



Communication

Interactions of JAZ Repressors with Anthocyanin Biosynthesis-Related Transcription Factors of *Fragaria × ananassa*

Adrián Garrido-Bigotes ¹ , Marcela Torrejón ², Roberto Solano ³ and Carlos R. Figueroa ^{4,*} 

¹ Laboratory of Plant Epigenetics, Faculty of Forest Sciences, Universidad de Concepción, Concepción 4070386, Chile; adrigarrido@udec.cl

² Laboratory of Signaling and Development, Department of Biochemistry and Molecular Biology, Faculty of Biological Sciences, Universidad de Concepción, Concepción 4070386, Chile; matorrejon@udec.cl

³ Plant Molecular Genetics Department, National Centre for Biotechnology, Spanish National Research Council (CNB-CSIC), 28049 Madrid, Spain; rsolano@cnb.csic.es

⁴ Institute of Biological Sciences, Campus Talca, Universidad de Talca, Talca 34655488, Chile

* Correspondence: cfigueroa@utalca.cl; Tel.: +56-71-220-0277

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Abstract: Strawberry fruits are rich in flavonoids like proanthocyanidins and anthocyanins. Their biosynthesis and accumulation are controlled by the MYB-bHLH-WD40 (MBW) transcriptional complex, which is mainly formed by basic helix-loop-helix (bHLH) and MYB transcription factors (TFs). In *Arabidopsis thaliana* both bHLH and MYB TFs are repressed by JASMONATE ZIM-DOMAIN (JAZ) proteins, the key repressors of the jasmonate-signaling pathway. The aim of this research was the characterization of the FaJAZ1/8.1/9/10 proteins and molecular targets of signaling components and anthocyanin biosynthesis-related TFs of *Fragaria × ananassa* by protein–protein interactions. For this, domain compositions were studied by multiple alignments and phylogenetic analyses, while interactions were analyzed by yeast two-hybrid (Y2H) assays. We detected high conservation of FaJAZ proteins and jasmonate-signaling components, as well as FabHLHs and FaMYB10 TFs. Moreover, we report the *F. × ananassa* YABBY1 (FaYAB1) TF, which is related to anthocyanin biosynthesis in *Arabidopsis*, showed high conservation of functional domains. We demonstrated that FaJAZ repressors interacted with *F. × ananassa* NOVEL INTERACTOR OF JAZ (FaNINJA), FaMYC2, and JASMONATE ASSOCIATED MYC2-LIKE (FaJAM) proteins. Besides, transcription factors of MBW-complex like FabHLH3, FabHLH33, and FaMYB10, together with FaYAB1, were molecular targets of FaJAZ repressors, exhibiting specificity or redundancy of interaction depending on particular FaJAZ protein. Overall, these results suggest that interactions of jasmonate-signaling components are fully conserved, and anthocyanin biosynthesis might be regulated by JAZ repressors in *F. × ananassa*.

Keywords: jasmonates; JA-signaling pathway; JAZ repressors; MBW-complex; MYC2 transcription factor; strawberry; YABBY genes

1. Introduction

Fleshy fruits of cultivated strawberry (*Fragaria × ananassa* Duch.) are highly valued by its taste, flavor, red color, and their multiple nutritional properties [1]. The nutritional value is given by flavonoid compounds like anthocyanins, which are in large amounts in red fruits [2] and have different benefits for human health [1]. In turn, other flavonoids like proanthocyanidins (PAs) are in a higher quantity at the immature stage of strawberry fruits [2], participating in the protection against pathogens of flowers and early fruit tissues [3] and also with antioxidant benefits for humans [4].

The physiology, metabolic pathways, and molecular networks for PAs and anthocyanins biosynthesis are well-understood in the model plant *Arabidopsis thaliana* (L.) Heynh [5] and is being understood in Rosaceae Juss. family's species like *F. × ananassa* [6–9] and *Malus × domestica* Bork. [10] among others. Strawberry is a model for the study of the physiology, metabolism, and genetics of non-climacteric fruits [11,12]. Firstly, the phytohormone abscisic acid (ABA) is the major regulator of strawberry ripening promoting the anthocyanin accumulation [13]. Exogenous applications of other phytohormones like jasmonates (JAs) also increase the fruit-associated PAs and anthocyanin contents both in laboratory and field conditions [14–17]. Secondly, ripe strawberry fruits are rich in anthocyanins, which are synthesized from leucoanthocyanidins by the sequential activity of anthocyanidin synthase (ANS) and uridine diphosphate (UDP) glucose:flavonoid 3-O-glucosyl transferase (UGT) enzymes [6,18]. Thirdly, these enzymes are under transcriptional control of the MBW-complex constituted by R2R3-MYB, basic helix-loop-helix (bHLH), and WD40 repeats transcription factors (TFs) [9,18]. In strawberry, two MBW complexes regulate differentially the expression of the genes encoding for the catalytic enzymes of PAs and anthocyanins biosynthesis [6,9,18]. On the one hand, the PA-related MBW complex is composed of TRANSPARENT TESTA GLABRA 1 (TTG1), MYB9/MYB11, and bHLH3, which have transcriptional control on *LAR* and *ANR* genes in *F. × ananassa* [6]. On the other hand, TTG1, MYB10, and EGL3 (bHLH33) control the transcription of *ANS* and *UGT* genes [6,8], being MYB10 the main factor controlling the anthocyanin biosynthesis [7,19]. In turn, these MBW complexes in *F. × ananassa* are regulated by negative TFs like MYB1 and MYB5 [6]. Besides, additional TFs like YABBY1/FILAMENTOUS FLOWER (YAB1/FIL) regulate the expression of *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1/MYB75*) involved in the anthocyanin biosynthesis in *Arabidopsis* [20]. Recent studies suggest that bHLH3 and bHLH33 could be forming the MBW either with complex with MYB10 and MYB9/MYB11, regulating anthocyanin and PA biosynthesis, respectively, being MYBs the TFs that give specificity to each metabolite biosynthesis regulation [9,15]. Moreover, PA and anthocyanin biosynthesis is regulated by endogenous signals, environmental stresses, and phytohormones [2]. The key functional role of JAs in the PA and anthocyanin accumulation has been demonstrated in *A. thaliana* [20–22] and has begun to be understood in Rosaceae-related species such as *M. × domestica* [10] and *F. × ananassa* [14,15].

Jasmonoyl-isoleucine (JA-Ile) is the bioactive JA in land higher plants [23] and mediates biotic and abiotic stress tolerance and fundamental development processes like secondary metabolism [24]. Previous studies have shown that JAs induce the accumulation of anthocyanins in leaf and fruit tissues of *Arabidopsis* [20–22] and strawberry [14,15], respectively. In this sense, the molecular JAs signaling pathway is involved in the JA-Ile sensing mechanism and the activation of JA responses [24,25]. The perception of JA-Ile is mediated by CORONATINE INSENSITIVE 1 (COI1) protein and JASMONATE ZIM-DOMAIN (JAZ) repressors, which interact when JA-Ile levels increase [23,26], and this mechanism is conserved in *Fragaria vesca* L. and *F. × ananassa* [27,28]. Then, JAZ proteins are degraded by the 26S proteasome, and MYC TFs are released to activate the expression of JA-responsive genes in *Arabidopsis* [29–32]. When JA-Ile levels are low, JAZ repressors interact with NOVEL INTERACTOR OF JAZ (NINJA) adaptor protein and MYC TFs to repress JA responses [31,33]. Additionally, JAZ repressors target JASMONATE ASSOCIATED MYC2-LIKE (JAM) transcription repressors, which compete with MYC transcription activators for negative regulation of the JA-transcriptional responses [34].

