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Structural characterization of the jasmonoyl-isoleucine perception complexes from *Fragaria vesca* by in silico molecular analysis

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Abstract

Background. The phytohormone jasmonates (JAs) regulate fundamental plant processes; such as the anthocyanin accumulation during ripening of strawberry, a non-climacteric fruit model. Jasmonoyl-isoleucine (JA-Ile), one of the bioactive JA molecules, mediates binding of the JAZ repressor protein to COI1, an F-box protein forming the SCF^{COI1} ubiquitin E3 ligase complex, in Arabidopsis. The COI1-JA-Ile-JAZ complex initiates the JA-signaling pathway leading to early jasmonate responses. Most of Arabidopsis JAZs contain a degron sequence at the Jas domain responsible for interaction with COI1 and JA-Ile. The woodland strawberry (*Fragaria vesca*) is a model plant for the Rosaceae family, in which the JA-signaling pathway is poorly understood at the molecular level. The aim of this work was to understand the molecular basis of the interaction between the *F. vesca* COI1 (FvCOI1) and JAZ1 (FvJAZ1) or JAZ8 (FvJAZ8) mediated by JA-Ile.

Methods. Multiple alignments of amino acid sequences and phylogenetic analyses were performed for FvCOI1 and FvJAZ1/8 and their ortholog sequences. The FvCOI1 and FvJAZ1/8 3D structures were built by homology modeling methods, which were further refined and validated by molecular dynamics simulation (MDS). A molecular docking approach along with MDS analysis were used to understand the interaction capacity between a putative degron-like present in FvJAZ1 and FvJAZ8 with the FvCOI1-JA-Ile and FvCOI1-JA complexes.

Results. FvCOI1 and FvJAZ1/8 showed high and moderate identity, respectively, with the corresponding ortholog proteins from other plant species including apple, grape, tomato and Arabidopsis. The resulting FvCOI1 structural model showed that the F-box and LRR domains were highly similar to that described in Arabidopsis COI1 (AtCOI1) crystal structure. Unexpectedly, we found that FvJAZ1 has a variant IPMQRK sequence respect to the canonical LPIAR(R/K) degron sequence observed in AtJAZ1. The MDS results showed that the FvCOI1-JA-Ile-FvJAZ1 complex was the most stable among all the analyzed ones, and the IPMQRK peptide of FvJAZ1 interacted directly with FvCOI1 and JA-Ile. In contrast, FvJAZ8 did not show a direct interaction with those two components, as expected from previous experimental results for the ortholog AtJAZ8.

Discussion. The present research provides novel insight into the molecular interactions between key JA-signaling components in the model plant *F. vesca*. Remarkably, we characterized the

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IPMQRK sequence present in FvJAZ1, a putative variant of the canonical degron previously described in AtJAZ1. We propose that the FvCOI1-JA-Ile-FvJAZ1 complex is stable, and that the degron-like sequence present in FvJAZ1 interacts in a steady manner with FvCOI1-JA-Ile. Up to now, this is the first structural characterization of molecular interactions that may be occurring between the core components of the JA-Ile perception complex in a fleshy fruit-related species.

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Introduction

Jasmonates (JAs) are phytohormones that regulate environmental adaptation and development in plants (Chini et al., 2016). Recently, it has been shown that JAs regulate early development and anthocyanin accumulation in grape and strawberry fruits, respectively (Concha et al., 2013; Böttcher et al., 2015) pointing out a role in development and ripening of non-climacteric fruits. Physiological effects of JAs are mediated by the bioactive molecule, (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) in Arabidopsis (Fonseca et al., 2009), which is structurally and functionally analogous to the bacterial phytotoxin coronatine (COR, Katsir et al., 2008). However, recently others bioactive (+)-7-*iso*-amino acid conjugates have been reported in Arabidopsis and other plants (Yan et al., 2016).

JA-Ile performs its physiological effects by activating the JA-signaling pathway, and this is now beginning to be well understood in Arabidopsis (Pérez & Goossens, 2013). The F-box CORONATINE INSENTIVE 1 (COI1) protein is part of the SKP1/CUL1/F-box (SCF^{COI1}) ubiquitin E3 ligase complex (Xu et al., 2002). The *coi1* null mutant has impaired JA responses in Arabidopsis (Xie et al., 1998). COI1 binds to JASMONATE ZIM-DOMAIN (JAZ) repressor protein when JA-Ile accumulates (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) to conform the COI1-JAZ co-receptor (Sheard et al., 2010). Then, the JAZ protein is degraded by the 26S proteasome after ubiquitination (Chini et al., 2007; Thines et al., 2007) and now, MYC transcription factors (Lorenzo et al., 2004; Fernández-Calvo et al., 2011; Niu, Figueroa & Browse, 2011; Figueroa & Browse, 2015) induce the expression of early-JA response genes (Chini et al., 2007). In the absence of JA-Ile, JAZ binds to MYCs repressing the expression of early JA response genes (Chini et al., 2007; Thines et al., 2007; Chung et al., 2008). These core components establish the initiation of the JA signaling pathway to regulate JA responses in terrestrial plants (Chini et al., 2016).

Arabidopsis COI1 (AtCOI1) amino acid sequence contains leucine-rich repeats (LRRs) and F-box domains, similar to the auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1) (Tan et al., 2007; Yan et al., 2009). JAZ belongs to the TIFY family with 12 members in Arabidopsis (Chini et al., 2007). These proteins contain the TIFY domain for binding themselves

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or to other JAZ and Novel Interactor of JAZ (NINJA) adaptor protein (Vanholme et al., 2007; Pauwels et al., 2010). Moreover, Arabidopsis JAZs (AtJAZs) contains a Jas domain (a CCT domain) that interacts with COI1 and transcription factors for its degradation and repression of early-JA responses, respectively (Chini et al., 2007, Katsir et al., 2008, Melotto et al., 2008). Most of AtJAZs contain the canonical LPIAR(R/K) degron sequence at the Jas domain that is responsible for interaction with COI1 and JA-Ile (Sheard et al., 2010). However, AtJAZ8 lacks this canonical degron sequence and its transcriptional repression activity is mediated by a N-terminal EAR motif allowing recruitment of TOPLESS co-repressors to repress the JA-signaling pathway by a NINJA-independent molecular mechanism in Arabidopsis (Shyu et al., 2012).

AtCOI1 interacts with JAZ in the presence of JA-Ile and its mimic COR molecule (Katsir et al., 2008). Its 3D structure was reported firstly using a molecular modeling approach (Yan et al., 2009) and then by a crystallographic study (Sheard et al., 2010). AtCOI1 consists of a packed structure composed by α-helix and β-sheets forming LRR domains, which are required for protein stability (Yan et al., 2009; Sheard et al., 2010). On the other hand, AtCOI1 contains a surface pocket with amino acid residues necessary for binding to JAZ in the presence of JA-Ile (Yan et al., 2009; Sheard et al., 2010). Recently, Sen et al. (2016) proposed the COI1 structural models for five monocot species (rice, wheat, maize, *Sorghum* and *Setaria*), and evaluated their interaction with JA-Ile and the JAZ1 degron sequence from the herbaceous plant finger millet. The authors showed that the five models were highly similar at structural level, indicating a highly conserved structure for COI1 in monocots (Sen et al., 2016). Experimental and in silico studies showed that the most likely interaction model among the COI1, JAZ and JA-Ile molecules consists in COI1 binding first to JA-Ile and then both to JAZ (Yan et al., 2009). However, a protein crystallographic study and additional experimental results reveal a different interaction model, in which COI1-JAZ acts as a co-receptor complex for perception of JA-Ile in Arabidopsis (Sheard et al., 2010). In this interaction model, COI1 LRR domains form a TIR1-like structure with a surface pocket for JA-Ile binding (Yan et al., 2009; Sheard et al., 2010). On the other hand, JAZ1 canonical LPIAR(R/K) degron sequence acts like a clamp closing the binding pocket to wraps JA-Ile (Sheard et al., 2010). Moreover, inositol pentakisphospate (InsP5) is a COI1 cofactor that increases the perception sensitivity to JA-Ile in Arabidopsis (Sheard et al., 2010).

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Until now, the molecular mechanisms underlying the JA-Ile perception and early responses are not well known in any other plant except Arabidopsis and at a lower extend in a heterologous co-receptor formed by finger millet JAZ1 and COI1 from several monocots. The aim of this research is to characterize the interaction between the *Fragaria vesca* COI1 (FvCOI1) and JAZ1/8 (FvJAZ1/8) mediated by JA-Ile to form the hormone-bound co-receptors to reveal the molecular mechanism involved in JA-Ile perception in a model organism for studies in *Fragaria* genus and climacteric fruits.

