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Genome-Wide Identification and Characterization of the ANS Gene Family in Pomegranate (*Punica granatum* L.)

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Abstract: Anthocyanidin Synthase (ANS) is a key enzyme in the later stages of the anthocyanin biosynthetic pathway, and its role is to convert colorless leucoanthocyanidins to colored anthocyanidins. In this study, a total of 75 members of the pomegranate ANS family were identified and divided into four groups (Group I, Group II, Group III and Group IV) based on evolutionary relationships. The 75 ANS gene family members were unevenly distributed on seven of the eight chromosomes of pomegranate. The results of the physical and chemical property analysis showed that 93.33% of the proteins were acidic proteins, 6.67% were alkaline proteins, 28% of the proteins were stable proteins and 72% were unstable proteins. Protein secondary structure analysis showed that α-Spiral and irregular curl are the main structural elements. Analysis of the conserved structural domains of the proteins showed that all 75 ANS family members contained one DIOX -N subfamily structural domain and one 2OG-FeII_Oxy subfamily structural domain. The results of subcellular localization showed that all 75 ANS family members of pomegranate were localized in the cytoplasm. Analysis of the transcriptome data showed that the expression of the pomegranate ANS genes were variety-specific and period-specific.

Keywords: anthocyanin synthase; genetic identification; co-linearity analysis; bioinformatics; expression analysis

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1. Introduction

Pomegranate (*Punica granatum* L.) belongs to the genus *Punica* in the family of *Pomegranate* and is an excellent fruit tree that combines ecological, economic and social benefits, ornamental value and health functions [1]. Pomegranates have a long history of cultivation and were one of the first known edible fruits [2]. The pomegranate originated in the Middle East [3] and gradually developed in China after Zhang Qian's mission to the west in the Han Dynasty. It has developed rapidly in China in recent years. Currently, Shandong Zaozhuang, Xinjiang Yecheng, Anhui Huaiyuan and Sichuan Huili are famous pomegranate cultivation areas in China [4]. Pomegranates are increasingly popular in the consumer market for their sweetness, high economic value, nutritional value, medicinal value and health functions [5]. The red color of pomegranate seeds is the result of the accumulation of anthocyanins, which have many physiological functions, not only for the plant itself to differentiate cells and prevent the occurrence of diseases, but also for human health, such as antioxidation and the prevention of cardiovascular diseases [6]. The results of Wang, D et al. [7] showed that pomegranate juice and peels have strong antioxidant effects, with pomegranate polyphenols and anthocyanins being the active substances that exert antioxidant effects. Therefore, it is important to clarify the mechanism of its regulation of pomegranate seed color.

The colors that appear in plants are determined by the presence of different pigment substances in the plant, which mainly include flavonoids, carotenoids, betaines and chlorophyll, among which anthocyanins are the most abundant type of flavonoid pigments [8].

Anthocyanins are secondary metabolites of plants and are water-soluble natural pigments of the flavonoid group. In their natural form, anthocyanins are extremely unstable and easily combine with sugar molecules to form anthocyanidins [9]. Anthocyanins are an important class of secondary metabolites in the flavonoid family, mainly found in the vesicles of epidermal cells; are responsible for the color development of leaves, flowers, fruits, seeds and other organs; giving flowers, fruits, seed coats and other organs of plants red, blue, purple and other colors; and are the main pigment substances for plant coloring [10,11]. They also play an important role in insect pollination, growth hormone transport, the protection of leaves from UV damage, pest and disease suppression and root tumor induction [12].

Anthocyanidin Synthase (ANS) is involved in a series of metabolic reactions and is a 2-ketoglutarate-dependent enzyme belonging to a family of glutamate-dependent oxygenases [13]. The main chain of ANS contains thirteen strands, of which eight form a jellyroll or double-stranded helix topology. The jellyroll forms a hydrophobic cavity, one end of which forms the active site [14]. Anthocyanidin Synthase is the key enzyme at the end of the anthocyanidin synthase pathway, and catalyzes the conversion of colorless leucoanthocyanidins to colored anthocyanidins. ANS belongs to the dioxygenase gene family in the flavonoid pathway and catalyzes a number of two-electron oxidations, such as hydroxylation, desaturations and oxidative ring closures [15]. Current studies have shown that ANS expression regulates the accumulation of anthocyanins and the color of fruit and flowers [16]. The ANS gene was first isolated from a mutant of maize by transposon tagging [17]. In addition, it has now been cloned in a variety of plants including *Arabidopsis thaliana* [14], *Litchi chinensis* [18], *Mangifera indica* [19], *Malus pumila* [20], *Camellia sinensis* [21] and *Vitis vinifera* [22]. Overexpression of ANS can increase anthocyanin accumulation, while reducing the expression of ANS significantly decreases the anthocyanin level in plants, and this leads to the production of white flowers [23,24].

Pomegranates are receiving more attention for their antioxidant and cardiovascular-disease-prevention properties, of which the main substance is anthocyanin. The ANS gene plays a crucial role in the synthesis of anthocyanins, and the identification and expression analysis of the ANS gene family in pomegranate has not been reported. In this study, members of the pomegranate ANS gene family were identified and characterized by bioinformatics tools, and their expression patterns in three different varieties of 'Hongyushizi', 'Baiyushizi' and 'Tunisia' and at different periods were analyzed to lay the foundation for studying the functions of the ANS gene family in pomegranate.

2. Materials and Methods

2.1. Plant Materials

The varieties of pomegranate tested were 'Hongyushizi', 'Baiyushizi' and 'Tunisia'. Samples were taken from pomegranate seeds at 40, 80, and 120 days after bloom, with three biological replicates for each variety and period. The seeds of pomegranates were quick-frozen in liquid nitrogen and stored at ultra-low temperatures at −80 °C. Samples were sent to Guangdong GENE DENOVO Company (Guangzhou, China) for transcriptome sequencing.

2.2. Identification of Members of the ANS Gene Family of Pomegranate

The whole genome sequence of pomegranate (ASM765513v2) was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 20 October 2021). Searching on NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 20 October 2021) yielded sequences of *Arabidopsis thaliana* containing the structural domain of Anthocyanidin Synthase (PF03171, PF14226). The obtained sequences were used as probes and homology searches were performed by local BLASTP (E-value less than 1×10^{-10}) against pomegranate's proteins in order to prevent the erroneous loss of sequences with low similarity to the probes but containing the structural domain of ANS [25]. We also used HMMER to validate our results. Then, we finally obtained all the members of the pomegranate ANS gene family.

2.3. Analysis of Physicochemical Properties and Prediction of Secondary Structure of Pomegranate ANS Proteins

The physicochemical properties, such as molecular weight and theoretical isoelectric point, of the pomegranate ANS proteins were predicted using the online tool Expasy (http://web.expasy.org/, accessed on 21 October 2021) [26].

Subcellular localization analysis of the pomegranate ANS family was conducted using the online tool Plant-mPLoc in Cell-Ploc 2.0 (http://Plant-mPLoc server (sjtu.edu.cn), accessed on 21 October 2021).

The online software SPOMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html, accessed on 22 October 2021) [27] was used to predict the secondary structure of the pomegranate ANS gene family proteins.

2.4. Construction of Phylogenetic Trees and Mapping of Gene Structures

The phylogenetic tree was constructed using MEGA X software for the pomegranate ANS gene family proteins according to the neighbor-joining (NJ) method, with the check parameter step (bootstrap) set to 1000 and all other parameters set to default values [28]. The gene structure was mapped by Gene Structure View in TBtools software (version number: v1.098685) [29].

