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Decoding Dwarfism: Gene Expression in Different Almond [*Prunus dulcis* (Mill.) D.A. Webb] Species

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Abstract: The hindered growth characteristics evident in almond [*Prunus dulcis* (Mill.) D.A. Webb] plants exert a significant influence on the yield. Nevertheless, the precise underlying mechanisms are still largely uncharted. In analogous botanical instances, pivotal regulators of growth and development have been recognized as gibberellin (GA) and brassinosteroid (BR) genes. Notwithstanding, these genetic determinants functions remain insufficiently probed within the context of almonds, a crop species of pronounced economic significance. Within the confines of this inquiry, we endeavoured to scrutinize the repercussions of GA and BR metabolic genes on the stunted growth phenomenon within almonds. This objective was pursued by exploring the impact of the administration of exogenous gibberellin 3 (GA3) on the stunted growth characteristics, accompanied by an exhaustive analysis of the transcriptional profiles pertaining to GA and BR genes in the aftermath of said treatment. The assessment of the expression levels of prospective genes associated with the stunted growth attributes was executed across both diminutive and semi-diminutive almond cultivars. The findings derived from our investigations have unequivocally unveiled heightened expression patterns of these select genes within the stem and root tissues of both modest and semi-modest almond cultivars. Such observations cogently suggest the plausible cardinal roles undertaken by these specific genetic elements in the orchestration of the stunted growth trait conspicuous in almond plants. Thus, it can contribute to regulating plant height, increasing productivity and reducing branch breakage.

Keywords: Almond growth, dwarfism, gibberellin genes, brassinosteroids genes, gibberellin 3 treatment

1. Introduction

Almond [*Prunus* dulcis (Mill.) D.A. Webb], which belongs to the Rosaceae family, is one of the largest produced nut crops worldwide. It is a temperate crop, grown in the United States of America, India, Pakistan, China, Türkiye, and other temperate regions of the world with an annual production of 3.2 million tons from the land area of 1.7 million hectares (Anonymous, 2023). Besides its fruit (nuts), it has expanded its place in the market with its milk production and as an alternative to wheat flour. Also, the use of milk in cosmetics further increases the demand for almond production.

Dwarfism in fruit orchards is an economically valuable agricultural trait that reduces the nutrient requirements of plants, thereby promoting fruit growth. Consequently, dwarf cultivars offer significant advantages ranging from increased crop productivity to reduced labor costs. Additionally, as this trait is limited to model plants, the development of dwarf cultivars/rootstocks has gained importance. As the mechanism of dwarfing becomes elucidated, the contribution of plant regulators in conjunction with practical applications will yield significant benefits.

Dwarfing is also of significant importance for almond, an economically valuable fruit tree, as it provides advantages such as making it easier for the producer to harvest the product, resulting in higher yields, and offering resistance against various environmental factors. Phytohormones, such as gibberellins (GAs) and brassinosteroids (BRs), which play an important role in plant growth and development also participate in the dwarfism process of plants. As is known that the process of

dwarfism is regulated by the expression of several genes and gene families, so defining these genes in terms of their functionality is of esteem importance. The mutant plants for these phytohormones display dwarf or semi-dwarf phenotypes proving that dwarfism is associated with the biosynthesis and signaling of various phytohormones in plants. However, a limited number of studies have been reported on the interactions of these phytohormone signaling pathways and there is no similar study in almonds.

The signaling pathways involved in the growth and development processes of plants interactively regulate these processes. Brassinosteroid and gibberellin are two main hormones that play a role in these processes and show similar effects. As the molecular mechanisms underlying these pathways have been elucidated, one or more common signaling elements have been shown to participate in the signaling of a single hormone. For example, both BR and GA regulate auxin responses and have been shown to play a common role in *Arabidopsis* to promote hypocotyl elongation. Besides, Gibberellic Acid Inhibitor (*GAI*), one of the negative regulators of the GA pathway, has been shown to regulate hypocotyl elongation in *Arabidopsis* by inactivating the transcription factor Brassinazole Resistant 1 (*BZR1*), which regulates gene expressions in BR response (Gallego-Bartolomé et al., 2012). Other similar studies have shown that dwarf phenotypes in plants are associated with BR, GA, and several auxins and strigolactone (SL) mutants (Ma et al., 2016). These mutants exhibit similar phenotypes such as stunted growth, poor seed germination, late flowering, and de-etiolation in the dark.

The genes responsible for stunting in plants began to be investigated with the rice semi-dwarf 1 (*sd1*) and wheat reduced height (*Rht*) genes (Spielmeyer et al., 2002; Hedden, 2003) and then GA-insensitive dwarf 1 (*GID1*) genes, which control plant height. Other genes such as GA oxidases (*GA3ox*, *GA2ox*, *GA20ox*) have also been identified. Deletion of *GA3ox* (Chen et al., 2014) and overexpression of *GA2ox* (Lo et al., 2008) have been shown to cause a decrease in plant height. The BR signaling pathway is controlled by a receptor kinase Brassinosteroid-Insensitive 1 (*BRI1*) and a transcription factor *BZR1*. BR-insensitive mutants (*bri1*) also display a stunted phenotype. In the presence of BR, activation of BRI1 leads to activation of BR-signal kinase and BRI1 suppressor 1 phosphatase. Defosforile BZR1 migrates to the nucleus and regulates the expression of many target genes in this pathway. To maintain growth and development and generate environmental responses, the stability of two important transcription factors BZR1 and Bri1-Ems-Suppressor 1 (*BES1*) is regulated by E3 ubiquitin ligases. The degradation of BES1 is promoted by strigolactones through the activation of the shoot branching-inhibition factor F-box protein known as More AXillary growth locus 2 (*MAX2*). Cytochrome P450 monooxygenases (P450s) associated with BR biosynthesis genes belong to a large gene family in plants, among which *CYP85*, *CYP90*, and *CYP724* [genes encoding cytochrome P450 (CYP450)] subfamilies are the most studied subfamilies. Deficient in these genes encoding oxidases in C-6 and C-22 oxidation pathways exhibit mutant phenotypes such as *dwarf 4* and *dwarf 11* (Wang et al., 2017; Fujiyama et al., 2019; Xing et al., 2020). In rice, miR396 targets Growth Regulating Factor 6 (*GRF6*), which participates in GA signaling, and the lines overexpressing miR396, the biosynthesis, and signalization of GA is impaired leading to a semi-dwarf phenotype. Besides, reduced lamina joint bending in BR signaling provided evidence of crosstalk between GA and BR (Ferrero-Serrano et al., 2019). However, although the BR and GA signaling pathways are well defined, especially in *Arabidopsis*, the relationship between them is still uncertain. Considering all these, in this study, the interaction of BR and GA biosynthesis and signaling pathways were investigated in almond, one of the important fruit trees, to bridge this important gap of information in the literature.