Materials and methods

Sequences analysis

The full-length inferred amino acid sequences of FvCOI1, FvJAZ1, and FvJAZ8 were obtained from *F. vesca* genome database version 2.0 (www.rosaceae.org; January 23, 2017) using the Arabidopsis AtCOI1, AtJAZ1 and AtJAZ8 sequences as queries. For FvCOI1, a single sequence (accession code: XP_004307613) with a high identity (69.8%) relative to AtCOI1 was found. In the case of FvJAZ1 and FvJAZ8, we selected the sequences that showed the highest identity respect to Arabidopsis orthologs. The amino acid sequences of FvJAZ1 (accession code: XP_004287655) and FvJAZ8 (accession code: XP_004293626) that exhibited 38.7% and 46.2% of sequence identity relative to AtJAZ1 and AtJAZ8, respectively, were selected for further studies (Table S1). Thus, in the present research, the XP_004307613, XP_004287655, XP_004293626 sequences were named as FvCOI1, FvJAZ1 and FvJAZ8, respectively. A search on the RCSB Protein Data Bank (January 3, 2017) confirmed that X-ray crystal structure for FvCOI1, FvJAZ1 and FvJAZ8 proteins were not publically available. A multiple alignment of amino acid sequences was performed using Clustal W and Bioedit Sequence Alignment Editor v7.0 software (Hall, 1999). Phylogenetic analyses were conducted using MEGA v7.0 software (Kumar, Stecher & Tamura, 2016), using the Neighbor-Joining methodology and a bootstrap analysis of 1000 replicates. The following GenBank accession numbers corresponding to the full-length amino acid sequences were used: FvCOI1 (*F. vesca* COI1, XP_004307613), FvJAZ1 (*F. vesca* JAZ1, XP_004287655), FvJAZ8 (*F. vesca* JAZ8, XP_004293626), AtCOI1

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(*Arabidopsis thaliana* COI1, NP_565919), AtJAZ1 (*A. thaliana* JAZ1, NP_564075), AtJAZ8 (*A. thaliana* JAZ8, NP_564349), VvCOI1 (*Vitis vinifera* COI1, AFF57759), VvJAZ9 (*V. vinifera* JAZ9, XP_002277157), VvJAZ3 (*V. vinifera* JAZ3, XP_003634826), SlCOI1 (*Solanum lycopersicum* COI1, NP_001234464), SlJAZ1 (*S. lycopersicum* JAZ1, XP_004243696), SlJAZ8 (*S. lycopersicum* JAZ8, XP_004244919), MdCOI1 (*Malus ×domestica* COI1, XP_008392915), MdJAZ1 (*M. ×domestica* JAZ1, XP_008388962), MdJAZ3 (*M. ×domestica* JAZ3, XP_008371611) and MdJAZ4 (*M. ×domestica* JAZ4, XP_008371611).

Building the protein structure using comparative modeling

The protein model for FvCOI1, FvJAZ1, and FvJAZ8 were built according to the method described by Morales-Quintana et al. (2011), using MODELLER 9v17 software (http://salilab.org/modeller/) by a comparative modeling methodology. The crystal structure (PDB code 3OGK) for Arabidopsis complex, which corresponds to COI1 protein co-crystalized with JAZ1 degron was selected as template for further studies. A SPC pre-equilibrated water model was used for each protein model, and then the system was neutralized by adding NaCl. After that, the system was equilibrated by molecular dynamics simulations (MDS) during 10 ns of using SCHRÖDINGER suite with OPLS v2005 force field (Jorgensen, Maxwell & Tirado-Rives, 1996). The protein protonation state was set to pH 7.2 since this value was reported in plant cell nucleus (Shen et al., 2013). To evaluate the model both PROCHECK (Laskowski et al., 1993) and ProSA2003 (Sippl, 1993) programs were employed.

Protein-ligand interactions

At first, a molecular docking method was used to predict the putative binding interaction modes. For this purpose, two different docking analyses were carried out. The first one used JA-Ile or jasmonic acid (JA) molecules (as a negative control) for binding to FvCOI1 using the AutoDock vina program (Trott & Olson, 2010); the last docking run was performed with FvJAZ1 or FvJAZ8 bound to FvCOI1-JA and FvCOI1-JA-Ile complexes separately using HADDOCK (Dominguez, Boelens & Bonvin, 2003; de Vries et al., 2007). The assembly order for the FvCOI1-JA-Ile-FvJAZ1, FvCOI1-JA-Ile-FvJAZ8, FvCOI1-JA-FvJAZ1, and FvCOI1-JA-

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FvJAZ8 complexes was performed according to Yan et al. (2009). Five independent docking runs were carried out, and 10 conformers were obtained in each case.

Then, MDS for each complex was studied. The initial coordinates for simulations were taken from the docking experiments described above. Each complex was embedded into a SPC pre-equilibrated water model and then neutralized by adding NaCl to the system. Each MD simulation was performed at constant pressure (1.01325 bar) and temperature (300 K) values, with a NVT ensemble. During 100 ns of each MDS, only the secondary structure for FvCOI1 had a 0.25 kcal mol^{-1} $Å^{-2}$ spring constant. Data were collected every 50 ps trajectory. Finally, all MDS were analyzed using the VMD software (Humphrey, Dalke & Schulten, 1996).

Results

FvCOI1 and FvJAZ1/8 sequence analysis

We compared COI1, JAZ1 and JAZ8 amino acid sequences among *Fragaria vesca* and three fleshy fruit-related species, aside from Arabidopsis. We analyzed sequences from *V. vinifera* and *S. lycopersicum* because their importance as models for non-climacteric and climacteric fruit ripening, respectively, and from *M. ×domestica* as representative species belonging to the Rosaceae family as *F. vesca*.

Multiple sequence alignments were performed to estimate the identity between FvCOI1 protein and its orthologs from the plant species described above. Then, a phylogenetic tree was constructed to reveal evolutionary relationships between these proteins. FvCOI1 sequence was highly conserved respect to AtCOI1, VvCOI1, SlCOI1 and MdCOI1 (Fig. 1A) displaying identity values higher than 69.8% (Table S2). Specifically, FvCOI1 exhibited the highest sequence identity with MdCOI1 (82.3%) (Table S2). FvCOI1 showed an F-box domain, 18 LRR domains and specific amino acid residues for binding to JA-Ile (R81, R345, Y383, R406, and R493), which are highly conserved between the analyzed COI1 orthologs (Fig. 1A). Phylogenetic analysis showed a close relationship between FvCOI1 and MdCOI1 or VvCOI1

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rather than SlCOI1 or AtCOI1 (Fig. 1B). These results indicate that FvCOI1, MdCOI1 and VvCOI1 diverged more recently from a common ancestor.

TIFY protein family includes JAZ proteins (Vanholme et al., 2007). The predicted TIFY protein sequences, TIFY10A and TIFY5A were found in *F. vesca* protein database and named as FvJAZ1 and FvJAZ8 respectively, according to their identities (Table S1). A multiple sequence alignment and phylogenetic analysis for JAZ1/8 ortholog proteins were performed to estimate the similarity degree and evolutionary relationships between them. We found that MdJAZ1, SlJAZ1 and VvJAZ9 were the orthologs for FvJAZ1 along with AtJAZ1 based on sequence identity (Table S2). FvJAZ1 showed higher sequence identity with MdJAZ1 (57.6%) (Table S2). On the other hand, FvJAZ8 showed higher sequence identity with VvJAZ3 (61.1%), MdJAZ3 (65.5%) and MdJAZ4 (65.5%) (Table S2). All analyzed JAZ sequences showed TIFY and Jas domains (Fig. 2B, C), while the canonical LPIAR(R/K) degron sequence was absent in all JAZ8 orthologs (Fig. 2C). Remarkably, FvJAZ1 contained an IPMQRK sequence instead of LPIAR(R/K) degron differing from the other ortholog sequences that contain the canonical LPIAR(R/K) sequence (Fig. 2C). The Jas domain central ($SLX_2FX_2KRX_2R$) and C-terminal (X_5PY) regions were highly conserved among all FvJAZ1/8 orthologs (Fig. 2C). In addition, a protein sequence alignment showed the absence of the LPIAR(R/K) sequence in FvJAZ8 and the respective orthologs, as expected in AtJAZ8 (Shyu et al., 2012) (Fig. 2C). AtJAZ8 presented the X_3SMK motif instead of the canonical degron sequence (Shyu et al., 2012). In our work, we found a conserved SMK sequence in all FvJAZ8 orthologs with the exception of SlJAZ10, which presented a TVK variant sequence (Fig. 2C). In turn, the EAR motif (LxLxL; Shyu et al., 2012) was present in all FvJAZ8 orthologs analyzed, with the exception of VvJAZ3 (Fig. 2A). Finally, a phylogenetic analysis grouped FvJAZ1/8 and ortholog proteins in groups II and I, respectively (Fig. 2D), exhibiting FvJAZ1 and FvJAZ8 an even more close evolutionary relationship with MdJAZ1 and AtJAZ8, respectively (Fig. 2D). Additionally, we analyzed 37 inferred JAZ sequences from different plant species (Fig. S1). We observed that the canonical [LPIAR(R/K)] degron sequence was present in 24 sequences while in the remaining 13 sequences it was less conserved, including the IPMQRK sequence found in FvJAZ1.