In detail, JAZ repressors are a subgroup of proteins within to TIFY family [29,35,36], which are present in whole land plants [37]. The number of JAZ proteins is different depending on the species [37]; for instance, the lower plant *Marchantia polymorpha* L. contains a single JAZ protein, while *A. thaliana* and *F. vesca* contain 13 and 12 members, respectively [37–39]. This is due to the fact that JAZ repressors have functional redundancy in higher plants, but specific JAZ-TF complexes allow to regulate only certain development events or abiotic and biotic constraints [40]. At the structural level, JAZ proteins are characterized by the presence of two well-conserved domains: TIFY involved in the interaction with NINJA adaptor protein and the formation of homo- and heteromeric JAZ-JAZ interactions [33,41]

and the Jas domain allowing the interaction with a high number of MYC and JAM TFs through JAZ-interaction domain (JID) domain [31,41]. Moreover, protein–protein interactions of JAZ to regulate some processes such as the secondary metabolism are also mediated by the Jas domain, which interacts with the bHLH domain of TT8 and EGL3, the CT domain of PAP1/MYB75 in Arabidopsis [22,42]. In some cases, TIFY is the main domain involved in the interaction with the C domain of NINJA protein [33,41] or with YABBY1 (YAB1) [20], although the TF domain involved in this interaction has not yet been reported. Finally, in *M. × domestica* the repressor role of JAZs-bHLH3 regulating the PA and anthocyanin biosynthesis have been demonstrated [10].

According to previously mentioned, this research aimed to characterize the interaction of strawberry JAZ proteins (FaJAZ1, FaJAZ8.1, FaJAZ9, and FaJAZ10) with canonical JA-signaling components (FaNINJA, FaMYC2s, and FaJAMs), as well as MBW complex-related components (bHLHs and MYBs). Specially, we characterize for the first time a YABBY gene in strawberry (*FaYAB1*) at the sequence level and the protein–protein interactions with FaJAZs. We found functional conservation of FaJAZ proteins with JA signaling- and anthocyanin biosynthesis-related TFs and proteins.

2. Materials and Methods

2.1. Identification of Ortholog Sequences

Full-length proteins of *A. thaliana* were obtained from TAIR database (v10, <https://www.arabidopsis.org/>, last accessed date: 16 June 2020) and were used as queries to search the ortholog sequences not previously characterized for *F. vesca*, *M. × domestica*, *Vitis vinifera* L., and *Solanum lycopersicum* L. in RefSeq Protein database (<https://www.ncbi.nlm.nih.gov/refseq/>, last accessed date: 16 June 2020) by BLASTP search using default parameters. Output sequences with higher identity, query cover, and lower e-value were selected as orthologs in each species. The Genbank Accession numbers are listed in Table S1.

2.2. Isolation and Cloning of Encoding Sequences

Total RNA was extracted from 4 g of strawberry fruits using the cetyltrimethylammonium bromide (CTAB) method and further RNA purification by RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to previously reported [14,39]. The cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, MA, USA) from 1 µg of total RNA, according to the manufacturer's instructions. Full-length coding sequences (CDS) of JA-signaling (NINJA, MYC2, MYC2-like, JAM1, JAM2, JAZ9) and YAB1 of *F. vesca*, and anthocyanin-related transcription factors (bHLH3, bHLH33, MYB10, MYB1, MYB5) of *F. × ananassa* were used for primer design containing attB1 and attB2 sites at 5' and 3' ends (Table S2), respectively, required for cloning by Gateway technology (Invitrogen, Carlsbad, CA, USA). Isolation of coding sequence was performed from cDNA by reverse transcription-PCR (RT-PCR) using Phusion Hot Start II high-fidelity DNA Polymerase (ThermoScientific, Waltham, MA, USA). The PCR conditions were as follows: 98 °C for 30 s; 35 cycles of 98 °C for 10s, 58 °C for 30 s and 72 °C for 30 s per kb; and 72 °C for 10 min. All PCR products were analyzed by 1% (*w/v*) agarose gel and purified using Zymoclean Gel DNA Recovery Kit' (Zymo Research, Irvine, CA, USA).

JA-signaling anthocyanin-related transcription factors full-length coding sequences containing attB1 and attB2 sites were recombined into pDONR207-Gateway donor vector by BP Clonase II (Invitrogen, Carlsbad, CA, USA) at 25 °C for 12 h. Constructs were verified by sequencing. Protein sequences were submitted to GenBank, and accession numbers are available in Table S1. Transcription factors (MYC2, MYC2-like, JAM1, JAM2, bHLH3, bHLH33, MYB10, YAB1) and MBW-complex repressors (MYB1, MYB5) were recombined into pGADT7-AD (activation domain)-Gateway expression vector as prey, and JAZ repressors (JAZ1, JAZ8.1, JAZ9, and JAZ10) were recombined into pGBKT7-DBD (DNA binding domain)-Gateway expression as bait, by LR Clonase II (Invitrogen, Carlsbad, CA, USA) at 25 °C for 12 h. These constructs were used for protein interactions by yeast-two hybrid assays.

2.3. Sequence Analysis

Predicted protein sequences of *F. × ananassa* JA-signaling (FaNINJA, FaMYC2, FaMYC2-like, FaJAM1, FaJAM2, FaJAZ1, FaJAZ8.1, FaJAZ9, and FaJAZ10), anthocyanin-related transcription factors (FabHLH3, FabHLH33, FaMYB10, FaMYB1, and FaMYB5), and FaYAB1 along with their orthologs in *F. vesca*, *M. × domestica*, *S. lycopersicum*, *V. vinifera*, and *A. thaliana* were used for protein sequence analysis (Tables S3 and S4). Multiple alignments were performed using the Clustal Omega tool (European Bioinformatics Institute, University College Dublin, Dublin, Ireland) [43] and visualized by Jalview software (University of Dundee, Scotland, UK) [44] to identify functional domains in proteins. Unrooted phylogenetic trees were constructed by Molecular Evolutionary Genetics Analysis X (MEGA X) software (Pennsylvania State University, University Park, PA, USA) [45] using the Neighbor-Joining method (Jones–Taylor–Thornton substitution model) and bootstrap analysis of 1000 replicates, and visualized using Evolview v2 software (Beijing Genomics Institute, Beijing, China) [46]. The Genebank Accessions for protein sequences are shown in Table S1.

2.4. Yeast Two-Hybrid Assays

Yeast two-hybrid assays were carried out using GAL4 Gateway System (Clontech, Palo Alto, CA, USA). The pGADT7-(AD) and pGBKT7-(DBD) constructs containing TFs and JAZ repressors, respectively, were cotransformed into AH109 yeasts to evaluate molecular interactions. *ADE2*, *HIS3*, and *lacZ* were used as reporter genes. Yeast transformants were selected on the SD/-Leu/-Trp medium. The interactions were tested on the SD/-Leu/-Trp (-2), SD/-Leu/-Trp/-His (-3), and SD/-Leu/-Trp/-Ade/-His (-4). Plates were incubated at 30 °C for 3 days. Empty Gateway vectors pGADT7-(AD) and pGBKT7-(DBD) were used as negative controls. To evaluate the activation of the *lacZ* reporter gene, cotransformed yeasts from the SD/-Leu/-Trp medium were transferred on a nylon filter. A Whatman filter paper was moistened with buffer Z (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH 7.0), β-mercaptoethanol, and X-gal (20 mg/mL). Plates were incubated at 30 °C under dark conditions for 24 h.