Gibberellins are one of the markers responsible for the dwarf phenotype, and plants with defected GA biosynthesis genes show reduced plant height. Compared to BRs the exogenous application of GA can reverse the effect of stunted phenotypes resulting from mutations in these pathways (Shen et al., 2019; Hu et al., 2020). While *GA2ox* makes biologically active GAs inactive, *GA20ox* mainly degrades C20 gibberellins. In most plants, *GA20ox* and *GA3ox* are down-regulated after exogenous GA application, while *GA2ox* genes that transform active GAs are up-regulated (He et al., 2019). It has been reported that the downregulation of *GA2ox* in tomatoes causes a significant increase in the active GA_4 content (Chen et al., 2016). *ClGA2ox1* and *ClGA2ox3* exhibit dwarf phenotypes with biological and morphological differences such as overexpression, decreased growth in *Camellia lipoensis*, late flowering, and smaller, rounded, and dark green leaves (Xiao et al., 2016). The expression levels of all these genes represent important markers for a complete understanding of the dwarfing mechanism. The production of dwarf or semi-dwarf rootstocks with genetic modifications becomes important in almond cultivation. Besides, wild species are used as a

source of genetic diversity for plant breeding programs.

Till now, it is still unclear how GA3 participates in the regulation of the complicated developmental processes of almond. To the best of our knowledge, in almond, no such study has been carried out so far. In the current study, expression profiles of some BR, GA and SL biosynthesis genes were investigated to investigate the effect of exogenous GA3 application on stunted phenotypes. In addition, expression profiles of some predicted genes thought to play a role in almond stunting mechanism were revealed. The effect of exogenous GA3 administration on the stunt phenotype and the expression profiles of these genes were evaluated by the expression analysis of some GA and BR genes after the application.

2. Materials and Methods

2.1. Plant material and growth conditions

Wild almond species *P. orientalis*, *P. webbii* and culture cultivar *P. dulcis* cv. Ferragnes seeds were obtained from Republic of Türkiye Ministry of Agriculture and Forestry Pistachio Research Institute. Almond seeds were germinated in controlled climatic chamber conditions and grown under the same conditions (25 °C 16 hours light/8 hours dark) until the seedlings reached a length of approximately 30 cm.

2.2. Exogenous application of GA3

Almond seeds were germinated under climatic chamber conditions and grown under the same conditions until they reached a length of 30 cm. Then, the seedlings were divided into two groups as control and test groups. In the test group, 1000 ppm of GA3 was sprayed on the leaves and soil every 3 days (for 12 days) In the control group, only water was sprayed at the same time periods. At the end of GA3 application, the seedlings were collected from the soil and the root, stem, and leaf tissues were separated for further analysis. Tissue samples were stored at -80 °C until use after being crushed in liquid nitrogen.

2.3. Gene expression analysis by quantitative PCR

Total RNA was extracted from leaf, stem, and root tissues of wild almond species (*P. orientalis* and *P. webbii*) as well as the cultivar (*P. dulcis* cv. Ferragnes) using the SV Total RNA Isolation System (Promega, Cat. No. Z3100). In brief, 30 mg of tissue samples were ground in liquid nitrogen and lysed with 175 µl of RNA lysis buffer containing 2-Mercaptoethanol. This was followed by the addition of 350 µl of RNA dilution buffer to the lysate. The resulting mixture was gently mixed and then centrifuged at 18.000 rpm for 10 minutes. Subsequently, 200 µl of 95% absolute ethanol was slowly added to the clean lysate in an Eppendorf tube, which was then centrifuged for 1 minute at 14.000 rpm. The RNA-binding column was washed with RNA washing solution twice, first with 600 µl and then with 250 µl, through centrifugation at 14,000 rpm for 1 minute each. For each sample, DNase-incubation mixture was prepared by combining 40 µl of Yellow Core Buffer, 5 µl of 0.09 M $MnCl_2$, and 5 µl of DNase I enzyme, which was added to the column and incubated at room temperature (23 °C) for 15 minutes. Following incubation, 200 µl of DNase stopping solution was added and centrifuged at 14.000 rpm for 1 minute. Two additional washing steps were conducted (600 µl and 250 µl of RNA washing solution), and then the column was eluted with 50 µl of nuclease-free water.

The quality and quantity of the isolated RNAs were determined using a NanoDrop® ND-1000 Spectrophotometer, and their integrity was visualized through agarose gel electrophoresis. Selected RNA samples were converted into first-strand cDNA using the GoScriptTM Reverse Transcription System (Promega, Cat. No. A5001). Specifically, 1 µg of RNA was mixed with Oligo (dT) primer and incubated at 70 °C followed by a cooling step. A reaction mixture containing GoScript ™ 5X Reaction Buffer, 25 mM $MgCl_2$, dNTP mix, Recombinant RNase® Ribonuclease Inhibitor, and GoScript ™ Reverse Transcriptase was added to the cooled RNA-primer mix. The transcription reaction underwent temperature cycles (25 °C for 5 minutes, 42 °C for 1 hour, and 70 °C for 15 minutes) inactivation.