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Globally, these results show that domains, motifs and amino acids residues, which participate in Arabidopsis COI1-JAZ interaction dependent on JA-Ile, are highly conserved in *F. vesca* and other orthologs, with the exception of the putative IPMQRK degron sequence that we observed in FvJAZ1.

3D structure of FvCOI1 based on comparative modeling

COI1 is a central component of the core module involved in jasmonate signaling and response. Until now, a 3D structure for FvCOI1 is not known. We used various in silico tools to obtain insights into the molecular mechanism that is responsible of FvCOI1 interaction with JA-Ile and FvJAZs. A 3D model for FvCOI1 was built based on sequence alignment between FvCOI1 and AtCOI1 template (69.8% identity). Two further optimization steps were performed to obtain a correct model for FvCOI1. First, an energy minimization procedure was performed, and after that, a short molecular dynamics simulation was run to achieve a final structural model for FvCOI1 (Fig. 3). A geometric and energetic model evaluation was performed to validate its quality. The RMSD values calculated between FvCOI1 and its template for the backbone was 10.8 Å (Fig. S2). The stereochemical quality of the 3D model was analyzed using Ramachandran plots generated by PROCHECK. It was found that the φ/ψ angles for most of the amino acid residues were at the favored region of 99.4% (including: most favorable regions, additional allowed regions and generously allowed regions) indicating a good stereochemical quality (Table S3). Finally, the FvCOI1 model showed a Z-score of -9.07 according to ProSA2003, which was close to -8.13 value obtained for the Arabidopsis template. Consequently, the final structure for FvCOI1 was acceptable for further analysis.

Regarding to the properties for the FvCOI1 structural model, it was formed by two domains, which revealed a TIR1-like overall architecture (Tan et al., 2007): a small N-terminal tri-helical F-box domain and a large LRR domain (Fig. 3A). The LRR domain included seventeen LRR domains, which adopted a tandem packed structure of staggered α-helix and β-sheets (Fig. 3B). FvCOI1 had a central cavity in the LRR domain; the top of the cavity surface was formed by three long loops (Fig. 3B), similar to AtTIR1 and AtCOI1 (Tan et al., 2007). This cavity is involved in hormone binding and to recognize the JAZ polypeptide substrate (Sheard et al.,

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2010).

3D structure for FvJAZ1/8 based on comparative modeling studies

JAZs are key components of the JA-Ile co-receptor by forming a hormone-dependent complex with COI1. To understand how this interaction works at molecular level, it was necessary to obtain the FvJAZ 3D structures. A search on the RCSB Protein Data Bank confirmed that was not publically available any X-ray crystal structure for any JAZ proteins, and only exists the AtJAZ1 Jas domain (including the degron peptide) co-crystalized with AtCOI1 (Sheard et al., 2010). Consequently, it was only possible to model the Jas domain of the FvJAZ1 and FvJAZ8 proteins. For this, a sequence alignment between the AtJAZ1 template fragment and our sequences was performed. Then, the non-aligned sequence was removed and only the sequence similar to the template fragment was considered for each FvJAZ (Fig. S3A). Similar to FvCOI1 model described above, two optimization steps were performed to obtain a correct model, followed by an energetic and geometric evaluation. The RMSD values for the backbone and calculated between AtJAZ1 and the two FvJAZ structures were 3.79 Å (Fig. S3D). Additionally, the RMSD value calculated between FvJAZ1 and FvJAZ8 was 2.9 Å (Fig. S3E). The PROCHECK showed that all amino acid residues were at the favored region respect to FvJAZ1 and FvJAZ8 (Table S3). Finally, the Z-score for FvJAZ1 and FvJAZ8 was -2.34 and -1.75 respectively, while the template showed a Z-score of -1.29. The final structures for FvJAZ1 and FvJAZ8 peptides were acceptable for further analysis.

Regarding to the structural characteristics of the FvJAZ1 and FvJAZ8 peptide fragments (Fig. S3B, C, respectively), they adopted a bipartite structure with a loop region followed by a small α-helix for assembling with the COI1–JA-Ile complex (Sheard et al., 2010).

FvCOI1-ligand interaction

To elucidate whether FvCOI1 binds directly to JA-Ile or JA (used as a negative control), we evaluated FvCOI1–JA-Ile/JA interactions by molecular docking methodology to generate each corresponding protein-ligand complex. As shown in Table 1, a negative energy was obtained for

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each tested ligand, indicating a favorable or likely protein-ligand interaction. However, the differences between ligands were significant, being the strongest binding interaction found between JA-Ile and FvCOI1 (Table 1).

To corroborate that FvCOI1 has a stronger interaction with JA-Ile than JA as we expected, MDS studies were performed. The time-course FvCOI1-ligand interaction for JA-Ile and JA was studied using MDS. The complex formed between FvCOI1 and JA-Ile showed a correct orientation as the previously described template structure for AtCOI1-JA-Ile complex. JA-Ile was oriented in the pocket entrance of FvCOI1, and sat in an 'upright' position with the keto group of its common cyclopentanone ring pointing up and forming a hydrogen bond (H-bond) with R493 and K8 residues from FvCOI1 (Fig. 4A). Additionally, other amino acid residues were important for the protein-ligand interaction, contributing to the FvCOI1 pocket entrance architecture (Fig. 4A). In contrast, the ligand in FvCOI1-JA complex (Fig. 4B) was incorrectly oriented in relation to the JA-Ile previously described by Sheard et al. (2010) and shown in Fig 4A, where its orientation was perpendicular with respect to JA-Ile, and located more distant to the K8 residue, forming a H-bond only with Y441.

FvJAZ1/8 binding to FvCOI1-JA-Ile and FvCOI1-JA

The previously generated FvCOI1-JA-Ile and FvCOI1-JA stable complexes were used to study protein-ligand-protein conformations by molecular docking simulation procedure using the FvJAZ1 and FvJAZ8 peptide fragments. As shown in Table 2, negative values of Haddock score were obtained for the four tested complexes, indicating a favorable or likely protein-ligand-protein interaction. The strongest binding interaction was found between FvJAZ1 and FvCOI1 with JA-Ile (Table 2), however, the two complexes with FvJAZ8 showed an unfavorable Haddock score.

The different formed complexes were evaluated by MDS procedure. First, the dependence on RMSD values was tested to check whether the convergence in calculations was obtained and if the equilibrated MD trajectory was stable. The RMSD value for FvCOI1-JA-Ile-FvJAZ1 complex was the lowest one, with a value around 0.9 Å (Fig. S4) indicating a conformational

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high stability for the protein structures. In the other three complexes the stability value was obtained only at the final part of the MD trajectory, observing higher RMSD values (around 1.5 and 2.0 Å) of RMSD respect to FvCOI1-JA-Ile-FvJAZ1 complex (Fig. S4).

Regarding to the orientation of the structures in the different complexes, the short structure for FvJAZ1 and FvJAZ8 was situated on top of the hormone-binding pocket (Fig. 5 and 6, respectively). Although, FvJAZ1 simultaneously interacted with both FvCOI1 and the ligand (JA-Ile or JA), its pocket only coordinated in a stable manner with JA-Ile (Video S1). This due to the fact that JA is more mobile and it started to separate from the complex (Fig. 5D, E; Video S2). In contrast, the two complexes with FvJAZ8 showed a stable coordination with the ligands (JA-Ile and JA) in the pocket. However, FvJAZ8 was more mobile during the MDS and it separated from the complexes formed by FvCOI1 and both jasmonate molecules (Fig. 6A, B, D, E; Video S3, S4).