2.5. Structural and Conserved Motif Domain Analysis of the Pomegranate ANS Protein

CD search (NCBI Conserved Domain Search (nih.gov), accessed on 23 October 2021) [30] and SMART were used for protein conserved domain analysis. The conserved motifs of the pomegranate ANS family of proteins were analyzed using the online tool MEME (http://meme-suite.org, accessed on 23 October 2021) [31], where the parameters of MEME were set to a maximum number of motifs of 10 and occurrences of a single motif of zero, or one per sequence [32].

2.6. Chromosome Positioning and Co-Linearity Analysis

Chromosomal localization of pomegranate ANS family genes based on pomegranate whole genome annotation information was performed using Gene Location Visualization from GTF/GFF in TBtools software [30]. MCScanX in TBtools software was used to identify gene duplication patterns and collinearity analysis. The Simple Ka/Ks Calculator in TBtools software was used for calculating the Ka/Ks values [33].

2.7. RNA Extraction, Library Construction and Sequencing

Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase free agarose gel electrophoresis. After total RNA was extracted, mRNA was enriched by Oligo (dT) beads. Then, the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse transcripted into cDNA with random primers [34]. Second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP and buffer. Then, the cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end repaired, poly(A) added and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.8. Raw Data Filtering, GO Enrichment and Transcriptome Expression Analysis

To obtain high quality clean reads, reads were further filtered by fastp [35] (version 0.18.0). The parameters were as follows: (1) removing reads containing adapters; (2) removing reads containing more than 10% of unknown nucleotides (N); (3) removing low quality reads containing more than 50% of low quality (Q-value $\leq$ 20) bases.

To analyze the expression characteristics of pomegranate ANS genes, Log2 based on the Fragments Per kb per Million reads (FPKM) value was used to create a heat map with the HeatMap tool in TBtools software.

3. Results

3.1. Identification, Physicochemical Characterization and Subcellular Localization of Pomegranate ANS Gene Family Members

A total of 75 candidate ANS genes from pomegranate were screened in this study, and the physicochemical properties of ANS proteins were analyzed by the online tool ExPASy. The results (Table 1) showed that the number of amino acids in the protein ranged from 289 to 442. The molecular weight of the pomegranate ANS family proteins ranged from 32,101.26 Da (*PgANS59*) to 49,422.13 Da (*PgANS62*), with an average molecular weight of 40,507.81 Da. The theoretical isoelectric point ranged from 4.98 (*PgANS58*) to 9.11 (*PgANS36*), with an average theoretical isoelectric point of 5.83. Of these, 93.33% were acidic proteins (theoretical PI < 7) and 6.67% were basic proteins (theoretical PI > 7), indicating that most of the pomegranate ANS exhibited acidic proteins. Hydrophilicity analysis showed that the pomegranate ANS proteins were all hydrophilic. Based on the instability factor, 28% of these were stable proteins (instability coefficient < 40) and 72% of these were unstable proteins (instability coefficient > 40), indicating that the majority of the pomegranate ANS were unstable proteins. The subcellular localization of all pomegranate ANS gene family proteins were localized in the cytoplasm, indicating that pomegranate ANS proteins were non-secretory and carried out metabolic activities within the cell.

Table 1. Information on the members of the pomegranate ANS gene family proteins.

Protein ID	Gene ID	Gene Name	Number of Amino Acids	Molecular Weight/(D)	Isoelectric Point	Total Average Hydrophilicity	Instability Factor	Subcellular Localization
XP_031403964.1	XM_031548104	*PgANS1*	356	40,324.31	5.84	−0.392	53.57	cytoplasm
XP_031393652.1	XM_031537792	*PgANS2*	336	38,173.35	5.50	−0.563	46.13	cytoplasm
XP_031384984.1	XM_031529124	*PgANS3*	336	38,357.03	5.79	−0.405	44.57	cytoplasm
XP_031390545.1	XM_031534685	*PgANS4*	334	37,853.40	5.75	−0.344	50.46	cytoplasm
XP_031376432.1	XM_031520572	*PgANS5*	357	40,044.51	5.66	−0.382	50.46	cytoplasm
XP_031377848.1	XM_031521988	*PgANS6*	356	38,836.67	5.62	−0.305	52.91	cytoplasm
XP_031393854.1	XM_031537994	*PgANS7*	356	40,019.21	5.37	−0.192	44.10	cytoplasm
XP_031393852.1	XM_031537992	*PgANS8*	356	40,047.29	5.46	−0.172	44.14	cytoplasm
XP_031376880.1	XM_031521020	*PgANS9*	362	41,430.45	5.17	−0.413	40.53	cytoplasm
XP_031393856.1	XM_031537996	*PgANS10*	356	40,055.22	5.14	−0.197	43.63	cytoplasm
XP_031393853.1	XM_031537993	*PgANS11*	384	43,440.10	6.03	−0.295	46.39	cytoplasm
XP_031393851.1	XM_031537991	*PgANS12*	390	43,645.10	5.77	−0.254	44.39	cytoplasm
XP_031395560.1	XM_031539700	*PgANS13*	356	40,391.35	6.12	−0.366	40.64	cytoplasm
XP_031380350.1	XM_031524490	*PgANS14*	358	40,565.39	5.29	−0.377	40.89	cytoplasm
XP_031382717.1	XM_031526857	*PgANS15*	347	39,631.90	5.26	−0.390	40.16	cytoplasm
XP_031386650.1	XM_031530790	*PgANS16*	366	41,504.68	5.75	−0.352	35.46	cytoplasm
XP_031378923.1	XM_031523063	*PgANS17*	359	40,562.30	5.07	−0.338	36.23	cytoplasm
XP_031382330.1	XM_031526470	*PgANS18*	339	38,929.06	5.13	−0.481	48.51	cytoplasm
XP_031394087.1	XM_031538227	*PgANS19*	362	41,139.64	5.30	−0.152	48.30	cytoplasm
XP_031378476.1	XM_031522616	*PgANS20*	359	39,580.33	6.09	−0.187	38.09	cytoplasm
XP_031395559.1	XM_031539699	*PgANS21*	376	42,663.06	6.77	−0.366	38.50	cytoplasm
XP_031391993.1	XM_031536133	*PgANS22*	338	38,634.90	5.78	−0.453	44.89	cytoplasm
XP_031393855.1	XM_031537995	*PgANS23*	361	40,534.63	5.07	−0.265	45.11	cytoplasm
XP_031401502.1	XM_031545642	*PgANS24*	366	41,173.19	5.26	−0.373	40.11	cytoplasm
XP_031391176.1	XM_031535316	*PgANS26*	350	38,763.98	5.39	−0.277	54.46	cytoplasm
XP_031378903.1	XM_031523043	*PgANS27*	353	39,091.58	5.80	−0.244	39.59	cytoplasm
XP_031388526.1	XM_031532666	*PgANS28*	367	41,013.40	5.28	−0.487	44.63	cytoplasm
XP_031390878.1	XM_031535018	*PgANS29*	369	41,918.31	5.38	−0.226	49.33	cytoplasm
XP_031388423.1	XM_031532563	*PgANS30*	358	40,381.98	5.62	−0.295	42.66	cytoplasm
XP_031372937.1	XM_031517077	*PgANS31*	388	43,418.39	6.28	−0.314	55.54	cytoplasm
XP_031388151.1	XM_031532291	*PgANS32*	372	41,873.01	5.42	−0.402	43.37	cytoplasm
XP_031383472.1	XM_031527612	*PgANS33*	380	43,218.42	5.38	−0.396	36.43	cytoplasm
XP_031389012.1	XM_03153315	*PgANS34*	350	38,489.70	5.22	−0.169	36.97	cytoplasm
XP_031391174.1	XM_031535314	*PgANS35*	353	39,037.42	5.34	−0.220	43.80	cytoplasm
XP_031385121.1	XM_031529261	*PgANS36*	367	40,709.85	9.11	−0.195	41.41	cytoplasm
XP_031383168.1	XM_031527308	*PgANS37*	358	41,381.08	6.75	−0.524	35.12	cytoplasm
XP_031383192.1	XM_031527332	*PgANS38*	354	39,158.46	5.62	−0.272	35.71	cytoplasm
XP_031376436.1	XM_031520576	*PgANS39*	370	41,879.97	5.48	−0.252	41.88	cytoplasm
XP_031379827.1	XM_031523967	*PgANS40*	358	41,371.03	5.91	−0.486	40.20	cytoplasm