Primer pairs were designed using Primer3 v4.1.0 (Table 1). Real-time PCR reactions were carried out on Roche Light Cycler® systems equipped with 96-multiwell plates using GoTaq® qPCR Master Mix (Promega, Cat. No. A6001). Templates for qPCR included cDNAs from leaf, stem, and root tissues, with actin serving as an internal control and nuclease-free water as a negative control. The qRT-PCR reactions consisted of 40 cycles with denaturation, amplification, and extension steps. Melting curve analysis was performed for quality control. Relative expression levels were determined using REST 2009 software and the 2-ΔΔCt algorithm (http://www.gene-quantification.de/rest-2009.html), normalized to almond actin levels. The reaction efficiency (RE) was set to 1, and a confidence interval (C.I.) of 95% was employed.

Table 1. The primer sequences used in the qPCR analysis*

Primers	Sequences (5'-3')	Melting temperature (°C)	Acc. No.
alm-dhc24-F	cagacagggagacacgcatt	52	NC_035438.2
alm-dhc24-R	catccagctcagacacagca		
alm-724b1-F	gcaagagctccatgcttgtg	50	XM_059741151.1
alm-724b1-R	tggctcatctggggtcaaac		
alm-eko-F	tatgtggaggagcttggcac	52	NM_001306026.1
alm-eko-R	tcattactgcactcctgcgg		
alm-ga20ox1d-F	gcctgatgaggaaaagccct	52	NM_001405785
alm-ga20ox1d-R	atagccacagtgctccccta		
alm-90b1-F	ccatggagatggcagcagaat	50	XM_008455515.3
alm-90b1-R	ccagtggaagttgaggacca		
alm-max2a-F	ccacaatcaacgacttgccg	52	XM_003540935
alm-max2a-R	aggggaagagcagagagtgg		
alm-bri1-F	tcatggcggagatggaaacc	52	NM_120100
alm-bri1-R	gtggtgaaggaaagcaagcc		
alm-hda19-F	gcaggtcctcaaaccctacc	55	NM_001085042.1
alm-hda19-R	ccaccagcataagtctggca		
alm-gai-F	gttacaaggttcgggcctca	52	NM_101361
alm-gai-R	aatcgtcgtggaaaacccga		
alm-85a-F	ggcagtgtccaggaaaggaa	50	XM_051051587
alm-85a-R	gacaccctaaggtgcagtcc		
alm-GA3ox1-F	atctgctggcagaagccttt	50	NM_101424.3
alm-GA3ox1-R	ctgccggcaagctttttcat		
alm-br6ox2-F	tctacaacctccatgatggctc	50	NM_113917
alm-br6ox2-R	acgtaaattctccaacctttttggg		
alm-actin-F	agcgggaaattgtccgtgat	58	AM491134.1
alm-actin-R	aagagaacttctgggcaccg		

*: The primers were designed to target regions that show homology with other almond species in which dwarfism genes have been studied.

2.4. Bioinformatic analysis

2.4.1. Prediction of miRNA-targeted dwarf genes

miRNAs targeting almond dwarf genes have been investigated using the psRNATarget (Dai and Zhao, 2011).

2.4.2. Phylogenetic analysis

Prunus dulcis GA and BR protein sequences were obtained from the National Center for Biotechnology Information (NCBI) Refseq Protein database, while their conserved domains were identified via the NCBI Conserved Domains Search Service. Employing MEGA X software, a phylogenetic examination of these proteins was executed, utilizing the maximum likelihood statistical method (Kumar et al., 2018). The robustness of the phylogenetic tree was assessed using the bootstrap method, involving 100 replications as outlined by Kumar et al. (2018). Additionally, the genomic positions of these GA proteins on the chromosome were determined and are visually presented in the analysis.

3. Results and Discussion

The genes responsible for encoding GA are distributed across half of the genome, encompassing the initial 8 chromosomes. Notably, there was found to be variation in the number of loci housing these GA genes among these chromosomes. Chromosome 1 emerges as the host for the greatest abundance of GA genes, totaling 8 instances, while the chromosomes 5, 7, and 8 exhibit the lowest count of GA genes (as depicted in Figure 1). Furthermore, instances of gene duplications have been detected, signifying a mechanism that influences the expansion of this gene family.

miRNAs play crucial roles in regulating normal plant growth and development, they are also known to play in response to environmental adversities such as various abiotic and biotic stresses. miRNAs that could target almond GA genes were predicted using psRNATarget analysis. These analyzes concluded that 12 of the 24 genes could be potential miRNA targets (Table 2).