During the MDS of FvCOI1-JA-Ile with FvJAZ1 different H-bonds were formed between amino acid residues of each protein and JA-Ile. As shown in the Figure 5C, the K8, K79, S145, and Y441 residues formed an H-bond with frequency values of 70%, 36%, 55%, and 67%, respectively. As described before, JA distanced itself from the FvCOI1-FvJAZ1 complex during the MDS (with a distance over 4 Å), explained the observed lower frequency value compared to JA-Ile as ligand. Using a 30% of frequency values as threshold, only Y441 residue form an H-bond with JA with 67% of frequency value (Fig. 5F up). Additionally, the total number of H-bonds was highest in the complex containing JA-Ile instead of JA, with an average of 3 or 4 H-bonds, while in the complex with JA and FvJAZ1 it contained 1 or 2 H-bonds (Fig. 5C down and 5F down). In the complexes formed between FvCOI1-JA-Ile or FvCOI1-JA with FvJAZ8 there was a long separation (more than 6-8 Å) between FvJAZ8 and the complex during a longer time of the MDS, and the H-bond formation was lower with one or two amino acid residues forming H-bonds (Fig. 6C up and 6F up). Finally, the total number of H-bonds formed between the different components of the FvCOI1-JA-Ile-FvJAZ8 complex was lower than the FvCOI1-JA-Ile-FvJAZ1 complex, which presented on average 1 or 2 H-bonds (Fig. 6C down and 6F down).

Discussion

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372

373 **Identification and characterization of FvCOI1 and FvJAZ1/8 amino acid sequences**

374

375 The perception of JA-Ile is critical for initiation of JA signaling pathway (Sheard et al., 2010).
376 COI1 is a highly conserved F-box protein in terrestrial plants, part of the SKP1/CUL1/F-box
377 (SCF^{COI1}) ubiquitin E3 ligase complex, that together with JAZs acts as a JA-Ile co-receptor (Han,
378 2016). The F-box and LRR domains present in AtCOI1 are critical for protein stabilization and
379 JAZ interaction, respectively (Sheard et al., 2010), and were highly conserved in FvCOI1 (Fig.
380 1A). On the other hand, R85, R348, Y386, R409 and R496 residues, which participate in binding
381 to JA-Ile in Arabidopsis (Sheard et al., 2010), were also conserved in FvCOI1 (represented by
382 R81, R345, Y383, R406 and R493) and ortholog proteins such as VvCOI1, SlCOI1 and MdCOI1
383 (Fig. 1A). The observed high conservation on F-box and LRR domains, presence of key amino
384 acid residues for JA-Ile binding, and evolutionary relationships between COI1 orthologs suggest
385 that FvCOI1 is a functional protein involved in the JA signaling pathway in strawberry.

386

387 On the other hand, JAZ are repressors of the JA-signaling pathway (Chini et al., 2007; Thines et
388 al., 2007; Yan et al., 2007). In *A. thaliana* and *S. lycopersicum* 12 JAZ proteins have been
389 described, whereas in *M. ×domestica* and *V. vinifera* the number is 18 and 11, respectively
390 (Zhang et al., 2012; Ishiga et al., 2013; Li et al., 2015). We analyzed the *F. vesca* protein
391 database and found two ortholog sequences to AtJAZ1 and AtJAZ8, named FvJAZ1 and
392 FvJAZ8, respectively (Table S1). FvJAZ1/8 proteins contain TIFY and Jas domains; the later
393 one interacts with COI1 in Arabidopsis (Melotto et al., 2008; Shyu et al., 2012). These domains
394 were highly conserved between *F. vesca* and other ortholog sequences (Fig. 2A-C). Surprisingly,
395 we observed that the putative IPMQRK degron sequence in FvJAZ1 was different respect to the
396 canonical sequence LPIAR(R/K) (Shyu et al. 2012) present in all analyzed JAZ orthologs (Fig.
397 2C). The JAZ degron is critical because together with COI1 acts as a co-receptor complex,
398 trapping JA-Ile with high affinity to the COI1 binding pocket (Sheard et al., 2010). On the other
399 hand, FvJAZ8 contains the same EAR motif sequence (LELRL) than AtJAZ8, which interacts
400 directly with TOPLESS co-repressor of JA-signaling pathway in a NINJA-independent manner
401 in Arabidopsis (Shyu et al., 2012). Particularly, neither VvJAZ3, SlJAZ10 and MdJAZ3/4
402 exhibited LELRL as EAR motif sequence (Fig. 2A). Phylogenetic analysis showed a close

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relationship between FvJAZ1 and *M. ×domestica* ortholog protein, suggesting a high conservation of FvJAZ1 orthologs in the Rosaceae family.

Structural model for FvCOI1

Using in silico approaches, we proposed a structural model for FvCOI1 (Fig. 3). First, we tested its quality using previously validated methodologies (Morales-Quintana et al., 2011; Morales-Quintana et al., 2012; Galaz et al., 2013), thus we obtained a high-quality structure. The final structural model for FvCOI1 was used to evaluate COI1 ability to bind two different jasmonate molecules (JA and JA-Ile) (Fig. 5, 6; and Table 2). We observed that FvCOI1 harbors a surface pocket, previously described as potential binding site for JA-Ile in AtCOI1 (Sheard et al., 2010).

The FvCOI1 sequence contained two typical domains, a F-box at the N-terminal region and the LRR domain at the C-terminal region (Fig. 3), similar to the observed in AtCOI1. The superposition between AtCOI1 and FvCOI1 structural model showed a high similarity at the F-box and LRR domains (Fig. S2). Interestingly, FvCOI1 and AtCOI1 did not show integrity at the LRR domain, because the LRR-8 domain has lost its helix conformation in both structures (Fig. 3B and S2). In contrast, Yan et al. (2009) showed a computational model for the AtCOI1 structure where the LRR-8 is formed by an α-helix, proposing that the LRR domain integrity is required for AtCOI1 stability in vivo (Yan et al., 2009). This was observed when authors evaluated four amino acid substitutions (G369E, G155E, D452A, and L490A) at the LRR domain, resulting in a reduction of the AtCOI1 stability in vivo. However, none of these substitutions were in LRR-8 residues. Although, the template and our model do not have an α-helix structure in LRR-8, they are stable. It was observed that the obtained trajectory and the resulting structure after the thermodynamic equilibrations were both stable when the MDS for FvCOI1 was analyzed. Finally, we noted that LRR-8 is not conserved among the different analyzed sequences (Fig. 1A) and it was not required for protein-ligand interaction (Fig. 5 and 6).

FvCOI1-JA-Ile/JA-FvJAZ1/8 complexes formation

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Using surface plasmon resonance (SPR) technology, Yan et al. (2009) found that AtCOI1, JA-Ile and AtJAZ1 were sufficient to form a complex, ruling out the possibility that other AtCOI1-interacting proteins can be involved in JA-Ile perception (Yan et al., 2009). Based on this results, we modeled the different complexes structures formed by the following components: FvCOI1, JA-Ile or JA, and FvJAZ1 or FvJAZ8. Thus, we obtained three possibilities for assembling each protein-ligand-protein complex. Yan et al. (2009) showed that AtCOI1 directly binds to JA-Ile, and subsequently binds AtJAZ1. Thus, in the present work, we used this previous evidence to set the temporal order for the conformation of the protein-ligand-protein complex.

In Arabidopsis, the Jas domain promotes hormone-dependent interaction between JAZ proteins and COI1 (Melotto et al., 2008; Chung & Howe, 2009). Structural studies showed that the JAZ1 degron is located at the N-terminal region of the Jas domain, and included five conserved LPIAR(R/K) residues that sealed JA-Ile in COI1 binding pocket (Sheard et al., 2010). Here, we showed that FvJAZ1 has a putative degron variant, the IPMQRK sequence (Fig. 2C, S1, S3A). Despite these differences, the FvJAZ1 showed a favorable HADDOCK score (Table 2), and the complex formed between FvJAZ1 and FvCOI1-JA-Ile was stable during all MDS (Video S1). We found that the FvJAZ1 putative degron formed H-bonds with JA-Ile at the C-terminal (QRK), whereas the N-terminal residues (IPM) interacted directly with FvCOI1, similar to that found by Sheard et al. (2010) with the AtJAZ1 canonical degron. Similar to FvJAZ1, finger millet JAZ1 (EcJAZ1) showed a variant sequence respect to the canonical degron (Sen et al., 2016; Fig. S1). Using an in silico approach, the authors showed that the interaction mode for five COI1 structural models from monocots was binding to JA-Ile and COR in the presence of EcJAZ1. The six residues that conform this degron were oriented to 5 Å from the ligand (either JA-Ile or COR), suggesting a likely interaction with the ligands (Sen et al., 2016). According to Shyu et al. (2012), alterations within the JAZ degron sequence could lead to a differential association of JAZ isoforms with COI1 based on JA-Ile levels, in order to activate broad range of JA responses. In this sense, FvJAZ1 may require a differential JA-Ile accumulation than other JAZ containing the canonical LPIAR(R/K) degron, to interact with COI1 for promoting FvJAZ1 ubiquitination and subsequently degradation by the 26S proteosome and activation of JA signaling pathway. Unexpectedly, several (+)-7-*iso*-amino acid conjugates such as (+)-7-*iso*-JA-Leu, (+)-7-*iso*-JA-Val, (+)-7-*iso*-JA-Ala and (+)-7-*iso*-JA-Met were recently reported as

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bioactive molecules in Arabidopsis, tomato, rice and tobacco (Yan et al., 2016), which could bring new ways to regulates JA responses in plants likely via differential interaction strength between COI1 and JAZ.