3. Results and Discussion

Fleshy fruits of strawberry are rich in anthocyanins, which gives high nutritional value and antioxidant capacity to human health [1,4]. Although many aspects of the metabolism and biochemical pathways of the anthocyanin biosynthesis are well known during the development of strawberry fruits, the knowledge about the role of some molecular pathways regulating its biosynthesis is still scarce. Some phytohormones are responsible for their biosynthesis and accumulation, such as ABA [13]. However, recently, the existence of possible regulatory mechanisms of the JA-signaling pathway through the interaction of JAZ repressors with TFs related to anthocyanin biosynthesis and accumulation in *M. × domestica* [10] and *A. thaliana* [19,21] have been demonstrated. In strawberry, anthocyanin accumulation is promoted by exogenous MeJA and a putative role of its JA-signaling pathway [14,15] have been proposed. In this work, we report that the JA-signaling pathway, through ortholog sequences for JAZ1, JAZ8.1, JAZ9, and JAZ10 repressors in *F. × ananassa* (Figure S1a), targets anthocyanin biosynthesis-related TFs suggesting that JAZs might be another regulator for anthocyanin accumulation in strawberry fruit.

3.1. The JA-Signaling Pathway is Structurally and Functionally Conserved in *Fragaria × ananassa*

The activation of JA-signal transduction triggers the regulation of developmental processes and responses to multiple stresses [23,39,40]. Essentially, the JA-signaling pathway regulates other molecular pathways by protein–protein interactions mediated by JAZ repressors and MYC TFs, and it is well described in Arabidopsis [29–32] and starting to be known in strawberry [15,27,28,39]. On the one hand, *F. vesca* and *F. × ananassa* contain a conserved perception mechanism at the structural and functional

levels [27,28]. However, on the other hand, components for downstream JA-signaling pathway like MYC or JAM TFs have only been characterized at the transcriptional level in *F. × ananassa* [39].

Noteworthy, most JAZ proteins are transcriptional repressors and are characterized by the presence of TIFY and Jas domains [29,35,36,41]. Although, some JAZ sequences like JAZ7 and JAZ8 contain a variant degron sequence avoiding the recognition by COI1 coreceptor and the continuous repression of MYC TFs for a fine-tuning of JA responses in Arabidopsis [47]. In *F. vesca* 12 JAZ proteins have been identified [27,37,39], and JAZ1, JAZ8.1, and JAZ10 orthologs were also recently characterized in *F. × ananassa*, showing high conservation in their structural and functional domains [28]. In this work, we isolated and characterized JAZ9 of *F. × ananassa*, which showed highly conserved TIFY and Jas domains (Figure S2a,b), similarly to *F. vesca* and *A. thaliana* orthologs [39], indicating that JAZ proteins maintain functional domains and possibly repression functions by protein–protein interactions with TFs in *F. × ananassa*.

Regarding other JA-signaling components, we detected high conservation for interaction domains of NINJA, MYC2, MYC2-like, JAM1, and JAM2 proteins in *F. × ananassa* (Figures S3–S5), similar to that reported for *F. vesca* [39], *A. thaliana* [33], and *Gossypium hirsutum* [48] among others. On one side, the NINJA adaptor is a corepressor of the JA-signaling pathway [33]. We isolated the *F. × ananassa* NINJA protein, which is the *A. thaliana* orthologous sequence (Figure S1b) and exhibited high conservation of amino acid residues in EAR and C domains (Figure S3a,b) for corepressor interaction of JA-signaling pathway with TOPLESS (TPL) and JAZ proteins, respectively [33]. According to this, *F. × ananassa* NINJA interacted with FaJAZ1, FaJAZ9, and FaJAZ10 (Figure 1), similar to that reported in Arabidopsis [33]. In contrast, FaJAZ8.1 did not display interaction with FaNINJA (Figure 1) according to previous reports in Arabidopsis orthologs through Y2H assays [33]. On the other side, MYC TFs displayed the highest conservation level for the bHLH domain (Figure S4c), which is involved in the binding to G-box regions of the DNA [31,49], and it is according with their roles as master regulators in the promotion of JA responses and anthocyanin accumulation in Arabidopsis leaves [31]. In *F. vesca*, MYC2 and MYC2-like were reported by Garrido-Bigotes et al. [39] and correspond to FvbHLH80 and FvbHLH96 [50], respectively. In the case of the JID domain, which is involved in the interaction with Jas domain of JAZ repressors [31] a lower amino acid residue conservation was detected because this domain is lacking in *V. vinifera* MYC2-like ortholog sequence (Figure S4a). An evident interaction was detected between all FaJAZs and FaMYC2/FaMYC2-like (Figure 1), similar to that reported for MYC2 ortholog in Arabidopsis [31]. Finally, JAM1/bHLH017, JAM2/bHLH013, and JAM3/bHLH003 are negative regulators of JA-associated responses in Arabidopsis and have been reported as inhibitors for anthocyanin accumulation [34,51]. In strawberry, these proteins contained JID and bHLH domains, like MYC TFs [31], but lack the conserved transactivation domain (TAD) (Figures S5 and S6) compared to MYC TFs [31]. Protein–protein interactions were observed between FaJAM1, FaJAM2, and all FaJAZ proteins (Figure 1), similar to previously reported in *A. thaliana*. It is important to highlight that the cotransformation with the controls constituted by pGADT7-(AD)-TFs and pGBKT7-(DBD) empty vector did not exhibit the activation of *ADE2* or *HIS3* reporter genes (Figure S7). Globally, the structural conservation of key domains in FaNINJA, FaMYC2, FaMYC2-like, FaJAM1, and FaJAM2 (Figures S3–S5) as well as in FaJAZ1, FaJAZ8.1, FaJAZ9, and FaJAZ10 (Figure S2), together with the interaction observed by Y2H assays indicate that the repressor capacity of JAZ proteins would be conserved in *F. × ananassa*.

3.2. bHLH and MYB Transcription Factors Interact with of JAZ Repressors in *Fragaria × ananassa*

The biosynthesis of anthocyanins is controlled by the MBW transcriptional complex in higher plants, including *A. thaliana*, *M. × domestica*, and *F. × ananassa* [5–10,42] between others. This complex is constituted by R2R3-MYB, bHLH, and WD40 TFs [5], and in *F. × ananassa* have been characterized two major complexes formed by different TFs and regulating differentially the PAs and anthocyanin biosynthesis [6] being MYB10 component, the key TF for anthocyanin accumulation in strawberry [7]. Besides, recent findings suggest that some TFs associated with PA biosynthesis like bHLH3 may be

involved in the anthocyanin biosynthesis [8,15]. Besides, the two MYB1 and MYB5 TFs act as repressors for the positive action of the MBW complex in the anthocyanin accumulation [6]. Noteworthy, *F. × ananassa* bHLH3, bHLH33, and MYB10 TFs have their orthologs TT8, EGL3, and PAP1/MYB75, respectively [5], which are molecular targets of JAZ proteins in *A. thaliana* [22].