Ferragnes, a semi-dwarf almond cultivar, has been compared to wild dwarf almond species (*P. orientalis* and *P. webbii*) in terms of the expression of selected GA genes. Results have revealed that the expression of *GA3ox* and *GA20ox1D* genes in leaf tissue of Ferragnes increased approximately 5 and 10 times compared

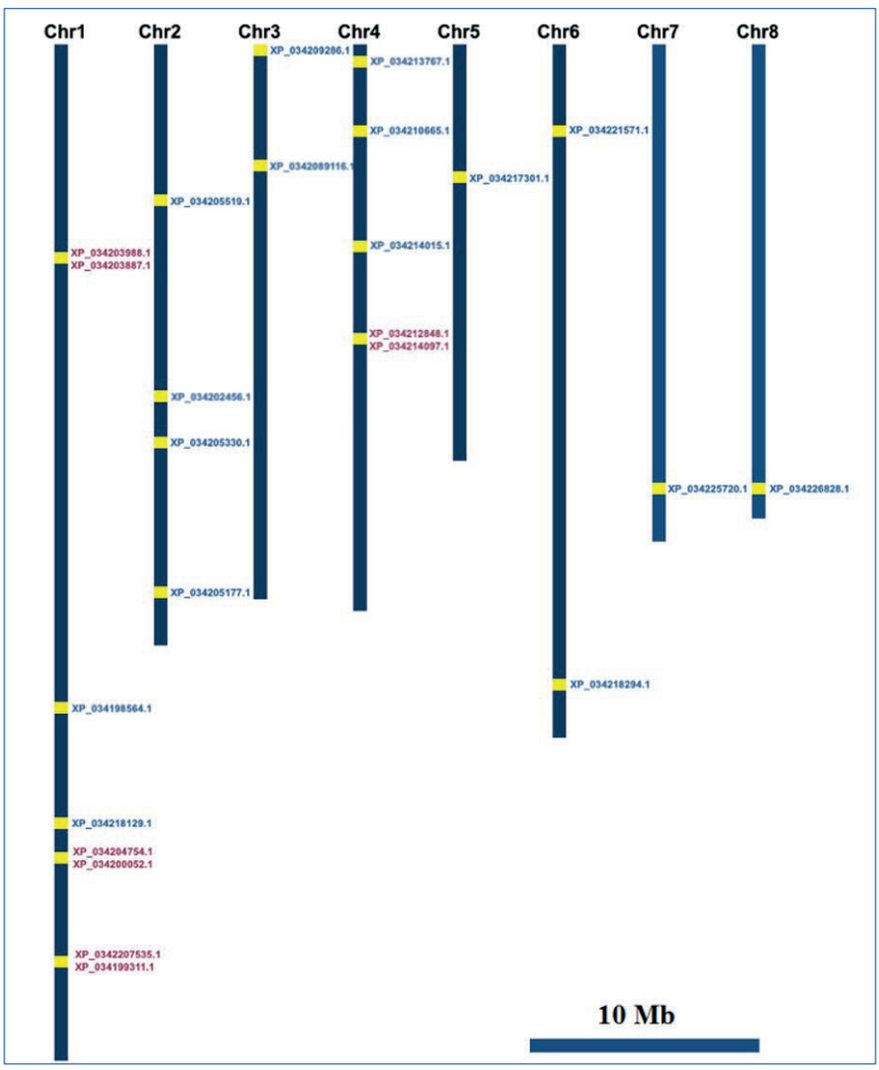

Figure 1. Chromosomal distribution of sixty GA genes in almond*.

*: Chromosome 1 (8 genes), chromosome 2 (4 genes), chromosome 3 (2 genes), chromosome 4 (5 genes), chromosome 5 (1 gene), chromosome 6 (2 genes), chromosome 7 (1 gene) and chromosome 8 (1 gene), regional copies are highlighted in purple

to *P. orientalis*. However, on the contrary, in root and stem tissues of *P. orientalis*, the expression of *GA3ox* and *GA20ox1D* genes was recorded higher; especially in *GA20ox1D* gene with a 24-fold increase in expression. A significant difference was observed in the *GAI* gene, with an increase of approximately 3-fold in leaf tissue and an approximately 2.5-fold increase in *P. webbii* stem tissue compared to Ferragnes. Compared to *P. orientalis*, in Ferragnes the expression of ent-kauren oxidase (*EKO*) gene, which is another key enzyme in GA biosynthesis, was recorded with a 6.5-fold increase.

The expression levels of GA genes that play a role in GA biosynthesis have been observed to increase in almost all species and tissues after GA3 application. For *GA20ox1D* the most significant increase was recorded in root and leaf tissues of wild dwarf almond species semi-dwarf almonds respectively. The only significant increase in the expression of the *GA3ox* gene was observed in *P. orientalis* stem tissue. No significant difference was noted in the expression level of this gene between other tissues and species compared to the control group.

Gibberellic acid signaling is controlled by GAIs characterized by a highly conserved *DELLA* protein domain. Since *GAI* transcript can be transported in phloem, it plays an active role in plant elongation. For this reason, *GAI* is often used to investigate plant height. Similarly, in current study, the expression level of the *GAI* gene was examined after the application of GA3. According to the results, a significant increase was recorded in the expression levels of *GAI* genes in the root tissue of wild almond species. On the contrary, no change in