Table 1. *Cont.*

Protein ID	Gene ID	Gene Name	Number of Amino Acids	Molecular Weight/(D)	Isoelectric Point	Total Average Hydrophilicity	Instability Factor	Subcellular Localization
XP_031383167.1	XM_031527307	*PgANS41*	358	41,503.05	5.81	−0.542	43.97	cytoplasm
XP_031395634.1	XM_031539774	*PgANS42*	373	41,126.14	6.35	−0.136	43.77	cytoplasm
XP_031394103.1	XM_031538243	*PgANS43*	320	36,348.78	5.18	−0.379	34.24	cytoplasm
XP_031391175.1	XM_031535315	*PgANS44*	296	32,917.57	5.87	−0.233	42.64	cytoplasm
XP_031385605.1	XM_031529745	*PgANS45*	358	40,276.11	5.26	−0.264	49.52	cytoplasm
XP_031398105.1	XM_031542245	*PgANS46*	380	42,899.82	5.08	−0.312	52.48	cytoplasm
XP_031399235.1	XM_031543375	*PgANS47*	365	41,639.61	5.27	−0.390	33.34	cytoplasm
XP_031380799.1	XM_031524939	*PgANS48*	346	39,478.70	5.42	−0.471	38.38	cytoplasm
XP_031397740.1	XM_031541880	*PgANS49*	336	40,882.18	5.33	−0.391	44.01	cytoplasm
XP_031390650.1	XM_031534790	*PgANS50*	319	36,283.50	7.06	−0.546	38.19	cytoplasm
XP_031384128.1	XM_031528268	*PgANS51*	319	35,943.26	5.34	−0.300	30.71	cytoplasm
XP_031390887.1	XM_031535027	*PgANS52*	393	44,373.41	6.11	−0.328	47.95	cytoplasm
XP_031383134.1	XM_031527274	*PgANS53*	378	43,069.06	6.82	−0.443	32.77	cytoplasm
XP_031390764.1	XM_031534904	*PgANS54*	395	44,855.05	5.43	−0.434	40.97	cytoplasm
XP_031399302.1	XM_031543442	*PgANS55*	434	48,533.05	6.38	−0.026	36.22	cytoplasm
XP_031407498.1	XM_031551638	*PgANS56*	356	39,585.56	6.71	−0.185	55.07	cytoplasm
XP_031389388.1	XM_031533528	*PgANS57*	394	44,472.40	6.50	−0.383	49.59	cytoplasm
XP_031380299.1	XM_031524439	*PgANS58*	369	42,082.85	4.98	−0.291	46.91	cytoplasm
XP_031407500.1	XM_031551640	*PgANS59*	289	32,101.26	9.08	−0.104	49.69	cytoplasm
XP_031374700.1	XM_031518840	*PgANS60*	354	39,399.37	5.76	−0.018	51.91	cytoplasm
XP_031378238.1	XM_031522378	*PgANS61*	393	44,385.28	6.15	−0.334	36.41	cytoplasm
XP_031399301.1	XM_031543441	*PgANS62*	442	49,422.13	6.56	−0.030	35.95	cytoplasm
XP_031373129.1	XM_031517269	*PgANS63*	379	42,826.43	5.05	−0.374	46.89	cytoplasm
XP_031374648.1	XM_031518788	*PgANS64*	354	39,488.26	6.05	−0.159	51.08	cytoplasm
XP_031388742.1	XM_031532882	*PgANS65*	304	34,270.08	6.11	−0.509	42.64	cytoplasm
XP_031383847.1	XM_031527987	*PgANS66*	371	41,943.06	8.93	−0.481	39.13	cytoplasm
XP_031373149.1	XM_031517289	*PgANS67*	369	42,122.84	5.01	−0.282	46.38	cytoplasm
XP_031390843.1	XM_031534983	*PgANS68*	429	48,221.93	8.02	−0.373	44.95	cytoplasm
XP_031377515.1	XM_031521655	*PgANS69*	372	42,320.19	5.47	−0.326	43.29	cytoplasm
XP_031397862.1	XM_031542002	*PgANS70*	376	42,150.93	5.41	−0.348	38.47	cytoplasm
XP_031377516.1	XM_031521656	*PgANS71*	372	42,394.42	5.58	−0.323	45.43	cytoplasm
XP_031377517.1	XM_031521657	*PgANS72*	309	35,244.09	5.51	−0.376	44.64	cytoplasm
XP_031400740.1	XM_031544880	*PgANS73*	303	33,706.66	5.28	−0.166	45.19	cytoplasm
XP_031400741.1	XM_031544881	*PgANS74*	301	33,484.22	5.06	−0.182	41.88	cytoplasm
XP_031395563.1	XM_031539703	*PgANS75*	317	36,302.44	5.44	−0.409	35.63	cytoplasm

3.2. Predicted Secondary Structure of the Pomegranate ANS Gene Family Proteins

Structural predictions (Table 2) showed that all members of the pomegranate ANS proteins were composed of α-helices, irregular coils, extended chains and β-turns. The lowest proportion of these was β-turns, all below 10%, with most around 6%. The majority of proteins had around 35% alpha-helices, with extended chains accounting for around 20% and the highest proportion of irregular coils at around 40%. From the results, it appeared that irregular coiling and α-helix were the main constituent forms of the secondary structure of the pomegranate ANS proteins, with β-turns and extended chains being the secondary constituent forms.

Table 2. Predicted secondary structure of the pomegranate ANS gene family proteins.