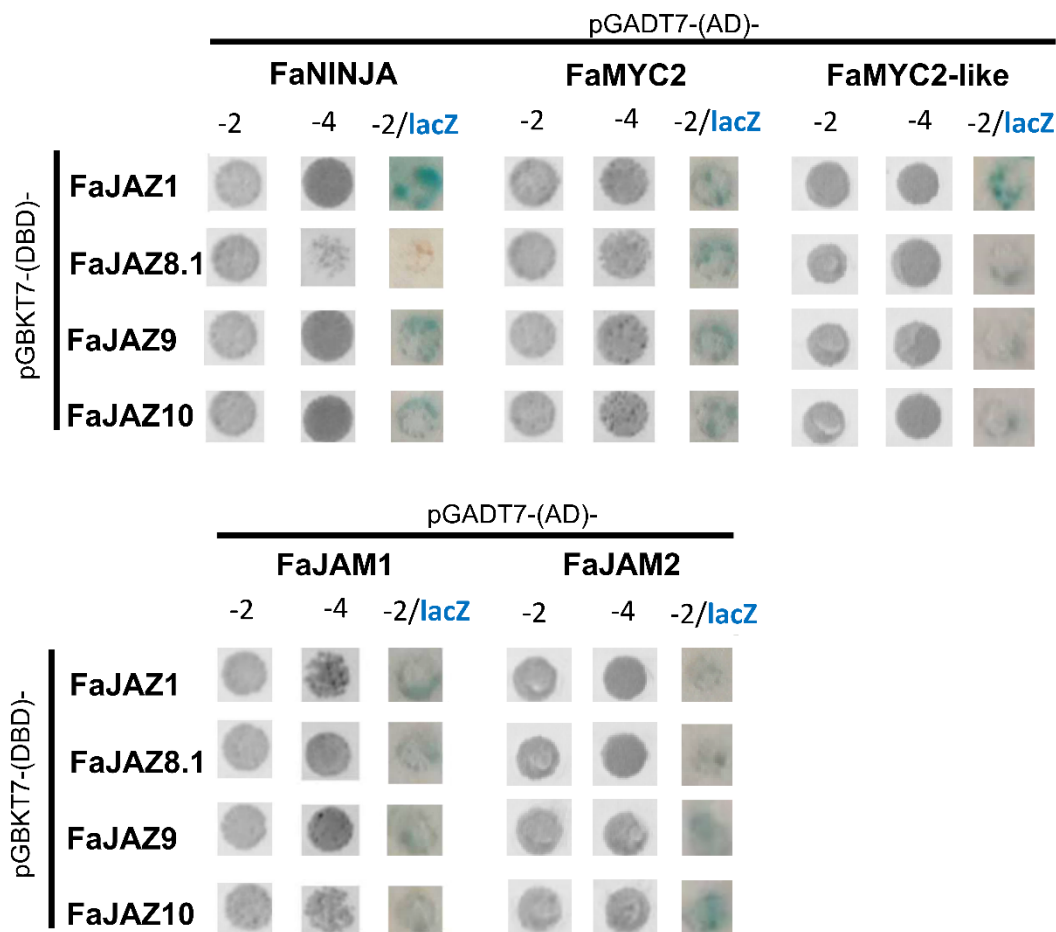


Figure 1. Protein–protein interactions of *Fragaria × ananassa* JASMONATE ZIM-DOMAIN (FaJAZs) repressors with components of the jasmonate (JA)-signaling pathway. FaJAZ1, FaJAZ8.1, FaJAZ9, and FaJAZ10 were cloned into pGBKT7-(DBD)-Gateway vector. *F. × ananassa* NOVEL INTERACTOR OF JAZ (FaNINJA), FaMYC2, FaMYC2-like, *F. × ananassa* JASMONATE ASSOCIATED MYC2-LIKE 1 (FaJAM1), and FaJAM2 were cloned into pGADT7-(AD)-Gateway vector. -2, SD-Leu-Trp; -4, SD-Leu-Trp-His-Ade; AD, activation domain; DBD, DNA binding domain; lacZ, β -galactosidase. FaJAZ1, *F. × ananassa* JAZ1; FaJAZ8.1, *F. × ananassa* JAZ8.1; FaJAZ9, *F. × ananassa* JAZ9; FaJAZ10, *F. × ananassa* JAZ10; FaNINJA, *F. × ananassa* NINJA; FaMYC2, *F. × ananassa* FaMYC2; FaMYC2-like, *F. × ananassa* FaMYC2-like; FaJAM1, *F. × ananassa* JAM1; FaJAM2, *F. × ananassa* JAM2.

In the present research, we isolated bHLH3, bHLH33, and MYB10 in *F. × ananassa* which showed high conservation of bHLH and CT domains (Figure S8a–c), and identity close to 100% (Table S4) according to previously characterized TFs in cultivated strawberry [6]. An important difference between our results compared to Schaart et al. [6], was the isolation of a larger sequence of FabHLH33 (Figure S9), although the key bHLH domain is completely conserved (Figure S8b). Early studies suggest that bHLH3/MYB10 and bHLH33/MYB10 regulate the transcriptional activation of *LAR/ANR* and *ANS/UGT* genes in *F. × ananassa* [6,8]. However, previous studies suggest that FaMYB10 in association with FabHLH3 could regulate the activation of *ANS/UGT* transcription for anthocyanin biosynthesis [15]. In the present research, FabHLH3 showed interaction with FaJAZ8.1, FaJAZ9,

and FaJAZ10 (Figure 2), and this TF is regulated by the same JAZ repressors and JAZ1 orthologs in *Arabidopsis* [22]. Otherwise, the interaction of JAZ2, JAZ3, and JAZ8 proteins with bHLH3 ortholog in *M. × domestica* was also reported [10]. Surprisingly, FabHLH33 only showed interaction with FaJAZ1 (Figure 2), while *A. thaliana* ortholog shows a clear interaction with JAZ8.1, JAZ9, and JAZ10 [22]. Possibly, the additional N-ter sequence presented in the FabHLH33 sequence isolated in this research (Figure S8) could interfere in the interaction with FaJAZ8.1, FaJAZ9, and FaJAZ10. In the case of FaMYB10, the interaction with FaJAZ1, FaJAZ8.1, and FaJAZ9 were observed (Figure 2), similar to that reported for its ortholog proteins in *A. thaliana* [22]. Finally, FaMYB1 and FaMYB5 (Figures S10 and S11) are R2R3 MYB TFs involved in the decrease of anthocyanin accumulation in strawberry fruits [52,53]. These negative TFs did not exhibit interaction with any JAZ protein in *F. × ananassa* (Figure 2), in contrast to that observed for some *A. thaliana* R2R3 related TFs like MYB21 and MYB24 that interact with JAZ1, JAZ8, and JAZ11 [54]. However, it is noteworthy, that *Arabidopsis* orthologs MYB6 (Figure S10e) and MYB5 (Figure S11d) are phylogenetically distant from MYB21, MYB24, and MYB10 transcription factors [55]. Moreover, the absence of interaction of FaMYB1 and FaMYB5 with JAZ repressors (Figure 2) may be because these proteins lack the CT interaction domain (Figures S10 and S11), which is also observed for MYB10, MYB21, and MYB24 [54]. Overall, JAZ proteins show specific and redundant roles [40] for interaction with MBW complex regulation, although other protein–protein interactions mediated by the other FaJAZ repressors described previously [39] should be evaluated.