Table 2. Target miRNAs associated with dwarfing in almond

miRNA	Target accession	Target start	Target end	miRNA aligned fragment	Target aligned fragment	Inhibition	Target description
aly-miR158a-5p	XM_034349286.1	427	447	CUUUGUCUACAAUUUUGGAAA	CCUCCACAAUUGUAGGCGAGG	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 20 oxidase 1 (LOC117619356), mRNA
ath-miR158a-5p	XM_034349286.1	427	447	CUUUGUCUACAAUUUUGGAAA	CCUCCACAAUUGUAGGCGAGG	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 20 oxidase 1 (LOC117619356), mRNA
bdi-miR7721-3p	XM_034349628.1	463	486	AAAGUUUGGCAUAGAAUUCAAUGC	CACUUGAACAUUAUGCCAAACUUU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 3-beta-dioxygenase 1-like (LOC117619631), mRNA
gma-miR9722	XM_034354774.1	1414	1434	UAAUAGAGGGAAGAAGAUGAA	AUCAUCUUCUUUCUUUUAUUU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase-like (LOC117623793), mRNA
tae-miR9773	XM_034348097.1	1295	1318	UUUGUUUUUAUGUUAUUUUGUGAA	AAAAGAAAAUAACAUAAAGGCAGU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618497), mRNA
tae-miR9773	XM_034347996.1	1295	1318	UUUGUUUUUAUGUUAUUUUGUGAA	AAAAGAAAAUAACAUAAAGGCAGU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618393), mRNA
aly-miR838-3p	XM_034358124.1	1097	1117	UUUUCUUCUUCUUCUUGCACA	CAUGAAAGGAGAGGAGGAAAG	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 1-like (LOC117626433), mRNA
aly-miR838-3p	XM_034354774.1	1167	1187	UUUUCUUCUUCUUCUUGCACA	UAUGGCAGAAGGAGAAGAAAG	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase-like (LOC117623793), mRNA
ath-miR5021	XM_034348097.1	1350	1369	UGAGAAGAAGAAGAAGAAAA	GUUUUUUCUUUUUCUUUUUU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618497), mRNA
ath-miR5021	XM_034347996.1	1350	1369	UGAGAAGAAGAAGAAGAAAA	GUUUUUUCUUUUUCUUUUUU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618393), mRNA
ath-miR5649a	XM_034362403.1	6	26	AUUGAAUAUGUUGGUUACUAU	CAAGUAAUUAACAAAUUUAAU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin receptor GID1C-like (LOC117629796), mRNA
ath-miR5649b	XM_034362403.1	6	26	AUUGAAUAUGUUGGUUACUAU	CAAGUAAUUAACAAAUUUAAU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin receptor GID1C-like (LOC117629796), mRNA
ath-miR5650	XM_034348097.1	591	611	UUGUUUUGGAUCUUAGAUACA	UUGCUCAAAGAUCCAAAACAA	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618497), mRNA
ath-miR5650	XM_034347996.1	591	611	UUGUUUUGGAUCUUAGAUACA	UUGCUCAAAGAUCCAAAACAA	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618393), mRNA

Table 2. (contiuned)

miRNA	Target accession	Target start	Target end	miRNA aligned fragment	Target aligned fragment	Inhibition	Target description
bdi-miR5174e-3p.2	XM_034342673.1	180	200	UUUAUGGAACGGAGGGAGUAG	GGACUCUCUUUGUUCCAUAUA	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 6-like (LOC117614008), mRNA
bdi-miR7712-5p	XM_034370937.1	1997	2020	UAGAGCUCUGAAGUUACCACCCAC	UGUUGUCGUAAUUUUAGAGUUCUG	Cleavage	PREDICTED: *Prunus dulcis* gibberellin receptor GID1B-like (LOC117636444), mRNA
gma-miR4415a-5p	XM_034349286.1	615	635	AAGUUGUGAUGAGAAUCAAUG	GGUAGAUUCUCGUCAAAACUU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 20 oxidase 1 (LOC117619356), mRNA
hvu-miR169	XM_034369829.1	1194	1214	AAGCCAAGGAUGAGUUGCCUG	AUUUCAACUCAUCUUUGGUUU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 3-beta-dioxygenase 1-like (LOC117635525), mRNA
mtr-miR5298a	XM_034370937.1	672	695	UGGAUAUGAUAUGAAGAUGAAGAA	UUUUCCAUUUUUCUAUCAUGUCCA	Cleavage	PREDICTED: *Prunus dulcis* gibberellin receptor GID1B-like (LOC117636444), mRNA
osa-miR1862e	XM_034348097.1	229	252	CUAGAUUUGUUUAUUUUGGGACGG	CAGCUCCAAAUUAAGCAAAUUUAC	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618497), mRNA
osa-miR1862e	XM_034347996.1	229	252	CUAGAUUUGUUUAUUUUGGGACGG	CAGCUCCAAAUUAAGCAAAUUUAC	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 2-like (LOC117618393), mRNA
osa-miR5145	XM_034342673.1	636	659	ACCUGUUUGGAUUCUUGAGGGCUA	GGCUUCUCAACAAUUCAUACAGGU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 2-beta-dioxygenase 6-like (LOC117614008), mRNA
osa-miR816	XM_034362403.1	220	239	GUGACAUAUUUUACUACAAC	AUUGUAGUGAGAUUUGUCAU	Cleavage	PREDICTED: *Prunus dulcis* gibberellin receptor GID1C-like (LOC117629796), mRNA
zma-miR395d-5p	XM_034346565.1	959	980	GUUCUAUGCAAGCACUUCACGA	ACAAGAGCUGCUUGCAUAGAGC	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 20 oxidase 1-like (LOC117617262), mRNA
zma-miR395g-5p	XM_034346565.1	959	980	GUUCUAUGCAAGCACUUCACGA	ACAAGAGCUGCUUGCAUAGAGC	Cleavage	PREDICTED: *Prunus dulcis* gibberellin 20 oxidase 1-like (LOC117617262), mRNA

the expression levels of *GAI* was recorded in the root tissues of semi-dwarf almonds. These results demonstrated that *GAI* gene expression increased only in root tissue with increasing GA. Leaf and stem did not reveal any change in the expression levels of *GAI* genes (Figure 2).

In the current study, the expression levels of some selected BR genes were evaluated. The expression results revealed the only significant increase in the expression of *CYP85A* gene from the cytochrome P450 gene family for Ferragnes leaf tissue. Expression of the *CYP90B1* gene from the same gene family showed a 2-fold significant increase in *P. orientalis* root and stem tissues and *P. webbii* leaf and root tissues. The most significant 15-fold increase in expression of *CYP90B1* gene was observed in Ferragnes leaf tissue compared to *P. orientalis*. Significant increases in expression of *CYP724B1* (another one from the same family) were observed in all tissues except *P. webbii* and *P. orientalis* root tissues. Expression of the brassinosteroid *BRI1* gene, which is one of the two kinase receptors that initiate the brassinosteroid response, was observed to increase in semi-dwarf almond tissue (5-fold in *P. webbii* and 10-fold in *P. orientalis*) compared to fully dwarf wild almonds.