On the other hand, COI1 has five important residues (R85, R348, Y386, R409 and R496), which are involved in binding to JA-Ile in Arabidopsis (Sheard et al., 2010). Interestingly, FvCOI1 contained these five conserved residues, but only four are involved in the protein-JA-Ile interaction, being R406 residue (equivalent to R409 in AtCOI1) not required for the complex formation. Respects to Arabidopsis JAZ, two adjacent arginine residues located in the degron motif have a critical role for the interaction with AtCOI1 in the presence of JA-Ile (Melotto et al., 2008). FvJAZ1 presented a change from R to K residue at the second position (Fig. 2C, S3A); however the two residues interacted strongly with the FvCOI1 without significant differences respect to the interaction mode observed for AtCOI1-AtJAZ1. On the contrary, FvJAZ8 contained M251 and K252 residues at this position (Fig. 2C) and the three first residues of its degron were different to the corresponding for FvJAZ1. Therefore, FvJAZ8 peptide only interacts with the FvCOI1-JA-Ile complex through the K residue that is the only one conserved residue in both FvJAZs degron sequences (Fig. 2C, S3A). As a consequence, the HADDOCK score was less favorable (Table 2) and the complex was unstable showing a high RMSD value (Fig. S4). During a long time MDS the interaction was lost (Fig. 6A-C) because FvJAZ8 was separated from the FvCOI1-JA-Ile complex (Video S2). This evidence is in agreement with previous results that showed a weaker interaction between AtJAZ8 and AtCOI1 in the presence of JA-Ile than between AtJAZ1 and AtCOI1 (Shyu et al., 2012).

Interestingly, we observed that JA did not leave the FvCOI1 pocket when FvJAZ8 peptide was present, whereas when FvJAZ1 degron-like peptide was present it did (Fig. 5, 6), supporting the idea that the presence of the putative degron sequence of FvJAZ1 could be an important feature for the selection of bioactive JAs, such as JA-Ile in strawberry.

Conclusions

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Based on the results presented in this work, we conclude that core components of the early JA signaling pathway in *F. vesca* such COI1 and JAZ1/8 are highly conserved compared to other ortholog sequences in plants. Remarkably, FvCOI1 showed a high identity respect to all COI1 orthologs analyzed, while the FvJAZ1/8 exhibited a moderate identity with the ortholog proteins from Arabidopsis. Particularly, we observed a putative degron sequence (IPMQRK) in FvJAZ1 that is different to the canonical LPIAR(R/K) degron sequence present in non-constitutively stable JAZ in Arabidopsis. Moreover, we propose that the FvCOI1-JA-Ile-FvJAZ1 complex is stable, and that the IPMQRK sequence present in FvJAZ1 interacts in a steady manner with FvCOI1-JA-Ile. Moreover, four amino acid residues observed in the binding site of FvCOI1 are identical to that observed in AtCOI1.

Finally, studying other possibilities on the nature of the interaction temporal order (i.e., FvCOI1-FvJAZ-JA-Ile and FvJAZ-JA-Ile-FvCOI1) and the use of cofactors to strength the complex stability will be subjects for future studies.

Funding statement

This work was supported by the National Commission for Scientific and Technological Research (CONICYT) of Chile [grants CONICYT, FONDECYT/Regular 1140663 to C.R.F. and CONICYT PAI/ACADEMIA 79140027 to L.M-Q.].

Acknowledgements

We thank the Center of Bioinformatics and Molecular Simulations (CBSM) at the University of Talca for providing SCHRÖDINGER suite.

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Figure 1

A

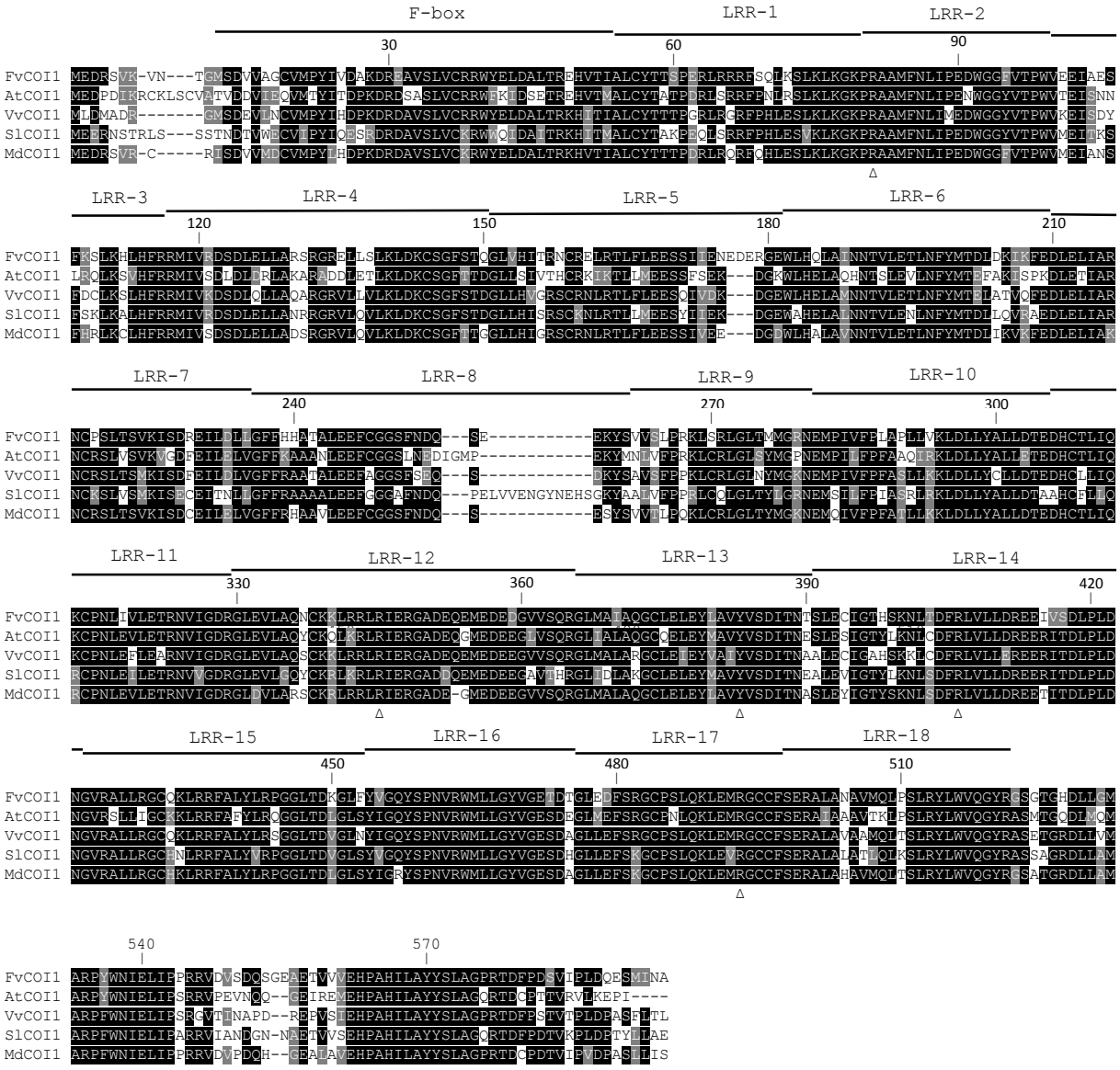

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Fig. 1 (continued)