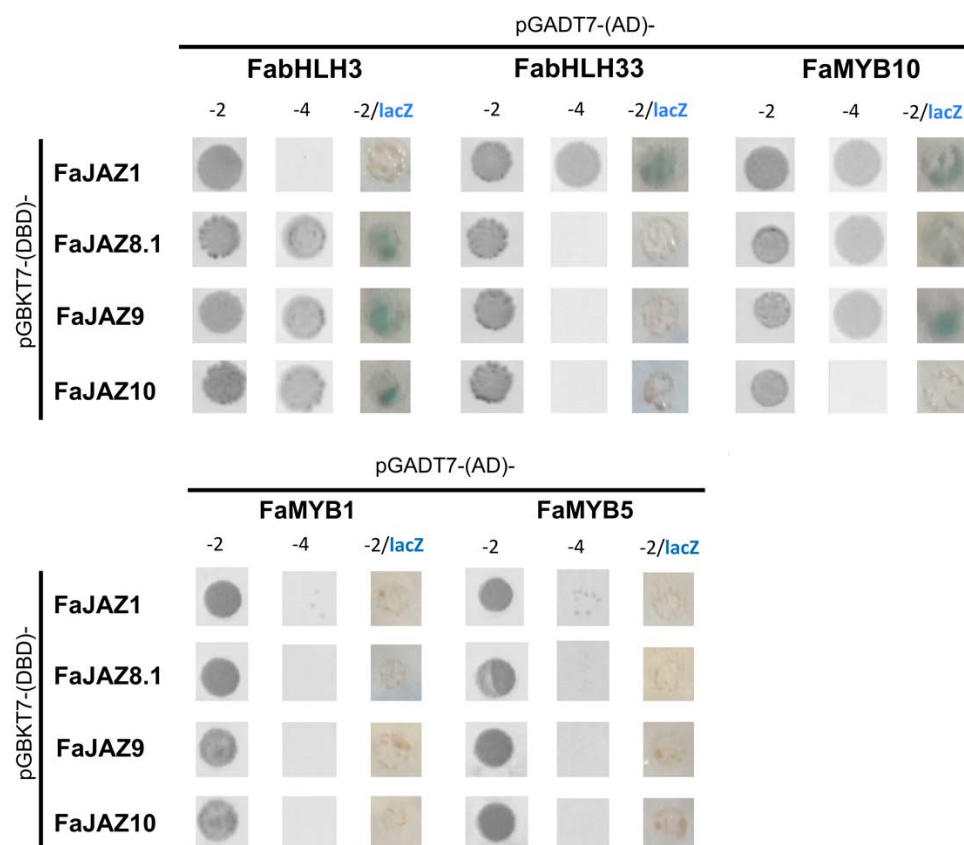


Figure 2. Protein–protein interactions of *Fragaria × ananassa* JASMONATE-ZIM DOMAIN (FaJAZs) repressors with anthocyanin-related transcription factors. FaJAZ1, FaJAZ8.1, FaJAZ9, and FaJAZ10 were cloned into pGBKT7-(DBD)-Gateway vector. *F. × ananassa* basic helix-loop-helix 3 (FabHLH3), FabHLH33, FaMYB10, FaMYB1, and FaMYB5 were cloned into the pGADT7-(AD)-Gateway vector. -2, SD-Leu-Trp; -4, SD-Leu-Trp-His-Ade; AD, activation domain; DBD, DNA binding domain; lacZ, β -galactosidase gene. FaJAZ1, *F. × ananassa* JAZ1; FaJAZ8.1, *F. × ananassa* JAZ8.1; FaJAZ9, *F. × ananassa* JAZ9; FaJAZ10, *F. × ananassa* JAZ10; FabHLH3, *F. × ananassa* bHLH3; FabHLH33, *F. × ananassa* bHLH33; FaMYB10, *F. × ananassa* MYB10; FaMYB1, *F. × ananassa* MYB1; FaMYB5, *F. × ananassa* MYB5.

3.3. The Transcription Factor YAB1 Interacts with JAZ Repressors in *Fragaria × ananassa*

Some TF-associated gene families have specific roles in development processes or stresses, but the major TFs show additional functions. For instance, the YABBY gene family is constituted by six TFs regulating the development of leaves and flowers [56,57]. However, some studies reported that the YAB1/FIL gene is related to anthocyanin biosynthesis through the control of the upregulation of the PAP1/MYB75 gene, while JAs through JAZ proteins are regulators of YAB1 in Arabidopsis [20]. Thus, YAB1 is an additional regulator of anthocyanin accumulation profile in Arabidopsis [20], and its presence and conservation in strawberry should be of great value for understanding more in-depth the regulation of anthocyanin biosynthesis.

As the YABBY gene family in strawberry is still unknown, we characterized and isolated YAB1 (Figure 3), which is the ortholog of YAB1/FIL in *A. thaliana* (Figure S1c) [20], YAB3 in *V. vinifera* [58], YAB1a and YAB1b in *S. lycopersicum* [59]. This protein family contains C2C2-zinc finger and YABBY domains [57], which displayed high conservation in *F. × ananassa* (Figure 3a,b), as well as orthologs of other fruits species like *M. × domestica*, *V. vinifera*, and *S. lycopersicum*. In detail, the conservation of amino acid residues is near to 100% with their ortholog sequences (Table S4), in both C2C2 (Figure 3a) and YABBY (Figure 3b) domains, similar to reported for its orthologs in other fruits like *V. vinifera* [58] and *S. lycopersicum* [59]. Moreover, FaYAB1 was clustered near to *F. vesca* YAB1s and *M. × domestica* YAB1a-like (Figure 3c), according to expected for plants of Rosaceae family [60]. These results indicate the structural conservation of YAB1 proteins in *F. × ananassa*.

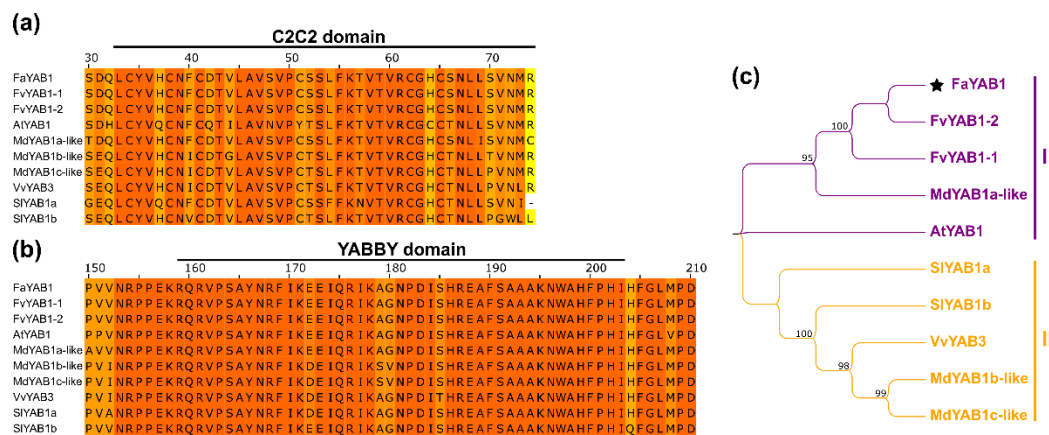


Figure 3. Characterization of *Fragaria × ananassa* YABBY1 (FaYAB1) protein and the comparison with their orthologs. (a) Multiple alignment of C2C2 domain, (b) YABBY domain, and (c) phylogenetic analysis for FaYAB1 protein and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acid residues, respectively. The unrooted phylogenetic tree was performed using full-length YAB protein sequences. FaYAB1 protein is indicated by a black star. 'I' and 'II' indicate the group number. Nodes with bootstrap values > 70% are labelled. C2C2, C2C2 zinc-finger domain. FaYAB1, *F. × ananassa* YAB1; FvYAB1-1, *F. vesca* YAB1-1; FvYAB1-2, *F. vesca* YAB1-2; AtYAB1, *A. thaliana* YAB1; MdYAB1a-like, *M. × domestica* YAB1a-like; MdYAB1b-like, *M. × domestica* YAB1b-like; MdYAB1c-like, *M. × domestica* YAB1c-like; VvYAB3, *V. vinifera* YAB3; SiYAB1a, *S. lycopersicum* YAB1a; SiYAB1b, *S. lycopersicum* YAB1b. The list of GenBank accessions is available in Table S1.

The interaction YABs-JAZs was reported in Arabidopsis between JAZ1, JAZ3, JAZ4, JAZ9 repressors, and YAB1, however, only JAZ3 shows a strong interaction [20]. We detected a weak interaction of FaJAZ1-FaYAB1 (Figure 4) similar to previously reported [20], but a stronger interaction was observed with FaJAZ9 (Figure 4). It is important to highlight that the AtJAZ3 ortholog is not present in *F. × ananassa*, however, FaJAZ9 is the closest ortholog [39], therefore might be supplying the

repressor activity of AtJAZ3 in *F. × ananassa*. Globally, these interactions suggest that *F. × ananassa* YAB1 ortholog could be regulated by JAZ9 protein.