Gene Name	α-Helix (%)	Extended Strand (%)	β-Turn (%)	Random Coil (%)
PgANS1	33.15	17.42	5.06	44.38
PgANS2	34.23	19.05	6.85	39.88
PgANS3	34.82	19.35	5.36	40.48
PgANS4	33.23	17.96	5.39	43.41
PgANS5	32.21	18.21	7.84	41.74
PgANS6	37.36	17.98	5.06	39.61
PgANS7	38.20	16.29	5.90	39.61
PgANS8	37.92	16.57	6.46	39.04
PgANS9	37.85	16.02	5.52	40.61
PgANS10	39.89	15.73	5.90	38.48
PgANS11	36.20	15.89	6.77	41.15

Table 2. *Cont.*

Gene Name	α-Helix (%)	Extended Strand (%)	β-Turn (%)	Random Coil (%)
PgANS12	33.85	16.41	4.10	45.64
PgANS13	35.67	15.73	6.46	42.13
PgANS14	34.08	16.20	5.03	44.69
PgANS15	35.16	17.29	5.48	42.07
PgANS16	33.61	18.03	7.38	40.98
PgANS17	42.90	15.60	6.96	34.54
PgANS18	38.05	16.52	5.31	40.12
PgANS19	38.40	15.19	6.35	40.06
PgANS20	30.92	19.78	6.41	42.90
PgANS21	36.44	16.49	6.12	40.96
PgANS22	34.91	17.75	6.21	41.12
PgANS23	36.57	16.07	5.26	42.11
PgANS24	37.98	18.58	5.74	37.70
PgANS25	34.39	15.61	6.35	43.65
PgANS26	35.43	16.57	6.29	41.71
PgANS27	34.28	17.56	5.38	42.78
PgANS28	35.97	14.99	5.45	43.60
PgANS29	38.21	15.18	7.05	39.57
PgANS30	38.55	17.04	5.87	38.55
PgANS31	34.54	16.49	5.93	43.04
PgANS32	36.83	17.47	5.65	40.05
PgANS33	40.53	16.32	7.11	36.05
PgANS34	35.14	18.00	6.00	40.86
PgANS35	34.56	19.55	5.10	40.79
PgANS36	32.43	16.35	6.27	44.96
PgANS37	33.52	17.60	5.59	43.30
PgANS38	35.88	18.08	5.08	40.96
PgANS39	37.84	16.49	5.95	39.73
PgANS40	30.73	19.27	4.47	45.53
PgANS41	31.01	17.60	5.59	45.81
PgANS42	30.29	16.62	4.83	48.26
PgANS43	40.62	17.19	6.88	35.31
PgANS44	37.84	17.57	6.76	37.84
PgANS45	36.31	19.27	6.98	37.43
PgANS46	40.53	16.32	6.05	37.11
PgANS47	37.81	16.99	5.48	39.73
PgANS48	35.26	16.76	4.62	43.35
PgANS49	39.34	16.39	6.28	37.98
PgANS50	40.44	17.87	7.21	34.48
PgANS51	43.26	17.55	7.52	31.66
PgANS52	33.59	14.76	5.34	46.31
PgANS53	34.39	18.25	6.61	40.74
PgANS54	32.41	17.47	4.30	45.82
PgANS55	38.94	20.97	5.99	34.10
PgANS56	34.55	14.61	5.06	45.79
PgANS57	31.98	18.53	5.58	43.91
PgANS58	36.04	17.62	5.69	40.65
PgANS59	38.75	18.69	5.88	36.68
PgANS60	36.16	14.41	5.65	43.79
PgANS61	36.64	16.28	6.36	40.71
PgANS62	38.69	20.81	4.75	35.75
PgANS63	37.73	17.68	5.54	39.05
PgANS64	37.29	15.25	5.93	41.53
PgANS65	37.83	17.76	7.57	36.84
PgANS66	38.27	17.25	6.20	38.27
PgANS67	37.40	18.97	5.42	38.21
PgANS68	35.43	16.32	5.13	43.12
PgANS69	38.98	16.40	6.18	38.44

Table 2. *Cont.*

Gene Name	α-Helix (%)	Extended Strand (%)	β-Turn (%)	Random Coil (%)
PgANS70	38.56	16.22	5.59	39.63
PgANS71	39.52	16.94	5.91	37.63
PgANS72	36.25	18.12	4.85	40.78
PgANS73	36.30	15.51	6.60	41.58
PgANS74	37.21	17.61	6.31	38.87
PgANS75	36.59	17.03	5.68	40.69

3.3. Phylogenetic and Genetic Structure Analysis of the Pomegranate ANS Family

To investigate the phylogenetic relationships of the pomegranate ANS proteins, a phylogenetic tree was constructed. The results (Figure 1a) show that the 75 ANS family members of pomegranate can be divided into four groups according to their distance of kinship, named Group I, Group II, Group III and Group IV, each containing 43, 13, 16 and 3 members, respectively. According to the analysis, the 75 pomegranate ANS proteins formed 29 paralogous proteins pairs. For example, *PgANS7* and *PgANS11*, *PgANS8* and *PgANS12*, *PgANS23* and *PgANS38*, *PgANS19* and *PgANS29* and *PgANS13* and *PgANS21* were paralogous proteins pairs. Of these, 21 pairs had 100% support from the 1000 bootstrapping test, except for 8 paralogous proteins pairs, including *PgANS8* and *PgANS12*, *PgANS23* and *PgANS39*, *PgANS17* 140 and *PgANS45*, *PgANS1* and *PgANS4*, *PgANS34* and *PgANS38*, *PgANS71* and *PgANS72*, *PgANS40* and *PgANS41*, *PgANS53* and *PgANS54*. This illustrates the robustness of the constructed phylogenetic tree, the similarity in protein sequences of the 21 pairs of pomegranate ANS proteins and their close affinity.

Structural mapping of the pomegranate ANS gene was carried out using Gene Structure View in TBtools software. The results (Figure 1b) showed that each member of the pomegranate ANS gene family had two–five CDSs (8 members had two CDSs, 29 members had three CDSs, 36 members had four CDSs and 2 members had five CDSs), with little variation in the number of CDSs between members and a relatively simple gene structure.

3.4. Analysis of Protein Structures and Conserved Motifs of Members of the Pomegranate ANS Family

Information on the location of the conserved structural domains of the pomegranate ANS proteins were analyzed using CD search and SMART online software and mapped using the "My Domains" function of the online software ProSite, in conjunction with a phylogenetic tree. The results (Figure 2a) showed that 75 pomegranate ANS protein sequences contained one DIOX -N [36] (non-haem dioxygenase in morphine synthesis N-terminal) subfamily structural domain and one 2OG-FeII_Oxy [37] (2-oxoglutarate Fe(II) oxygenase, 2-ketoglutarate-Fe^{2+} oxidase) subfamily with a characteristic polypeptide sequence of the Anthocyanidin Synthase family. The ANS enzyme oxidized colorless leucoanthocyanidins to colored anthocyanidins via Fe^{2+} and (2-oxoglutarate) ions and belonged to the family of dioxygenases.

Analysis of the conserved motifs of the pomegranate ANS proteins was conducted using the online tool MEME, which yielded 10 potentially conserved motifs (Figure 2b), with different motifs indicated by different colored boxes. The 10 obtained motifs were named as Motif 1–Motif 10. All members of the pomegranate ANS family contained Motif 1, Motif 3, Motif 4 and Motif 6 conserved motifs, and all protein motifs were highly conserved in their order of arrangement. Distribution of motifs in Group I: (1) all had Motif 8, excepting *PgANS44*; (2) all had Motif 3, Motif 6, Motif 4, Motif 2 and Motif 5; (3) Motif 7, except for *PgANS6*, *PgANS43*, *PgANS51*, *PgANS65* and *PgANS66*; (4) all had Motif 9, excepting *PgANS24*, *PgANS32*, *PgANS50*, *PgANS43*, *PgANS65* and *PgANS66*; (5) all had Motif 10, excepting *PgANS29*, *PgANS24*, *PgANS32*, *PgANS43*, *PgANS65* and *PgANS66*. The distribution of motifs in Group II: (1) all had Motif 8, Motif 3, Motif 7, Motif 9, Motif 6, Motif 1 and Motif 4; (2) all had Motif 2, excepting *PgANS71* and *PgANS72*; (3) all had Motif 5, excepting *PgANS49* and *PgANS72*; (4) all had Motif 10, excepting

PgANS55, *PgANS62* and *PgANS72*. Distribution of motifs in Group III: (1) all had Motif 8, excepting *PgANS59*; (2) all had Motif 3, Motif 9, Motif 6, Motif 1, Motif 4, Motif 2 and Motif 5; (3) all had Motif 7, excepting *PgANS57*; (4) all had Motif 10, excepting *PgANS52*, *PgANS68*, *PgANS53* and *PgANS54*. Distribution of motifs in Group IV: (1) all had Motif 8, excepting *PgANS75*; (2) all members of Group IV contained Motif 3, Motif 7, Motif 9, Motif 6, Motif 1, Motif 4, Motif 2, Motif 5 and Motif 10.