B

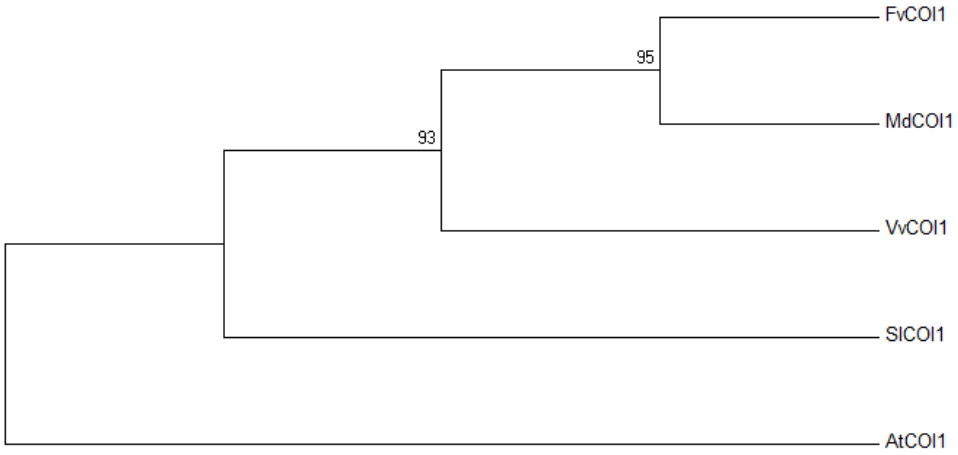

Figure 1. Comparison of FvCOI1 with ortholog proteins. (A) Alignment of the inferred amino acid sequences of FvCOI1 with other orthologs. Gaps are indicated by dashes, letters with black background represent identical amino acid residues, and letters with grey background represent similar residues. Open triangles (Δ) indicate conserved residues for JA-Ile binding in COI1. F-box and the 18 LRR domains are shown. (B) Phylogenetic analysis of FvCOI1 sequence with other orthologs. For GenBank accession numbers see Material and Methods section. FvCOI1, *Fragaria vesca* COI1; AtCOI1, *Arabidopsis thaliana* COI1; VvCOI1, *Vitis vinifera* COI1; SlCOI1, *Solanum lycopersicum* COI1; and MdCOI1, *Malus ×domestica* COI1. F-box: F-box domain, LRR: leucine-reach repeat domain.

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Figure 2

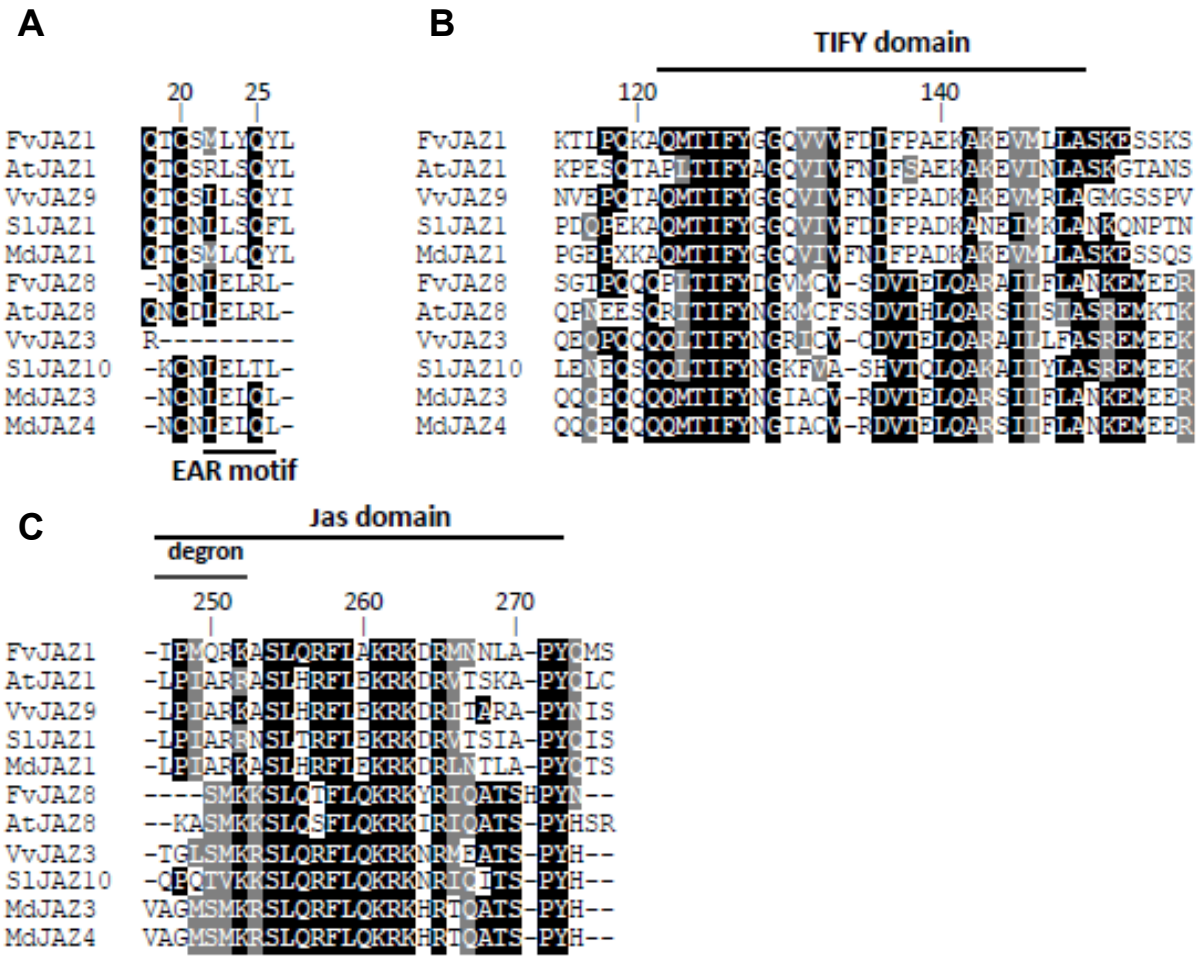

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Fig. 2 (continued)

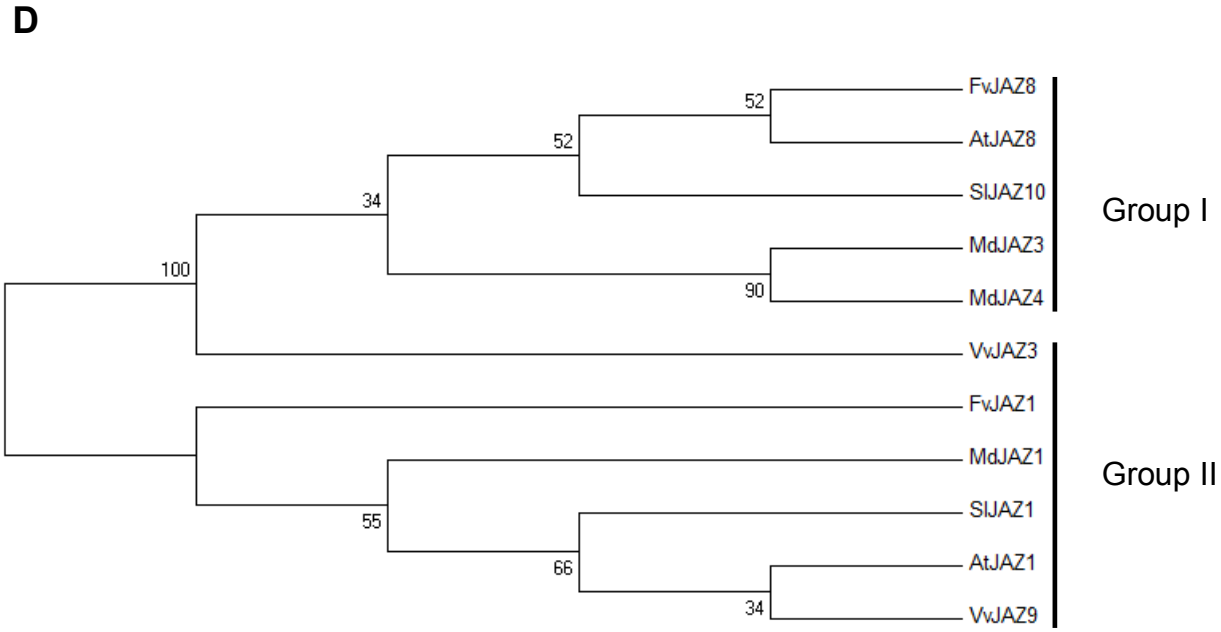

Figure 2. Comparison of main domains and motifs of FvJAZ1/8 with ortholog sequences. (A), (B) and (C) Alignments of the inferred full-length amino acid sequences of EAR motif, TIFY and Jas domains of FvJAZ1 and FvJAZ8 with their orthologs, respectively. The degron sequences are shown within the Jas domain. Gaps are indicated by dashes, letters with black background represent identical amino acid residues, and letters with grey background represent similar amino acid residues. (B) Phylogenetic analysis of FvJAZ1/8 amino acid sequence with orthologs ones. Group I and group II include orthologs for FvJAZ8 and FvJAZ1, respectively. For GenBank accession numbers see Material and Methods section. FvJAZ1/8, *Fragaria vesca* JAZ1/8; AtJAZ1/8, *Arabidopsis thaliana* JAZ1/8; VvJAZ9/3, *Vitis vinifera* JAZ9/3; SlJAZ1/10, *Solanum lycopersicum* JAZ1/10; and MdJAZ1/3/4, *Malus ×domestica* JAZ1/3/4.