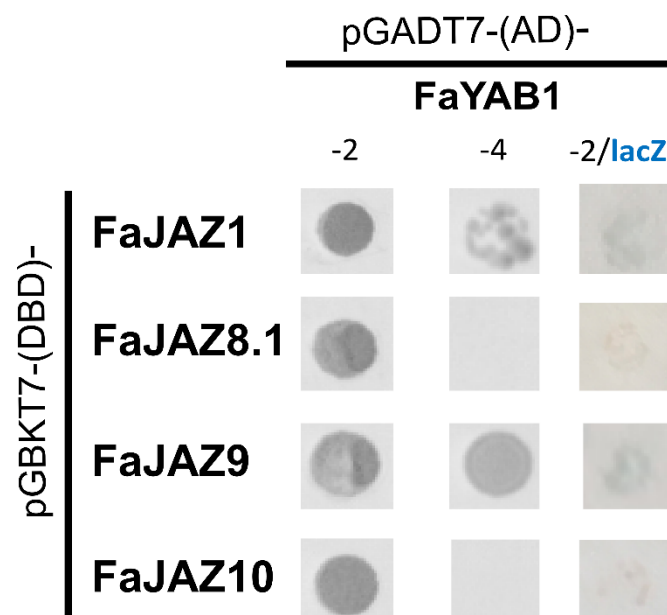


Figure 4. Protein–protein interactions of *Fragaria × ananassa* JASMONATE ZIM-DOMAIN (FaJAZs) repressors with the *F. × ananassa* YABBY1 (FaYAB1) transcription factor. FaJAZ1, FaJAZ8.1, FaJAZ9, and FaJAZ10 were cloned into pGBKT7-(DBD)-Gateway vector. FaYAB1 was cloned into the pGADT7-(AD)-Gateway vector. -2, SD-Leu-Trp; -4, SD-Leu-Trp-His-Ade; AD, activation domain; DBD, DNA binding domain; lacZ, β -galactosidase gene. FaJAZ1, *F. × ananassa* JAZ1; FaJAZ8.1, *F. × ananassa* JAZ8.1; FaJAZ9, *F. × ananassa* JAZ9; FaJAZ10, *F. × ananassa* JAZ10; FaYAB1, *F. × ananassa* YAB1.

4. Conclusions

In summary, interactions of JAZ proteins with components of the JA-signaling pathway and TFs related to anthocyanin biosynthesis were presented. *F. × ananassa* JAZ repressors are conserved at the structural level, including JAZ1, JAZ8.1, JAZ9, and JAZ10. Additionally, these proteins interact with TFs like MYC2, MYC2-like, JAM1, and JAM2, together with the NINJA adaptor protein in the same manner to that observed for *A. thaliana* orthologs, indicating that the function of the JA-signaling pathway would be conserved in *F. × ananassa*. JAZ proteins target TFs involved in the anthocyanin biosynthesis and showed functional redundancy, indicating anthocyanin biosynthesis could be fine-tuning by the JA-signaling pathway. Otherwise, MYB1 and MYB5 do not interact with JAZ1, JAZ8.1, JAZ9, or JAZ10, showing that JAZ repressors would not regulate these negative regulators of the anthocyanin accumulation. Finally, YAB1 protein was characterized for the first time in strawberry, which is a molecular target of FaJAZ9, suggesting that the anthocyanin biosynthesis could also be regulated by interactions between JAZs and YAB1 in *F. × ananassa*. Furthermore, these results provide additional information about JAZ-molecular targets related to anthocyanin biosynthesis in strawberry fruits. However, further studies will be necessary to know the putative *in vivo* regulation of the anthocyanin accumulation by JAZ repressors during the ripening of strawberry fruit.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/10/1586/s1>; Table S1. GenBank accessions of protein sequences used in this research. bHLH, basic helix-loop-helix; EGL3, ENHANCER OF GLABRA 3; JAZ, JASMONATE ZIM-DOMAIN; NINJA, NOVEL INTERACTOR OF JAZ; JAM, JASMONATE ASSOCIATED MYC2-LIKE; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT 1; TT8, TRANSPARENT TESTA 8; Fa, *Fragaria × ananassa*; Fv, *Fragaria vesca*; At, *Arabidopsis thaliana*; Md, *Malus × domestica*; Vv, *Vitis vinifera*; Sl, *Solanum lycopersicum*. Table S2. Primer sequences used for isolation and cloning of full-length coding sequences of *Fragaria × ananassa* used in this research. Orange and blue colors indicate attB1 and attB2 sites, respectively, and black letters indicate specific regions for hybridization