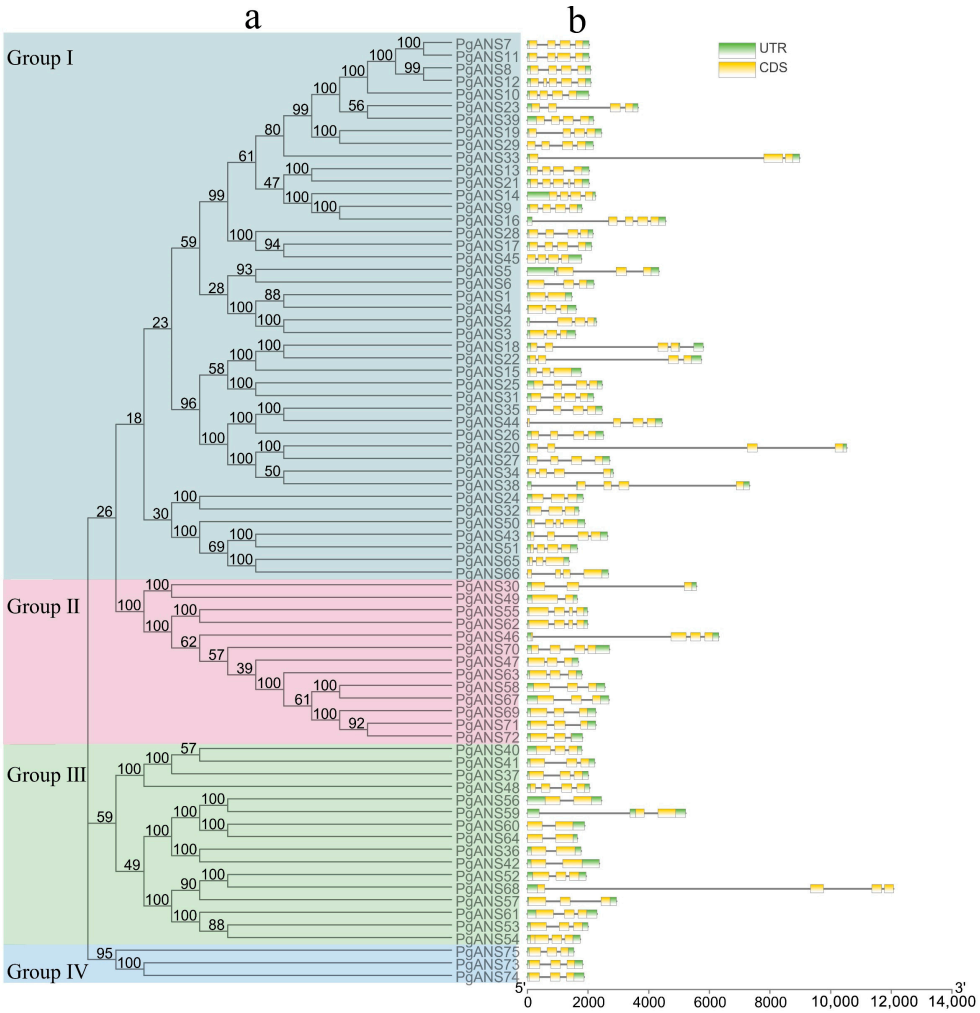

Figure 1. (**a**) Phylogenetic tree of the pomegranate ANS proteins and (**b**) genetic structure of the pomegranate ANS genes. UTR represents untranslated region, while CDS represents coding sequence.

3.5. Chromosome Positioning

Analysis of the chromosomal positioning of the 75 pomegranate ANS family genes based on the gene location file showed (Figure 3) that the 75 ANS genes were unevenly distributed on seven of the eight pomegranate chromosomes, Chr1, Chr2, Chr3, Chr4, Chr5, Chr6 and Chr8, with no genes being positioned on Chr7. The largest number of pomegranate ANS genes were distributed on Chr4, with 19 gene members; Chr2 had 15 gene members; Chr1 had 14 gene members; Chr5 had 10 gene members; Chr3 had 9 gene members; Chr8 had 5 gene members; and Chr6 had the lowest number of ANS genes distributed on it, with only 3 gene members.