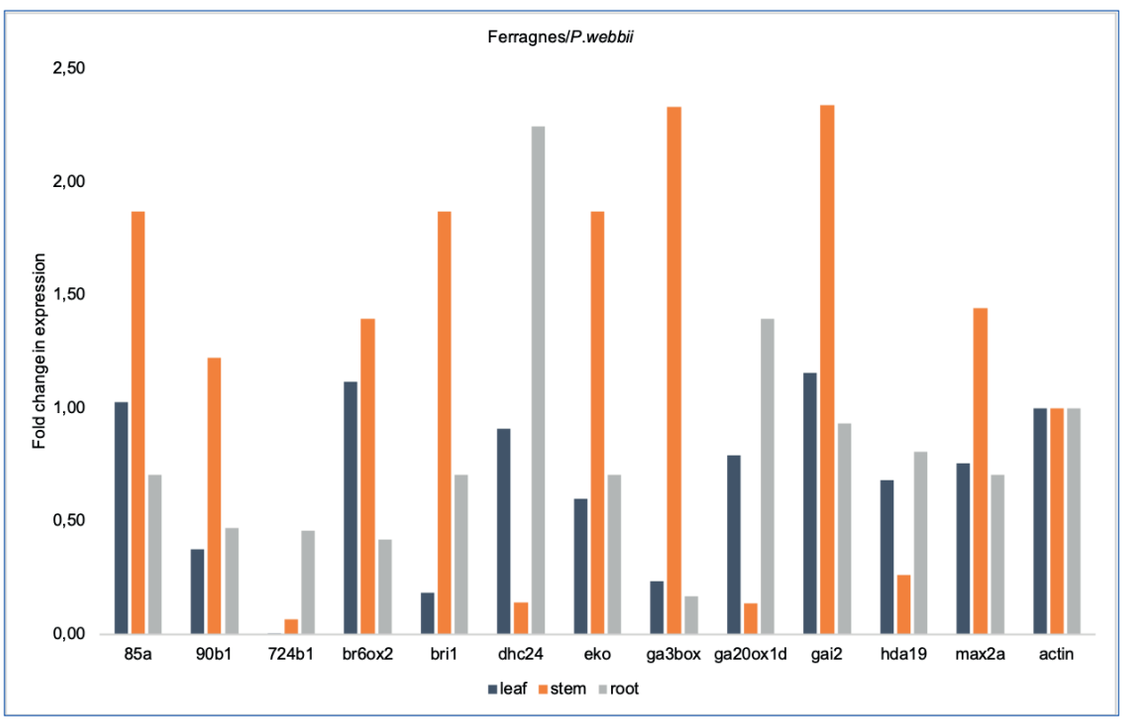

Figure 2. Expression pattern of the almond semi-dwarf/dwarf genes, in stem, leaf and root*

*: Relative expression level of these genes was determined using real-time quantitative RT-PCR normalized to expression of the actin gene, the results demonstrate that, compared to other tissues, the expression levels of these genes in the root tissue are increased for many of the genes investigated.

After applying of exogenous GA3, the expression profile of *CYP85A*, which plays an important role in BR biosynthesis, showed upregulation except in Ferragnes leaf tissues. The highest expression difference was observed in *P. orientalis* stem and Ferragnes root tissue with an approximately 20-fold increase. The most important expression difference of delta (24) -sterol reductase (*dhc-24*), another BR-related gene, was noted in Ferragnes leaf, *P. orientalis* stem and root tissues, respectively (Figure 3).

Apart from BR and GA, the *MAX2* gene, one of the SL genes that play an active role in shoot elongation and branching, exhibited a 4-fold increase in expression in the stem tissues of *P. orientalis*. Following exogenous GA3 application, the *MAX2* gene was expressed in Ferragnes leaf tissue. Looking at the histone deacetylase (HDA19) expression profile from the histone deacetylase family, a significant increase in expression was recorded in *P. orientalis* root and *P. webbii* stem tissues (Figure 4).

In plant breeding, theoretical and practical knowledge is needed to grow plants suitable for yield efficiency. Many studies show that most of the stunted phenotypes of plants are associated with GA and BR, and a few are associated with auxin (indole acetic acid, IAA) or SL. Brassinosteroids and

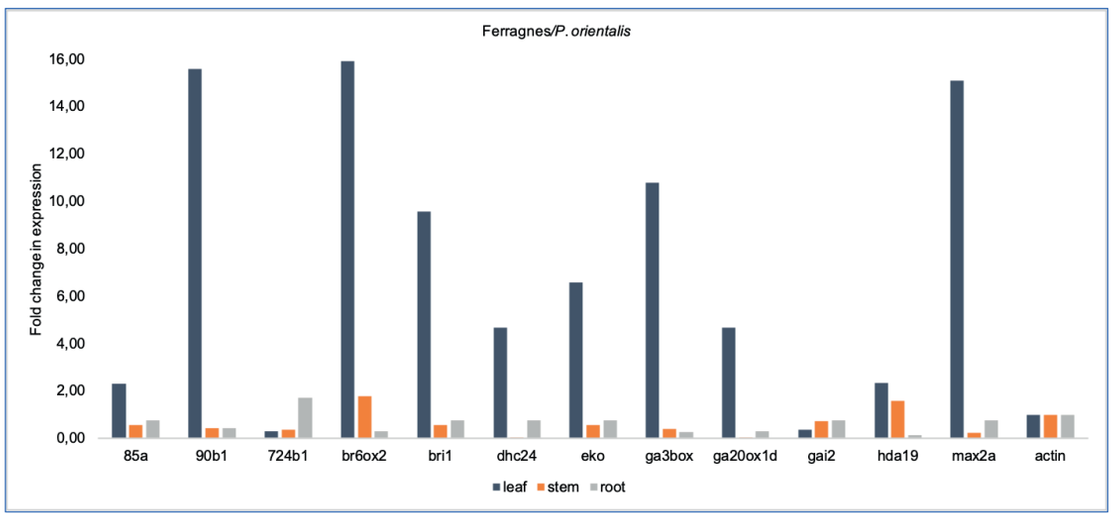

Figure 3. The comparison of gene expression levels responsible for dwarfism between the Ferragnes (semi-dwarf) and the *P. orientalis* (dwarf)*

