected in a main cluster composed of 13 and 14 players, respectively. In this main cluster, five additional partner proteins to the AREB/ABF/ABI5 members were identified as hub SnRK2 proteins potentially involved in the ABA-dependent signaling pathway (Kobayashi et al. 2005) both in chickpea and lentil.

The gene expression data obtained from meta-analysis of RNA-Seq datasets revealed that *AREB/ABF/ABI5* subfamily genes are dynamically modulated in different tissues of unstressed plants in both chickpea and lentil, but with some genes assuming a certain tissue-specific expression manner. However, this expression dynamic in unstressed tissues is significantly altered for all these genes when plants are kept under any stress conditions. Overall, the expression levels of *AREB/ABF/ABI5* genes showed a tendency to increase as cultivar tolerance or resistance increases for determined stress conditions. Although up-regulation of these genes has been observed under stress conditions in all tissues, some of these tissues showed higher expression compared to other tissues. In accordance, gene expression data obtained from different organs of unstressed chickpea and lentil plants, as well as from plants under different abiotic and biotic stress conditions, such as drought, salinity, heat, alkalinity, and fungi disease, support the previous findings both at the level of sequence and protein–protein interaction network. A similar gene expression profile in different tissues of plants kept under stress conditions compared to unstressed tissues was also observed with *AREB/ABF/ABI5* genes identified in *Corchorus olitorius* (Fiallos-Salguero et al. 2023). In *Solanum tuberosum*, all seven *AREB/ABF/ABI5* genes identified were up-regulated under ABA, drought, and high salinity conditions, except for the *StABL2* gene (Liu et al. 2019). Furthermore, these gene expression results are also in agreement with the previous reports showing the broad defense action mediated by ABA against both abiotic and biotic stresses (Bharath et al. 2021).

The ABA quantification data revealed that tolerant chickpea and lentil cultivars accumulate less ABA at the onset of PEG-induced drought stress but increasing or greater amounts of ABA were observed at 3 and 8 days after drought stress. This slightly reduced amount of ABA in tolerant cultivars at the onset of stress can confer greater plant development and biomass yield compared to the susceptible cultivars under drought stress, since ABA can inhibit plant growth and development while improving drought tolerance (Basso et al. 2023b). On the other hand, the expression profile of the stress marker genes (*CaGOLS2* and *LcGOLS2*) suggested that the activation of drought-related defense mechanisms is similar (in chickpea) or higher (in lentil) in tolerant cultivars at 3–8 days after the onset of drought stress compared to sensitive cultivars. The real-time RT-PCR data showed that *Ca_06458*, *Lcu.2RBY.1g044680.1*, *Lcu.2RBY.6g046660.1*, and *Lcu.2RBY.7g020780.1* were consistently considered the major genes involved in the chickpea and lentil defense response to PEG-induced drought stress. Nonetheless, *Ca_01547*, *Ca_00791*, *Ca_15706*, and *Ca_07056* genes showed a more distant relation with a defense response to drought stress in chickpea. Similarly, *Lcu.2RBY.2g048550.1*, *Lcu.2RBY.4g037060.1*, and *Lcu.2RBY.2g084200.1* genes showed also a more distant relation with a defense response to drought stress in lentil. Although this gene subfamily is relatively conserved between chickpea and lentil, the major genes showed a slightly different expression profile in plants kept under drought stress in these two species, as well as compared to *AREB/ABF/ABI5* orthologous genes of *Ipomoea batatas* (Mathura et al. 2023) and *Solanum tuberosum* (Liu et al. 2019). Although *Lcu.2RBY.6g046660.1* and *Lcu.2RBY.7g020780.1* genes are considered more important in regulating seed maturation, dormancy, and germination, they appear also to be modulated in the shoot tissues of lentil in response to drought stress. Therefore, these data highlight the complex regulatory role of these subfamily members in modulating the ABA-dependent defense response from shoots to seeds (Dröge-Laser et al. 2018). The biotechnological potential of these five main genes associated with improved plant tolerance to drought stress is emphasized for future validation via transgenic overexpression or transcriptional modulation through the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/dCas9 system in chickpea and lentil. In addition, CRISPR/Cas9-mediated knockout of genes that encode negative regulators of AREB/ABF/ABI5 proteins is also a potential alternative for use in chickpea and lentil (Chen et al. 2021).

Conclusion

This study identified and comprehensively characterized the AREB/ABF/ABI5 subfamily members in chickpea and lentil. These collective data showed that these members contain several conserved features, such as C1–C4 motifs, bZIP and NLS domains, subcellular localization, structural domains, phylogenetic relationships, 3D protein structures, *cis*-acting regulatory elements associated with plant defense against abiotic stresses, and gene expression modulated in a tissue-specific and abiotic stress-induced manner. The expression profiles of almost all *AREB/ABF/ABI5* subfamily

genes identified in this study showed a tendency to increase with chickpea and lentil tolerance levels to abiotic and biotic stresses. The *Ca_06458*, *Lcu.2RBY.1g044680.1*, *Lcu.2RBY.4g037060.1*, *Lcu.2RBY.6g046660.1*, and *Lcu.2RBY.7g020780.1* genes were considered the major players involved in chickpea and lentil defense response to PEG-induced drought stress. This study provides substantial support for further focused research on specific *AREB/ABF/ABI5* genes. Therefore, these findings are important for the development of biotechnological tools to improve chickpea and lentil tolerance or resilience to drought stress through the use of RNAi (Basso et al. 2025), transgenesis (Basso et al. 2020), and genome editing (Paes-de-Melo et al. 2020).

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Author contributions MFB performed the in silico analysis, drought stress, and molecular experiments. PI, MC, FI, and FelM performed the plant assay and ABA determination. FC performed the meta-analysis. FLC and GB performed the protein 3D structures. MFB wrote the original manuscript. PI and MC provided input for the manuscript. FedM provided financial resources. All authors approved the final version.

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Declarations

Competing interests The authors declare that the research was conducted without commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for publication or ethics approval and consent to participate Not applicable.

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