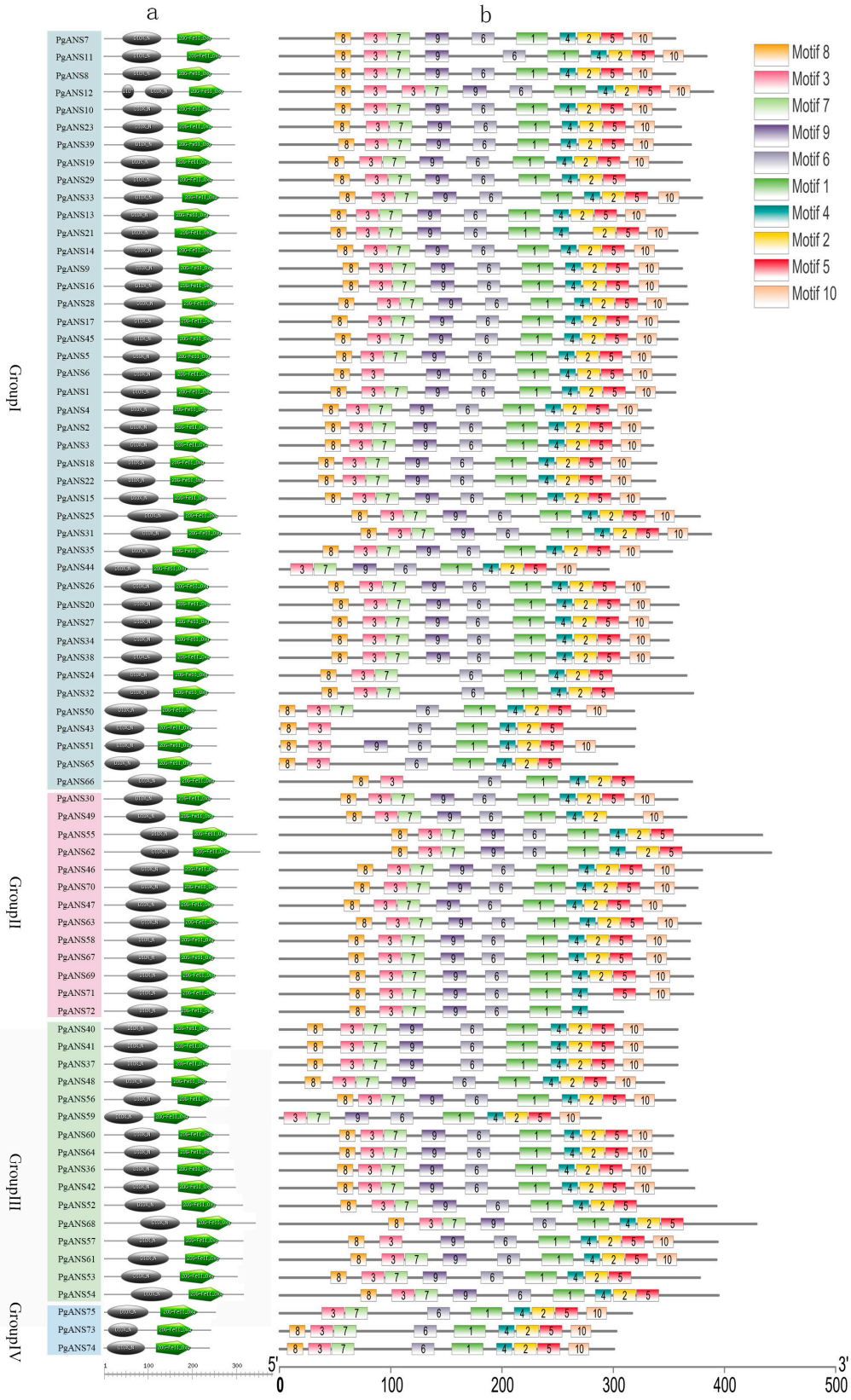

Figure 2. Conserved structural domains of the pomegranate ANS proteins (**a**) and conserved motifs of the pomegranate ANS proteins (**b**).

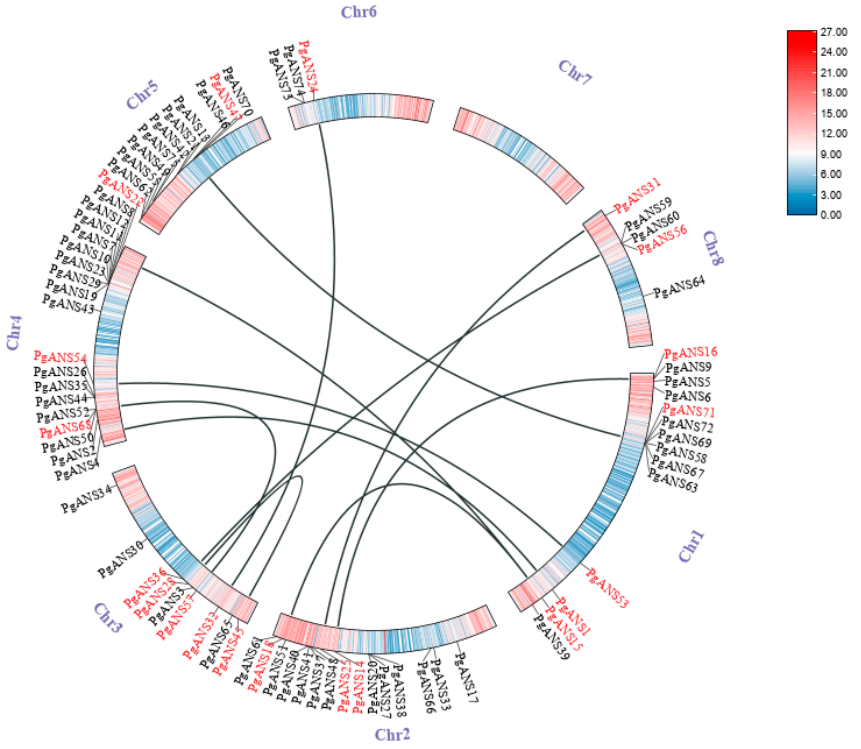

Figure 3. Location of the pomegranate ANS genes on the chromosome and gene duplication events. The red-colored names were the presence of co-linear genes.

Further analysis revealed tandem duplication between *PgANS45* and *PgANS28*, as well as fragment duplication. Ten pairs of fragment duplication genes were identified, which were *PgANS16* and *PgANS14*, *PgANS15* and *PgANS18*, *PgANS1* and *PgANS4*, *PgANS53* and *PgANS54*, *PgANS15* and *PgANS22*, *PgANS71* and *PgANS47*, *PgANS25* and *PgANS31*, *PgANS57* and *PgANS68*, *PgANS32* and *PgANS24* and *PgANS36* and *PgANS56*, suggesting possible functional similarities between some gene members of the pomegranate ANS family.

In addition, we calculated Ka/Ks ratios for 11 pomegranate ANS gene pairs to assess the selective pressure between duplicated pomegranate ANS genes. The results showed that the Ka values of the 11 pomegranate ANS gene pairs ranged from 0.10 to 0.51, and the Ks values ranged from 1.20 to 4.38. Additionally, all the values of Ka/Ks were less than one (Table 3).

Table 3. Estimated divergence period of the *PgANS* gene pairs. Ks, synonymous substitution rate; Ka, non-synonymous substitution rate.

Gene Pairs	Ka	Ks	Ka/Ks
PgANS1–PgANS4	0.48	1.33	0.36
PgANS45–PgANS28	0.51	1.73	0.29
PgANS16–PgANS14	0.47	4.38	0.11
PgANS15–PgANS18	0.36	3.87	0.09
PgANS53–PgANS54	0.20	2.90	0.07
PgANS15–PgANS22	0.33	1.70	0.19
PgANS71–PgANS47	0.38	2.18	0.17
PgANS25–PgANS31	0.28	3.12	0.09
PgANS57–PgANS68	0.38	2.40	0.16
PgANS32–PgANS24	0.10	1.20	0.08
PgANS36–PgANS56	0.38	1.66	0.23

3.6. Summary of RNA Sequencing Data

The output of RNA-seq date is shown in Table 4. After filtering low-quality, adapter-polluted and high content of unknown base (N) reads, we acquired a total of 1,200,021,858 clean reads (from 35,721,370 to 53,391,468 for each sample) and 178,302,270,781 clean bases (from 5,322,204,950 to 7,882,028,946 for each sample) in 27 libraries. An average quality value of Q20 (sequencing base quality score > 20) was 96.89% for each library.

Table 4. Summary of RNA Sequencing Data, Clean Reads Q20—Sequencing base quality score > 20 and GC Content–GC ratio of sequence bases before filtering.