with coding sequence. attB1/attB2, sites for BP recombination into the pDONR207-Gateway vector. CDS, coding sequence; bHLH, basic helix-loop-helix; Fa, *F. × ananassa*; JAM, JASMONATE ASSOCIATED MYC2-LIKE; JAZ, JASMONATE ZIM-DOMAIN; NINJA, NOVEL INTERACTOR OF JAZ. Table S3. Identity (%) between JASMONATE ZIM-DOMAIN (JAZs), NOVEL INTERACTOR OF JAZ (NINJA), MYC2s, and JASMONATE ASSOCIATED MYC2-LIKE (JAMs) protein sequences of *Fragaria × ananassa* with their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Fa, *F. × ananassa*; Fv, *F. vesca*; At, *A. thaliana*; Md, *M. × domestica*; Vv, *V. vinifera*; Sl, *S. lycopersicum*. Table S4. Identity (%) between basic helix-loop-helix 3 (bHLH3), bHLH33, MYB10, MYB1, MYB5, and YABBY1 (YAB1) protein sequences of *Fragaria × ananassa* with their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. EGL3; ENHANCER OF GLABRA 3; Fa, *F. × ananassa*; Fv, *F. vesca*; At, *A. thaliana*; Md, *M. × domestica*; Vv, *V. vinifera*; Sl, *S. lycopersicum*. Figure S1. Phylogenetic analyses for JASMONATE ZIM-DOMAIN (JAZ), NOVEL INTERACTOR OF JAZ (NINJA), and YABBY (YAB) proteins of *Fragaria × ananassa* with their orthologous proteins of *Arabidopsis thaliana*. (a) Unrooted phylogenetic tree for FaJAZs, (b) FaNINJA, and (c) FaYAB1. Full-length protein sequences were used for the construction of the unrooted phylogenetic tree. FaJAZ, FaNINJA, and FaYAB1 proteins are indicated by a black star. 'I', 'II', and 'III' indicate the group number. Nodes with bootstrap values > 70% are labelled. AtJAZ1, *A. thaliana* JAZ1; AtJAZ2, *A. thaliana* JAZ2; AtJAZ3, *A. thaliana* JAZ3; AtJAZ4, *A. thaliana* JAZ4; AtJAZ5, *A. thaliana* JAZ5; AtJAZ6, *A. thaliana* JAZ6; AtJAZ7, *A. thaliana* JAZ7; AtJAZ8, *A. thaliana* JAZ8; AtJAZ9, *A. thaliana* JAZ9; AtJAZ10, *A. thaliana* JAZ10; AtJAZ11, *A. thaliana* JAZ11; AtJAZ12, *A. thaliana* JAZ12; AtJAZ13, *A. thaliana* JAZ13; FaJAZ1, *F. × ananassa* JAZ1; FaJAZ8.1, *F. × ananassa* JAZ8.1; FaJAZ9, *F. × ananassa* JAZ9; FaJAZ10, *F. × ananassa* JAZ10; AtAFP1, *A. thaliana* ABI-Five binding Protein 1; AtAFP2, *A. thaliana* ABI-Five binding Protein 2; AtAFP3, *A. thaliana* ABI-Five binding Protein 3; AtAFP4, *A. thaliana* ABI-Five binding Protein 4; AtNINJA, *A. thaliana* NINJA; FaNINJA, *F. × ananassa* NINJA; AtCRC, *A. thaliana* CRC; AtINO, *A. thaliana* INO; AtYAB1, *A. thaliana* YAB1; AtYAB2, *A. thaliana* YAB2; AtYAB3, *A. thaliana* YAB3; AtYAB5, *A. thaliana* YAB5; FaYAB1, *F. × ananassa* YAB1. Figure S2. Characterization of *Fragaria × ananassa* JASMONATE ZIM-DOMAIN 1 (FaJAZ1), FaJAZ8.1, FaJAZ9, and FaJAZ10 proteins and the comparison with their orthologs. (a) Multiple alignment of TIFY domain, (b) Jas domain, and (c) phylogenetic analysis for FaJAZ proteins and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acid residues, respectively. The unrooted phylogenetic tree was performed using full-length JAZ protein sequences. FaJAZ proteins are indicated by a black star. 'I', 'II', and 'III' indicate the group number. Nodes with bootstrap values > 70% are labelled. NLS, Nuclear Location Signal. FaJAZ1, *F. × ananassa* JAZ1; FaJAZ8.1, *F. × ananassa* JAZ8.1; FaJAZ9, *F. × ananassa* JAZ9; FaJAZ10, *F. × ananassa* JAZ10; FvJAZ1, *F. vesca* JAZ1; FvJAZ8.1, *F. vesca* JAZ8.1; FvJAZ9, *F. vesca* JAZ9; FvJAZ10, *F. vesca* JAZ10; AtJAZ1, *A. thaliana* JAZ1; AtJAZ8, *A. thaliana* JAZ8; AtJAZ9, *A. thaliana* JAZ9; AtJAZ10, *A. thaliana* JAZ10; MdJAZ1, *M. × domestica* JAZ1; MdJAZ3, *M. × domestica* JAZ3; MdJAZ4, *M. × domestica* JAZ4; MdJAZ13, *M. × domestica* JAZ13; MdJAZ14, *M. × domestica* JAZ14; MdJAZ17, *M. × domestica* JAZ17; VvJAZ2, *V. vinifera* JAZ2; VvJAZ3, *V. vinifera* JAZ3; VvJAZ9, *V. vinifera* JAZ9; VvJAZ11, *V. vinifera* JAZ11; SlJAZ1, *S. lycopersicum* JAZ1; SlJAZ5, *S. lycopersicum* JAZ5; SlJAZ10, *S. lycopersicum* JAZ10; SlJAZ11, *S. lycopersicum* JAZ11. The list of GenBank accessions is available in Table S1. Figure S3. Characterization of *Fragaria × ananassa* NOVEL INTERACTOR OF JAZ (FaNINJA) protein and the comparison with their orthologs. (a) Multiple alignment of ethylene-responsive element binding factor-associated amphiphilic repression (EAR) domain, (b) C domain, and (c) phylogenetic analysis for FaNINJA protein and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acid residues, respectively. The unrooted phylogenetic tree was performed using full-length NINJA protein sequences. FaNINJA protein is indicated by a black star. 'I' and 'II' indicate the grouping number. Nodes with bootstrap values > 70% are labelled. FaNINJA, *F. × ananassa* NINJA; FvNINJA, *F. vesca* NINJA; AtNINJA, *A. thaliana* NINJA; MdNINJA, *M. × domestica* NINJA; VvNINJA, *V. vinifera* NINJA; SlNINJA, *S. lycopersicum* NINJA. The list of GenBank accessions is available in Table S1. Figure S4. Characterization of *Fragaria × ananassa* MYC2 (FaMYC2) and FaMYC2-like proteins and the comparison with their orthologs. (a) Multiple alignment of JAZ-interaction domain (JID domain), (b) transactivation domain (TAD domain), (c) basic helix-loop-helix (bHLH) domain, and (d) phylogenetic analysis for FaMYC2 and FaMYC2-like proteins and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acid residues, respectively. The unrooted phylogenetic tree was performed using full-length MYC2, MYC2-like, MYC3, MYC4, and MYC5 protein sequences. FaMYC2 and FaMYC2-like proteins are indicated by a black star. 'I' and 'II' indicate the group number. Nodes with bootstrap values > 70% are labelled. JAZ, JASMONATE ZIM-DOMAIN. FaMYC2, *F. × ananassa* MYC2; FaMYC2-like, *F. × ananassa* MYC2-like; FvMYC2, *F. vesca* MYC2; FvMYC2-like, *F. vesca* MYC2-like; AtMYC2, *A. thaliana* MYC2; AtMYC3, *A. thaliana* MYC3; AtMYC4, *A. thaliana* MYC4; AtMYC5, *A. thaliana* MYC5; MdMYC2a, *M. × domestica* MYC2a; MdMYC2b, *M. × domestica* MYC2b; MdMYC2-like1, *M. × domestica* MYC2-like1; MdMYC2-like2, *M. × domestica* MYC2-like2; MdMYC2-like3, *M. × domestica* MYC2-like3; VvMYC2, *V. vinifera* MYC2; VvMYC2-like, *V. vinifera* MYC2-like; SlMYC2, *S. lycopersicum* MYC2. The list of GenBank accessions is available in Table S1. Figure S5. Characterization of *Fragaria × ananassa* JASMONATE-ASSOCIATED MYC2-LIKE1 (FaJAM1) and FaJAM2 transcription repressors and the comparison with their orthologs. (a) Multiple alignment of JAZ-interaction domain (JID domain), (b) basic helix-loop-helix (bHLH domain), and (c) phylogenetic analysis for FaJAM1 and FaJAM2 proteins and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acid residues, respectively. Black squares in (a) indicate residues involved in the interaction with JAZ proteins