*: The results showed significant increases in gene expression levels, particularly in leaf tissue.

gibberellins are two groups of phytohormone that regulate many common growth and development processes throughout the plant life cycle. However, no similar studies are investigating the regulatory effects of these genes in regulating plant height in almonds. Therefore, in this study, the expressions of some important BR and GA metabolism genes in different almond species/types and tissues were investigated quantitatively.

Almond homologs of these biosynthesis genes, which show homology with model plants such as *Arabidopsis*, wheat, corn, barley, *Brassica* sp. and rice, have been identified by various bioinformatics tools. This study investigated the effects of six GA and six BR genes on plant height regulation. The changing gene expressions of these 2 pathways, which are in close interaction with each other, were evaluated after exogenous GA3 application.

DELLA proteins are negative regulators of GA signaling (Feng et al., 2008). The reversibility of the stunted phenotype after the administration of exogenous GA3 indicates that the active hormone pathway is blocked due to hormone deficiency. A study on a stunted banana mutant (Williams 8818-1) evaluated the expression levels of the *GA3ox*, *GA20ox*, and *GA2ox* genes by increasing the GA3 content. It was demonstrated that these GA genes with significant expression differences may be important regulatory genes in GA metabolism (Chen et al., 2016). After GA3 treatment, the expression levels of the *GA3ox* gene increased and decreased at certain time intervals by transient expression analysis in peony buds. This study concluded that *GA3ox* and *GA20ox* genes, which are involved in GA biosynthesis, are important regulators in plant height regulation. In addition, after GA application, the expression of these genes was observed to increase in all tissues examined (Guan et al., 2019). Similarly, in the current study, the genes related to GA and BR were upregulated even though they showed different expression levels in different tissues after GA3 application. Especially, this increase in expression was recorded in root and stem tissues of wild almond species and semi-dwarf almond leaf tissue.

BRs and GAs stand as pivotal regulators of plant growth, each following distinct signaling pathways. However, their interplay often furnishes insights into their synergistic contribution to overall plant development. Notably, the dependency of GAs on BRs for enhanced hypocotyl elongation underscores their collaborative influence on growth dynamics (Shahnejat-Bushehri et al., 2016). Within brassinosteroid biosynthesis, a crucial role is attributed to the cytochrome P450 monooxygenase gene family. This gene ensemble includes the Plant *CYP85A* members responsible for encoding BR-6-oxidases, enzymes catalyzing the generation of two active brassinosteroid forms, BL and CS. Notably, mutations in these genes have been associated with stunted growth across various plant species. For instance, experimental evidence has demonstrated that knockout mutations in the *BR6ox1* gene, encoding the *CYP85A1* enzyme, induce a stunted phenotype in tomato. Intriguingly, while sharing considerable polypeptide homology, these mutations do not elicit comparable phenotypic effects in *Arabidopsis*, a discrepancy potentially stemming from the divergence between monocot and dicot plants (Nomura et al., 2005). Likewise, the current study discerns a notable surge in expression levels, particularly within the stem