Sample	Raw Reads	Raw Bases (bp)	Clean Reads	Clean Bases (bp)	Clean Reads Q20 (%)	Clean Reads Ratio (%)	GC Content (%)
Baiyushizi-T1(1)	48,667,638	7,300,145,700	48,379,952	7,219,608,238	97.14%	99.41%	50.05%
Baiyushizi-T1(2)	47,486,554	7,122,983,100	47,186,944	7,050,368,799	96.74%	99.37%	49.77%
Baiyushizi-T1(3)	47,593,812	7,139,071,800	47,305,900	7,039,879,175	96.95%	99.40%	49.60%
Baiyushizi-T2(1)	36,016,766	5,402,514,900	35,721,370	5,322,204,950	96.42%	99.18%	50.36%
Baiyushizi-T2(2)	41,189,404	6,178,410,600	40,865,830	6,095,053,040	96.56%	99.21%	50.53%
Baiyushizi-T2(3)	39,571,150	5,935,672,500	39,300,102	5,862,500,285	97.00%	99.32%	50.59%
Baiyushizi-T3(1)	40,037,072	6,005,560,800	39,938,146	5,907,058,376	96.64%	99.75%	50.42%
Baiyushizi-T3(2)	42,861,892	6,429,283,800	42,742,380	6,337,181,505	96.83%	99.72%	50.31%
Baiyushizi-T3(3)	45,807,760	6,871,164,000	45,679,274	6,745,209,770	96.87%	99.72%	50.40%
Hongyushizi-T1(1)	48,234,574	7,235,186,100	47,911,350	7,148,996,855	96.80%	99.33%	49.68%
Hongyushizi-T1(2)	39,476,844	5,921,526,600	39,184,588	5,847,426,009	96.58%	99.26%	49.58%
Hongyushizi-T1(3)	41,389,884	6,208,482,600	41,102,138	6,137,884,644	96.64%	99.30%	49.66%
Hongyushizi-T2(1)	48,913,026	7,336,953,900	48,584,658	7,251,639,210	96.76%	99.33%	50.48%
Hongyushizi-T2(2)	38,788,642	5,818,296,300	38,486,532	5,744,610,574	96.52%	99.22%	50.08%
Hongyushizi-T2(3)	48,650,276	7,297,541,400	48,296,112	7,208,671,024	96.70%	99.27%	50.36%
Hongyushizi-T3(1)	43,736,804	6,560,520,600	43,660,662	6,324,509,067	97.56%	99.83%	49.25%
Hongyushizi-T3(2)	42,770,708	6,415,606,200	42,642,452	6,357,074,521	97.33%	99.70%	50.57%
Hongyushizi-T3(3)	43,698,610	6,554,791,500	43,576,138	6,466,042,710	97.44%	99.72%	50.76%
Tunisia-T1(1)	38,816,494	5,822,474,100	38,516,272	5,739,905,920	96.28%	99.23%	49.61%
Tunisia-T1(2)	47,502,710	7,125,406,500	47,163,990	7,039,556,375	96.39%	99.29%	49.70%
Tunisia-T1(3)	51,473,316	7,720,997,400	51,172,936	7,638,003,169	96.98%	99.42%	49.64%
Tunisia-T2(1)	48,979,242	7,346,886,300	48,609,708	7,242,609,081	96.62%	99.25%	50.15%
Tunisia-T2(2)	49,197,628	7,379,644,200	48,851,404	7,265,839,885	96.79%	99.30%	50.18%
Tunisia-T2(3)	49,308,242	7,396,236,300	48,974,856	7,303,980,121	96.86%	99.32%	50.22%
Tunisia-T3(1)	40,240,650	6,036,097,500	40,170,000	5,888,045,002	97.52%	99.82%	50.17%
Tunisia-T3(2)	53,530,512	8,029,576,800	53,391,468	7,882,028,946	97.56%	99.74%	49.20%
Tunisia-T3(3)	42,669,448	6,400,417,200	42,606,696	6,236,383,530	97.56%	99.85%	49.59%

3.7. Expression Analysis of the Pomegranate ANS Gene Family

The expression characteristics of pomegranate ANS genes were analyzed in three different varieties (Honhyushi, Baiyushizishi and Tunisia) 40, 80 and 120 days after the blooming period (Figure 4). Out of 75 aforementioned pomegranate ANS genes, we only detected the expression of 64 genes in the transcriptome datasets, while *PgANS5*, *PgANS7*, *PgANS8*, *PgANS13*, *PgANS18*, *PgANS38*, *PgANS44*, *PgANS55*, *PgANS59*, *PgANS69* and *PgANS72* were not detected in any samples, possibly due to special expression patterns that cannot be examined in our libraries. The 21 genes (*PgANS27*, *PgANS73*, *PgANS36*, *PgANS2*, *PgANS50*, *PgANS31*, *PgANS64*, *PgANS21*, *PgANS25*, *PgANS56*, *PgANS23*, *PgANS10*, *PgANS43*, *PgANS60*, *PgANS46*, *PgANS54*, *PgANS33*, *PgANS34*, *PgANS35*, *PgANS42* and *PgANS62*) had a similar expression pattern in the three different varieties; that is, the expression level of the genes tended to increase and then decrease as the growth period progressed, with the highest expression level at 80 days after the blooming period (T2). This suggested that most *PgANS* genes had a period-specific expression pattern.

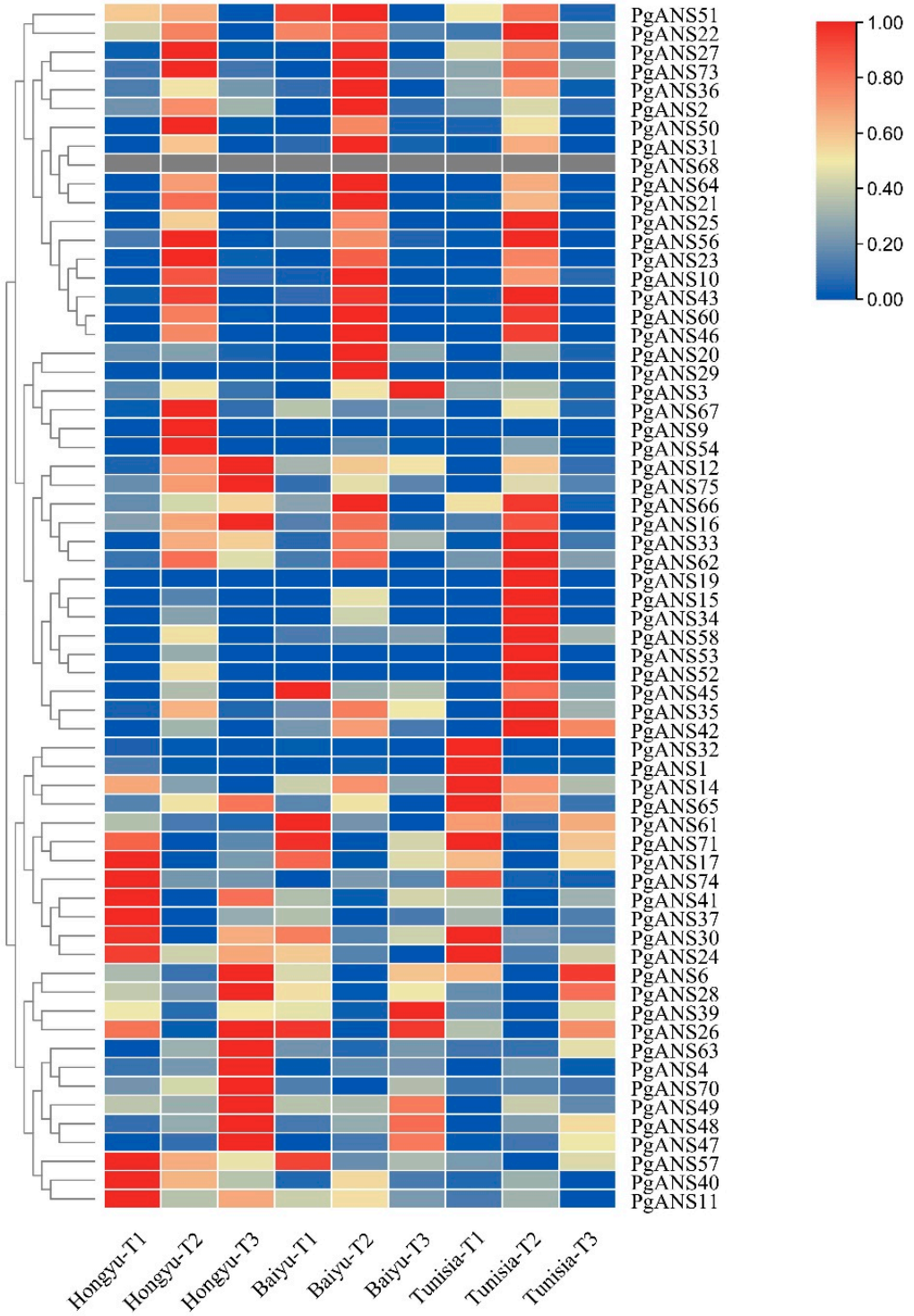

Figure 4. Heat map of the expression of pomegranate ANS genes in 3 species at different growth periods. T1—40 days after blooming period, T2—80 days after blooming period, T3—120 days after blooming period, 'Hongyu'—Hongyushizishizi, 'Baiyu'—Baiyushizi.