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Figure 3

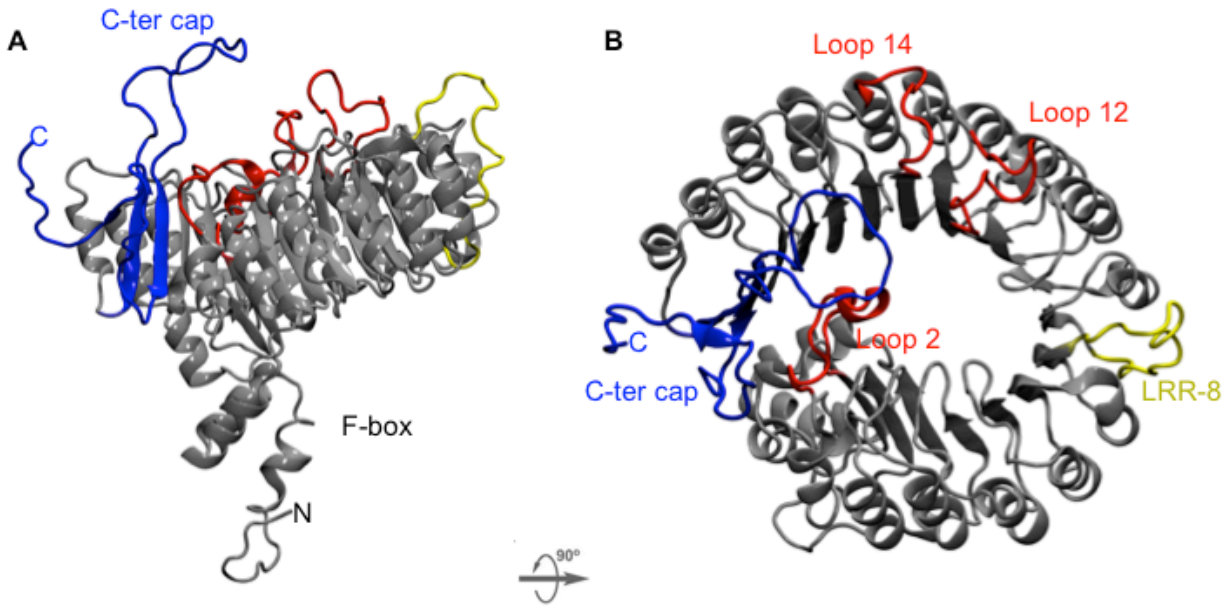

Figure 3. Structural model for COI1. Two views of the FvCOI1 structural model are shown as NewCartoon representation (A and B). The F-box and LRR domains of FvCOI1 are shown in gray color. The C-terminal cap is shown in blue while the three important loops forming the hormone pocket in FvCOI1 are shown in red. The LRR-8 domain is represented in yellow.

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796 **Figure 4**
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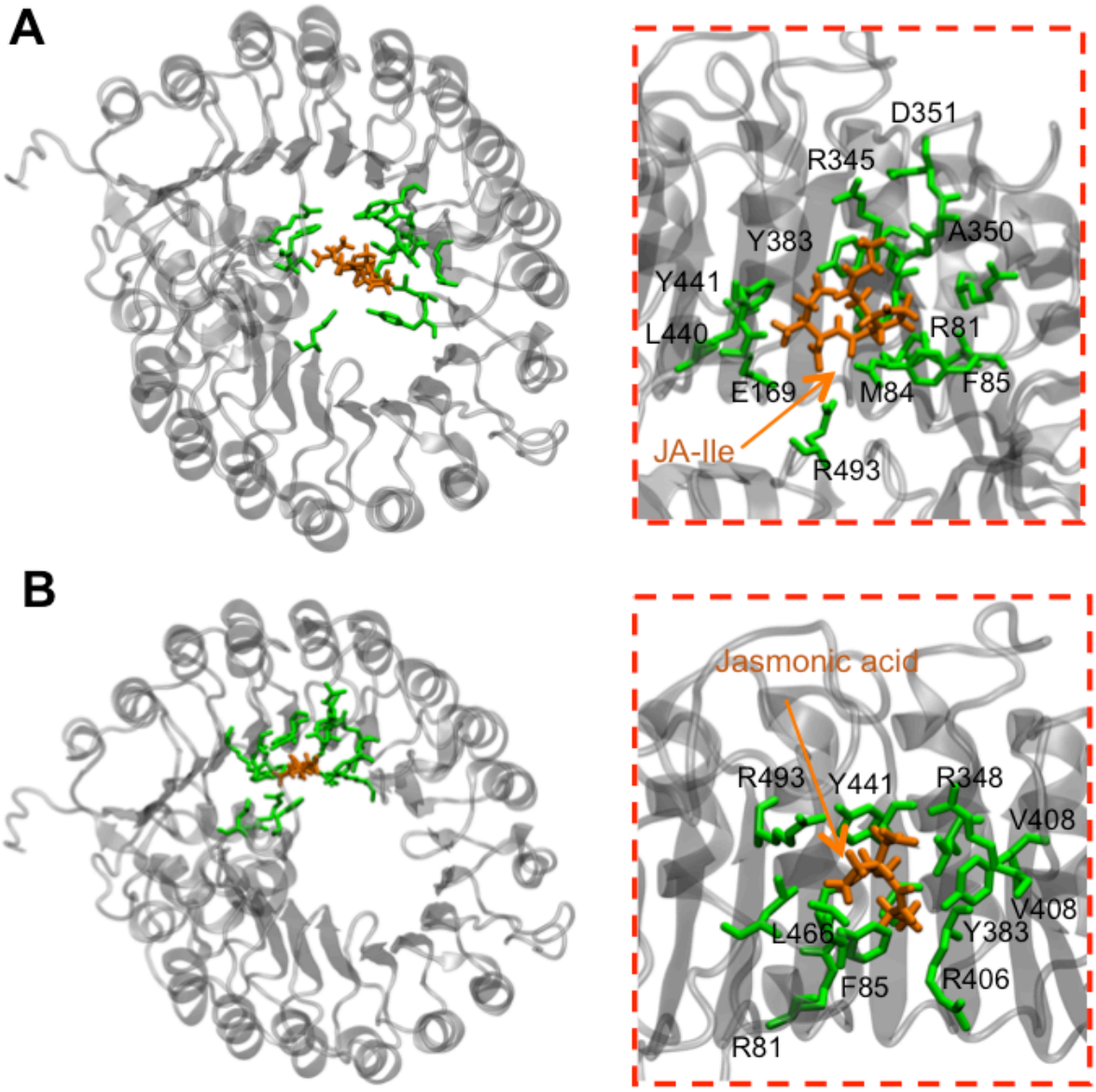

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800
801 **Figure 4. Ligand binding analysis based on a refined FvCOI1 model.** A detailed view of the
802 hormone-binding pocket site of FvCOI1 showing the residues involved in the interaction
803 (represented in green) with JA-Ile (A), and jasmonic acid (B) (represented in orange).
804 Magnifications of the hormone binding pockets are shown at the right side.