according to Goossens et al. 2015 (New Phytologist 206, 1229). The unrooted phylogenetic tree was performed using full-length JAM protein sequences. FaJAM1 and FaJAM2 proteins are indicated by a black star. 'I' and 'II' indicate the group number. Nodes with bootstrap values > 70% are labelled. JAZ, JASMONATE ZIM-DOMAIN. FaJAM1/bHLH013, *F. × ananassa* JAM1/bHLH013; FaJAM2/bHLH003, *F. × ananassa* JAM2/bHLH003; FvJAM1/bHLH013, *F. vesca* JAM1/bHLH013; FvJAM2/bHLH003, *F. vesca* JAM2/bHLH003; AtJAM1/bHLH017, *A. thaliana* JAM1/bHLH017; AtJAM2/bHLH013, *A. thaliana* JAM2/bHLH013; AtJAM3/bHLH003, *A. thaliana* JAM2/bHLH003; MdbHLH003-like1, *M. × domestica* bHLH003-like1; MdbHLH003-like2, *M. × domestica* bHLH003-like2; MdbHLH013, *M. × domestica* bHLH013; MdbHLH013-like, *M. × domestica* bHLH013-like; VvbHLH003, *V. vinifera* bHLH003; SlbHLH003, *S. lycopersicum* bHLH003; SlbHLH003-like, *S. lycopersicum* bHLH003-like; SlbHLH013-like, *S. lycopersicum* bHLH013-like. The list of GenBank accessions is available in Table S1. Figure S6. Protein region of *Fragaria × ananassa* JASMONATE-ASSOCIATED MYC2-LIKE1 (FaJAM1) and FaJAM2 corresponding to transactivation domain (TAD domain) contained in MYC activators. FaJAM1/bHLH013, *F. × ananassa* JAM1/bHLH013; FaJAM2/bHLH003, *F. × ananassa* JAM2/bHLH003; FvJAM1/bHLH013, *F. vesca* JAM1/bHLH013; FvJAM2/bHLH003, *F. vesca* JAM2/bHLH003; AtJAM1/bHLH017, *A. thaliana* JAM1/bHLH017; AtJAM2/bHLH013, *A. thaliana* JAM2/bHLH013; AtJAM3/bHLH003, *A. thaliana* JAM2/bHLH003; MdbHLH003-like1, *M. × domestica* bHLH003-like1; MdbHLH003-like2, *M. × domestica* bHLH003-like2; MdbHLH013, *M. × domestica* bHLH013; MdbHLH013-like, *M. × domestica* bHLH013-like; VvbHLH003, *V. vinifera* bHLH003; SlbHLH003, *S. lycopersicum* bHLH003; SlbHLH003-like, *S. lycopersicum* bHLH003-like; SlbHLH013-like, *S. lycopersicum* bHLH013-like. List of GenBank accessions is available in Table S1. Figure S7. Interaction controls of yeast two hybrid (Y2H) assays. (a) Interaction control of *Fragaria × ananassa* JASMONATE ZIM-DOMAIN 1 (FaJAZ1), FaJAZ8.1, FaJAZ9, and FaJAZ10 cloned into pGBKT7-(DBD)-Gateway vector and pGADT7-(AD) empty Gateway vector. (b) *F. × ananassa* NOVEL INTERACTOR OF JAZ (FaNINJA), FaMYC2, FaMYC2-like, JASMONATE ASSOCIATED MYC2-LIKE 1 (FaJAM1), and FaJAM2 cloned into pGADT7-(AD)-Gateway vector and pGBKT7-(DBD) empty Gateway vector. (c) *F. × ananassa* basic helix-loop-helix 3 (FabHLH3), FabHLH33, FaMYB10, FaMYB1, FaMYB5, and *F. × ananassa* YABBY1 (FaYAB1) cloned into pGADT7-(AD)-Gateway vector and pGBKT7-(DBD) empty Gateway vector. FaJAZ1, *F. × ananassa* JAZ1; FaJAZ8.1, *F. × ananassa* JAZ8.1; FaJAZ9, *F. × ananassa* JAZ9; FaJAZ10, *F. × ananassa* JAZ10; FaNINJA, *F. × ananassa* NINJA; FaMYC2, *F. × ananassa* MYC2; FaMYC2-like, *F. × ananassa* MYC2-like; FaJAM1, *F. × ananassa* JAM1; FaJAM2, *F. × ananassa* JAM2; FabHLH3, *F. × ananassa* bHLH3; FabHLH33, *F. × ananassa* bHLH33; FaMYB10, *F. × ananassa* MYB10; FaMYB1, *F. × ananassa* MYB1; FaMYB5, *F. × ananassa* MYB5; FaTTG1, *F. × ananassa* TTG1; FaYAB1, *F. × ananassa* YABBY1. Figure S8. Characterization of *Fragaria × ananassa* basic helix-loop-helix 3 (FabHLH3), FabHLH33, and FaMYB10 proteins and the comparison with their orthologs. (a,b) Multiple alignment of bHLH domain, (c) CT domain, and (d–f) phylogenetic analysis for FabHLH3, FabHLH33, and FaMYB10 proteins, respectively, and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acidic residues, respectively. The unrooted phylogenetic tree was performed using full-length bHLH3, bHLH33, and MYB10 orthologs sequences. FabHLH3, FabHLH33, and FaMYB10 proteins are indicated by a black star. 'I' and 'II' indicate the group number. Nodes with bootstrap values > 70% are labelled. EGL3, ENHANCER OF GLABRA 3; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT 1; TT8, TRANSPARENT TESTA 8. FabHLH3, *F. × ananassa* bHLH3; FvbHLH3, *F. vesca* bHLH3; AtTT8, *A. thaliana* TT8; MdbHLH3, *M. × domestica* bHLH3; VvbHLH3, *V. vinifera* bHLH3; SlbHLH3, *S. lycopersicum* bHLH3; FabHLH33, *F. × ananassa* bHLH33; FvbHLH33, *F. vesca* bHLH33; AtEGL3, *A. thaliana* EGL3; MdbHLH33, *M. × domestica* bHLH33; VvbHLH33, *V. vinifera* bHLH33; SlbHLH33, *S. lycopersicum* bHLH33; FaMYB10, *F. × ananassa* MYB10; FvMYB10, *F. vesca* MYB10; AtPAP1/MYB75, *A. thaliana* PAP1/MYB75; MdMYB10, *M. × domestica* MYB10; VvMYB10, *V. vinifera* MYB10; SIMYB10, *S. lycopersicum* MYB10. The list of GenBank accessions is available in Table S2. Figure S9. Pairwise alignment between two *Fragaria × ananassa* basic helix-loop-helix 33 (FabHLH33) transcription factors. The asterisk indicates FabHLH33 previously reported by Schaart et al. (2013). The list of GenBank accessions is available in Table S2. Figure S10. Characterization of *Fragaria × ananassa* MYB1 (FaMYB1) protein and the comparison with their orthologs. (a,b) Multiple alignment of R2 and R3 domains, (c,d) C1 and ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs, and (e) phylogenetic analysis for FaMYB1 protein and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acidic residues, respectively. The unrooted phylogenetic tree was performed using full-length MYB1 ortholog sequences. FaMYB1 protein is indicated by a black star. 'I', 'II', and 'III' indicate the group number. Nodes with bootstrap values > 70% are labelled. FaMYB1, *F. × ananassa* MYB1; FvMYB3-like, *F. vesca* MYB3-like; AtMYB6, *A. thaliana* MYB6; MdMYB6, *M. × domestica* MYB6; VvMYB4a, *V. vinifera* MYB4a; SIMYB1-like, *S. lycopersicum* MYB1-like. The list of GenBank accessions is available in Table S2. Figure S11. Characterization of *Fragaria × ananassa* MYB5 (FaMYB5) protein and the comparison with their orthologs. (a,b) Multiple alignment of R2R3 domains and C1 motif, (c) C3 motif, and (d) phylogenetic analysis for FaMYB5 protein and their orthologs in *Fragaria vesca*, *Arabidopsis thaliana*, *Malus × domestica*, *Vitis vinifera*, and *Solanum lycopersicum*. Orange and yellow colors in the multiple alignment indicate higher and lower conservation of amino acidic residues, respectively. The unrooted phylogenetic tree was performed using full-length MYB5 ortholog sequences. FaMYB5 protein is indicated by a black star. 'I' and 'II' indicate the group number. Nodes with bootstrap values > 70% are labelled. FaMYB5, *F. × ananassa* MYB5; FvMYB5, *F. vesca* MYB5; AtMYB5, *A. thaliana* MYB5; MdMYB5, *M. × domestica* MYB5; MdMYB5-like, *M. × domestica* MYB5-like; VvMYB5b, *V. vinifera* MYB5b; SIMYB5-like, *S. lycopersicum* MYB5-like. The list of GenBank accessions is available in Table S2.

Author Contributions: Conceptualization: C.R.F. Methodology: A.G.-B., M.T., R.S., and C.R.F. Validation: A.G.-B., M.T., R.S., and C.R.F. Formal analysis: A.G.-B., M.T., R.S., and C.R.F. Investigation: A.G.-B., M.T., R.S., and C.R.F. Resources: A.G.-B., M.T., R.S., and C.R.F. Data curation: A.G.-B. Writing—original draft preparation: A.G.-B. and C.R.F. Writing—review and editing: A.G.-B., M.T., R.S., and C.R.F. Visualization: A.G.-B. and C.R.F. Supervision: C.R.F. Project administration: C.R.F. Funding acquisition: C.R.F. All authors have read and agreed to the published version of the manuscript.

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