Analysis of the expression of ANS genes in the three varieties at T1 (40 days after the blooming period) showed that *PgANS17*, *PgANS74*, *PgANS41*, *PgANS37*, *PgANS57*, *PgANS40* and *PgANS11* were expressed at the highest levels in the red variety, 'Hongyushizi'. *PgANS51*, *PgANS22*, *PgANS45*, *PgANS61* and *PgANS26* were expressed at the highest levels in 'Baiyushizi'. Additionally, *PgANS32*, *PgANS1*, *PgANS14* and *PgANS65* had the highest expression levels in Tunisia.

Analysis of the expression of ANS genes in the three varieties at T2 (80 days after the blooming period) showed that *PgANS23*, *PgANS67*, *PgANS9*, *PgANS54*, *PgANS57* and *PgANS40* were expressed at the highest levels in 'Hongyushizi'. *PgANS51*, *PgANS36*, *PgANS2*, *PgANS31*, *PgANS64*, *PgANS21*, *PgANS20* and *PgANS29* were expressed at the highest levels in 'Baiyushizi'. Additionally, *PgANS42* and *PgANS71* were expressed at the highest levels in Tunisia.

Analysis of the expression of ANS genes in the three varieties at T3 (120 days after the blooming period) showed that *PgANS12*, *PgANS75*, *PgANS16*, *PgANS65*, *PgANS41*, *PgANS30*, *PgANS24*, *PgANS6*, *PgANS28*, *PgANS26*, *PgANS63*, *PgANS4*, *PgANS70*, *PgANS49*, *PgANS48*, *PgANS47*, *PgANS66*, *PgANS33* and *PgANS62* were expressed at the highest levels in the red variety, 'Hongyushizi'. Additionally, *PgANS42* was expressed at the highest levels in Tunisia. However, *PgANS3* and *PgAMS39* had the highest expression levels in the white variety, 'Baiyushizi'.

4. Discussion

The metabolism and regulation of plant anthocyanins have become a hot spot in scientific research in recent years. Anthocyanidin Synthase (ANS), a key enzyme in the later stages of the anthocyanidin synthesis pathway, has received widespread attention. The ANS gene has been cloned from most plants and has been shown to be an important structural gene for anthocyanin biosynthesis [38]. Anthocyanins readily combine with glycosides to form anthocyanidins, which, for the plant itself, not only give the plant a bright color and thus attract pollinators and foragers, facilitating pollination and seed dispersal [39], but also play a protective role in plant growth and development. In humans, anthocyanins have antioxidant, anti-aging and anti-vascular sclerosis effects [40,41]. ANS catalyzes the conversion of colorless anthocyanins to colored anthocyanins, which affects the accumulation of anthocyanins and determines the formation of flower and fruit coloration [42]. ANS is a family of iron- and 2-O-ketoglutarate-dependent dioxygenases, and the binding site for iron ions and 2-O-ketoglutarate in the conserved structural domain is the active central structure present in the cytochrome P450 family of genes [43]. In this study, a total of 75 members of the pomegranate ANS genes were screened by sequence alignment to predict and analyze the composition, structural characteristics and physicochemical properties of the proteins they encode. The results showed that members of the pomegranate ANS proteins contain conserved structural domains of the 2-ketoglutarate-Fe^{2+}-dioxygenase family, typical of the plant dioxygenase family of genes.

We performed covariance analysis of the members of the pomegranate ANS gene family and identified 11 co-linear gene pairs. Further analysis of these 11 pairs of genes showed that all gene pairs had Ka/Ks values less than one, indicating that they underwent strong purifying selection and played a key role in the evolution of the ANS genes [44].

The results of GO enrichment analysis showed that 64 genes were involved in metabolic processes, single organism processes and catalytic activities, and 3 genes were involved in the cell and cell part, respectively. It is conjectured that these genes may be involved in the growth and development of pomegranate fruits.

In this study, we examined the transcriptome data of 'Hongyushizi', 'Baiyushizi' and 'Tunisia' varieties at different fruit ripening periods using transcriptome sequencing technology. Studies have shown that the expression of the ANS gene was species-specific, with expression levels of dark > light > white/no color varieties; for example, in Saussurea medusa, ANS expression was higher in the red line than in the white and green lines of the healing tissue [45]. The expression level of purple kale (*Brassica oleracea var. Capitata Linnaeus*) ANS was significantly higher than that of green kale [46]. The expression of ANS was significantly higher in the pink petal line of peach (*Prunus persica*) than in the white petal line [47]. In this study, *PgANS12*, *PgANS75*, *PgANS16*, *PgANS65*, *PgANS41*, *PgANS30*, *PgANS24*, *PgANS6*, *PgANS28*, *PgANS26*, *PgANS63*, *PgANS4*, *PgANS70*, *PgANS49*, *PgANS48*, *PgANS47*, *PgANS66*, *PgANS33* and *PgANS62* were expressed at the highest levels in the red variety, 'Hongyushizi', confirming that the ANS gene was expressed at a higher level in the darker

varieties than in the white/stainless varieties. We speculated that these genes played an important role in the formation of pomegranate seed color. However, *PgANS39* and *PgANS3* were expressed at the highest levels in 'Baiyushizi' during the T3 period. We speculated that the expression of these genes may have inhibited the accumulation of anthocyanins, thereby giving pomegranate seeds a white color.

A comparison of the expression pattern of the ANS genes in the same variety at different periods revealed that there was period of specificity in the expression pattern of the ANS genes in pomegranate. The 21 genes (*PgANS27*, *PgANS73*, *PgANS21*, et al.) showed a trend of increasing and then decreasing expression as the growth period progressed. The highest expression levels were found at 80 days after the bloom period, which is consistent with the study by Wang Chunhui et al. [48] on structural genes related to ANS in the fruit of the 'Hongyang' kiwi fruit mutant. It suggested that the regulatory mechanisms of the relevant structural genes differ in the synthesis of anthocyanosises and play different roles in the anthocyanidin synthase pathway. Further experiments are needed to verify the effect of *PgANS* expression on the color of pomegranate seeds.

5. Conclusions

In this study, a genome-wide analysis of the phylogenetic relationships, intron/exon structures, motif composition and expression characteristics of *PgANS* genes was performed. A total of 75 ANS members from pomegranate were identified and divided into four groups. The results of gene expression analysis indicated that the *PgANS* genes have both a variety-specific and a period-specific expression pattern. The results of this study further elucidated the expression pattern of pomegranate ANS genes in pomegranate seeds, and ultimately lay a certain theoretical foundation for the breeding of new anthocyanin-rich pomegranate varieties.

Author Contributions: Methodology, X.Z.; software, L.H. and M.Z.; validation, H.N. and H.S.; formal analysis, H.N.; data curation, H.N. and F.Y.; writing—original draft preparation, H.N. and H.S.; writing—review and editing, S.Z.; visualization, X.Z. and H.N.; supervision, S.Z.; funding acquisition, S.Z.; H.S was the co-first author of this article. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original transcriptome data used in this study have been submitted to the NCBI, and the data are stored in the SRA database. the accession number is "PRJNA914887".

Conflicts of Interest: The authors declare no conflict of interest.

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