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Figure 5

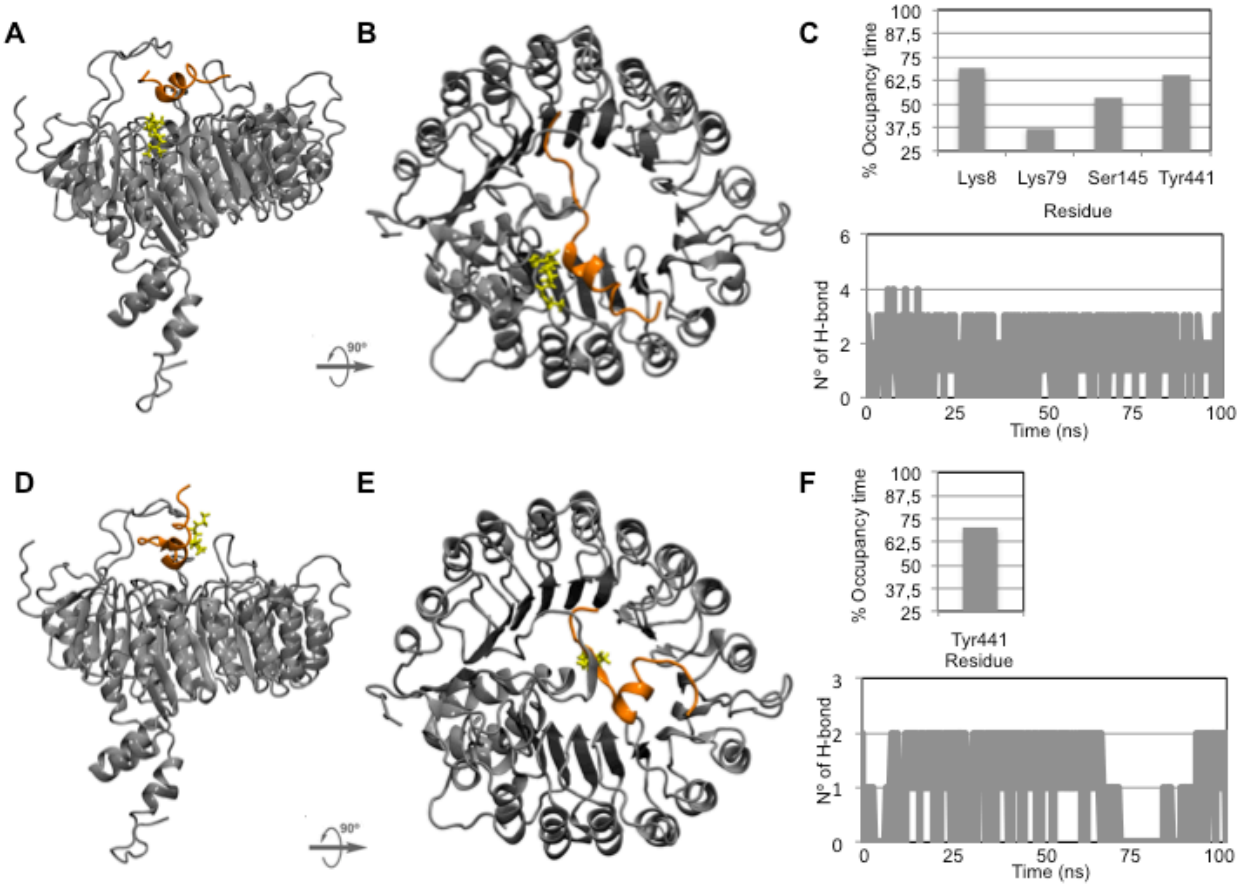

Figure 5. MDS analyses of the interactions in FvCOI1-JA-Ile-FvJAZ1 (A, B, C) and FvCOI1-JA-FvJAZ1 complexes (D, E, F). A frontal (A) and top (B) views of interaction between the FvCOI1-JA-Ile complex and FvJAZ1. (C) Top graph shows the time percentage that a hydrogen bond between a particular amino acid residue of FvCOI1 and the JA-Ile or FvJAZ1 is established. Only values greater than or equal to 30% frequency are shown in the graph. Lower graph shows the number of total H-bonds formed by the complex. A frontal (D) and top (E) views of the interaction between the FvCOI1-JA complex and FvJAZ1. (C) Top graph shows the time percentage that hydrogen bonds between Y441 of FvCOI1 and the JA or FvJAZ1 is established. Lower graph shows the number of total H-bonds formed by the complex.

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820 **Figure 6**
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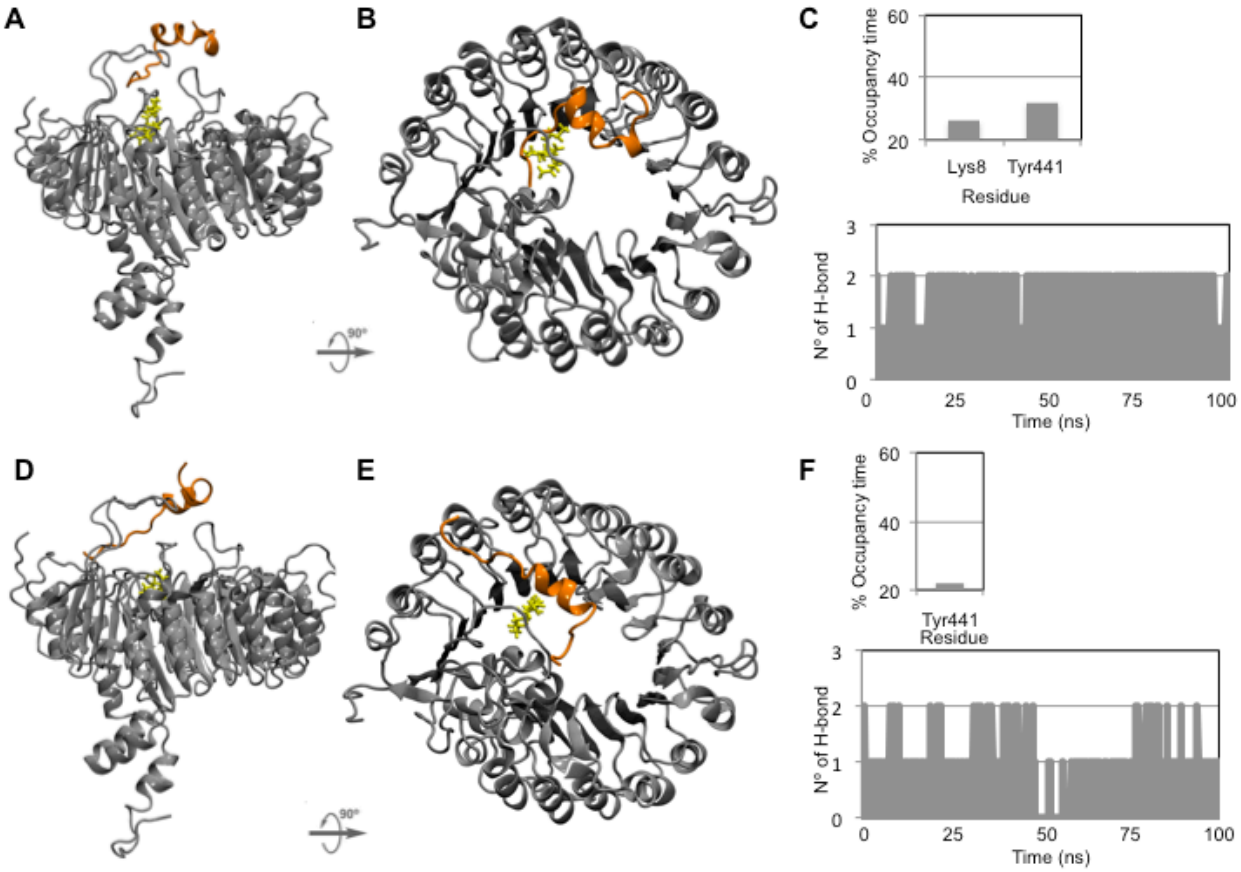

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825

826 **Figure 6. MDS analyses of the interactions in FvCOI1-JA-Ile-FvJAZ8 (A, B, C) and**
827 **FvCOI1-JA-FvJAZ8 complexes (D, E, F).** A frontal (A) and top (B) views of interaction
828 between the FvCOI1-JA-Ile complex and FvJAZ8. (C) Top graph shows the time percentage that
829 a hydrogen bond between a particular amino acid residue of FvCOI1 and the JA-Ile or FvJAZ1 is
830 established. Only values greater than or equal to 30% frequency are shown in the graph. Lower
831 graph shows the number of total H-bonds formed by the complex. A frontal (D) and top (E)
832 views of the interaction between the FvCOI1-JA complex and FvJAZ8. (C) Top graph shows the
833 time percentage that hydrogen bonds between Y441 of FvCOI1 and the JA or FvJAZ8 is
834 established. Lower graph shows the number of total H-bonds formed by the complex.

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Tables

Table 1. Affinity energy for the interaction between FvCOI1 protein and JA or JA-Ile ligands

Ligands	FvCOI1 Affinity energy (kcal mol^{-1})
Jasmonic acid (JA)	-1.3[a] ±0.2
(+)-7-*iso*-jasmonoyl-isoleucine (JA-Ile)	-11.1[b] ±0.2

Data correspond to the mean ± SE for the best protein-ligand conformation of five independent docking runs for each ligand. Different lower-case letters indicate significant differences between each ligand (Tukey HSD test, $p \leq 0.05$).

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Table 2. HADDOCK score obtained for the interaction between FvJAZ1 and FvJAZ8 with FvCOI1-ligand complexes

Protein	HADDOCK score	
	FvCOI1-JA-Ile	FvCOI1-JA
FvJAZ1	-71.3[a] ±14.7	-43.3[b] ± 5.3
FvJAZ8	-37.1[a] ± 3.3	-30.3[a] ± 8.4

Data correspond to the mean ± SE for the best protein-ligand-protein conformation of three independent docking runs for each ligand. Different lower-case letters indicate significant differences between each FvJAZ protein with the FvCOI1-ligand complex (Tukey HSD test, $p \leq 0.05$).

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