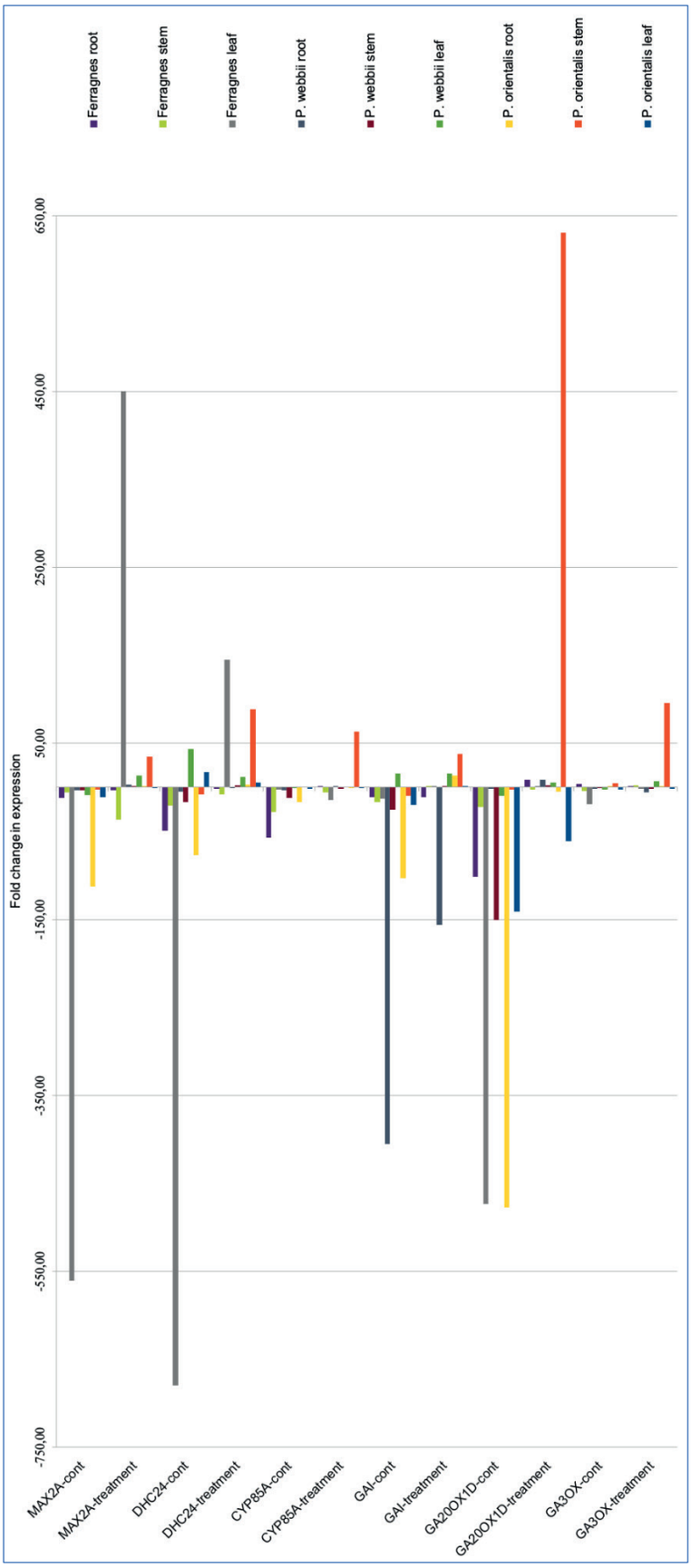

Figure 4. In dwarf and semi-dwarf almonds, the expression levels of genes involved in the BR biosynthesis pathway were evaluated after gibberellin treatment

tissue of *P. orientalis* and the root tissue of Ferragnes, subsequent to the administration of exogenous GA3. These findings establish a foundation for comprehending the intricate crosstalk between these two pathways, governing plant growth and development and the intricate correlation of these genes with the stunted growth phenotype. *BES1* and *MAX2*, key regulators of BR and SL signaling pathways, have been shown to interact and act as a substrate for regulating SL-sensitive gene expression (Wang et al., 2013; Stefanowicz et al., 2015). It has also been observed that the SL receptor *AtD14* plays a role in the degradation of BES1, and this degradation suppresses the shoot branching of *max2-1* mutants (Wang et al., 2013). In *Arabidopsis* and rice, SL receptors have been shown to regulate SL-sensitive gene expression by targeting the *BES1/D53* transcription factors for *SCFMAX2/D3* degradation (SCF, S-phase kinase-associated protein 1-Cullin1-F-box; D3, Dwarf3) (Challis et al., 2013). Similarly, the current study recorded significant expression of the *MAX2A* gene in *P. orientalis* stem and Ferragnes leaf tissue. After GA3 application, this gene was upregulated with high expression levels in stem and leaf tissues. Similarly, this study provided primitive evidence that the interaction between BR-GA pathways indirectly affects the SL pathway.

Populus MAX gene mutants show partial shoot branching compared to *Arabidopsis* mutants, providing evidence for the conservation of SL genes in woody plants. Application of GA3 can increase the hypocotyl length and, in later stages, completely restore the reduced plant height and delay in flowering time. According to our findings, increased expression of *MAX2* gene was recorded in Ferragnes leaf tissue among other vegetative tissues. This provides evidence that both Ferragnes have a semi-dwarf phenotype, this phenotypic feature can be reduced by GA application, and that these almond species used in the study may have a function like the orthologs of the *MAX2* gene. There was no significant difference between *MAX2* gene expression levels in vegetative tissues of *P. orientalis* and *P. webbii*.

GA-20-oxidases are a limiting enzyme in GA biosynthesis, and for *GA3ox* to form bioactive GAs, some GAs are used as substrates and catalyze sequential oxidation events. *GA20ox1*, which plays a role in growth and fertility, mutation of this gene causes stunted phenotype and infertility, while its overexpression increases growth with GA accumulation. Similarly, *GA3ox* deletion causes a decrease in plant height (Guo et al., 2020). Expression of this gene can completely reverse the stunted and highly branched *Arabidopsis* max2-1 mutant phenotype. This shows that *GhMAX2s* have preserved functions with *AtMAX2*, and these two genes can be used in plant height regulation by suppressing branching (Zhao et al., 2017). In the current study, a 15-fold increase was recorded in an expression of *MAX2A* in the leaf tissues of Ferragnes. Besides, after the application of GA3, although the *MAX2A* gene is upregulated in tissues of all species/cultivars an obvious difference was recorded for the leaf tissues of Ferragnes. These results show us that *MAX2A*, one of the SL, can improve plant height by preventing side branching after GA3 application. A similar study revealed that after *Medicago trunculata* GA3 application, GA genes were upregulated in the stunted phenotype compared to the wild phenotype, resulting in a significant difference in leaf size and petiole length (Guo et al., 2020). Another similar study with a super dwarf cotton mutant (named AS98) gave an important response to exogenous hormone applications, particularly GA3, by rearranging the plant height (Zhang et al., 2011). These findings supported the main hypothesis of our study that exogenous GA3 can play a vital role in reversing the dwarf phenotype of almond.

4. Conclusions

Herein, the expression levels of candidate genes responsible for plant stunting were evaluated comparatively in the wild and cultivated almonds. The interactions of the GA, BR, and SL pathways in regulating plant growth after exogenous GA3 application are investigated at the gene level. This study will contribute to future breeding programs for high crop yield in dwarf almond species/cultivars. Exogenous hormone application resulted in up or down regulation of the growth-regulatory GA and BR genes at the gene expression level. These applications demonstrated an impact on the dwarfing mechanism but could not completely reverse it. Findings have been obtained that will contribute to the transition from research to practical application of the dwarfing trait.

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Declaration of Conflicts of Interest

No conflict of interest has been declared by the author.

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