Alternative oxidase (AOX) constitutes a small family of proteins in *Citrus clementina* and *Citrus sinensis* L. Osb

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Abstract

The alternative oxidase (AOX) protein is present in plants, fungi, protozoa and some invertebrates. It is involved in the mitochondrial respiratory chain, providing an alternative route for the transport of electrons, leading to the reduction of oxygen to form water. The present study aimed to characterize the family of AOX genes in mandarin (*Citrus clementina*) and sweet orange (*Citrus sinensis*) at nucleotide and protein levels, including promoter analysis, phylogenetic analysis and *C. sinensis* gene expression. This study also aimed to do the homology modeling of one AOX isoform (CcAOXd). Moreover, the molecular docking of the CcAOXd protein with the ubiquinone (UQ) was performed. Four AOX genes were identified in each citrus species. These genes have an open reading frame (ORF) ranging from 852 bp to 1150 bp and a number of exons ranging from 4 to 9. The 1500 bp-upstream region of each AOX gene contained regulatory cis-elements related to internal and external response factors. CsAOX genes showed a differential expression in citrus tissues. All AOX proteins were predicted to be located in mitochondria. They contained the conserved motifs LET, NERMHL, LEEEA and RADE-H as well as several putative post-translational modification sites. The CcAOX protein was modeled by homology to the AOX of *Trypanosoma brucei* (45% of identity). The 3-D structure of CcAOXd showed the presence of two hydrophobic helices that could be involved in the anchoring of the protein in the inner mitochondrial membrane. The active site of the protein is located in a hydrophobic environment deep inside the AOX structure and contains a diiron center. The molecular docking of CcAOXd with UQ showed that the binding site is a recessed pocket formed by the helices and submerged in the membrane. These data are important for future functional studies of citrus AOX genes and/or proteins, as well as for biotechnological approaches leading to AOX inhibition using UQ homologs.
Introduction

The term oxidase refers to any enzyme that catalyzes the oxidation–reduction reaction involving molecular oxygen as an electron acceptor. In these reactions, the oxygen is reduced to water or to hydrogen peroxide. The alternative oxidase (AOX) protein is present in plants, fungi, protozoa and some invertebrates, but it has not been found in mammals. It is located on the matrix side of the inner mitochondrial membrane and is involved in the mitochondrial respiratory chain, providing an alternative route for the passage of electrons. The main electron transport route in eukaryotes passes through the complex IV (known as cyanide-sensitive cytochrome oxidase) of the respiratory chain, but in some organisms the electron transport route goes through the AOX protein (known as cyanide-insensitive and hydroxamic acid-sensitive terminal oxidase). Both routes lead to the transportation of electrons and the reduction of oxygen to form water \[1, 2\]. However, the transportation through the AOX protein occurs without the pumping of protons into the intermembrane space and consequently is not coupled with ATP synthesis and energy conservation \[3\]. The AOX catalyzes the four-electron oxidation of ubiquinol (reduced form of ubiquinone \([UQ]\)) by oxygen, and the energy of ubiquinol oxidation by oxygen is released as heat \[3–5\].

The AOX proteins (32–36 kDa) are encoded by a family of nuclear genes \[6\], and several studies report that, in plants, variations of environmental factors such as abiotic stresses, pathogen infection and oxidative stress may influence the expression of AOX genes \[3, 7–10\]. Moreover, AOX has been proposed to play a role in homeostasis and plant growth \[11\] and in maintaining metabolic flexibility for rapid adaptation to stress \[12\]. In citrus plants, the only studies of AOX proteins have been related to abiotic stresses (e.g., drought, boron tolerance) \[13–15\], and no genome-wide characterization of the AOX family has yet been performed for this genus. The availability of the data from the recent sequencing of the genome of some citrus species (https://www.citrusgenomedb.org/) allowed for the genome-wide analysis of gene families as a pre-requisite for functional and/or pre-breeding studies. The present study aimed to characterize the family of AOX genes in mandarin (\(C. clementina\)) and sweet orange (\(C. sinensis\)) at nucleotide and protein levels, including promoter analysis. The study also aimed to construct the homology modeling of one AOX isoform (CcAOXd). Moreover, the molecular docking of the CcAOXd protein with the \([UQ]\) was performed.

Material and methods

In silico analysis of AOX citrus genes and proteins

The identification and structural analysis of the AOX genes (introns/exons) were performed using the Citrus Genome Database (https://www.citrusgenomedb.org/). Open reading frame (ORF) analysis was performed using the ORFinder software (http://www.ncbi.nlm.nih.gov/orffinder/). The prediction of the theoretical isoelectric point (pI) and the molecular weight (MW) was obtained using the pl/Mw tool (www.expasy.org). Conserved domain and family protein were analyzed using the Pfam (http://pfam.sanger.ac.uk/search/sequence) and InterProScan software \[16\]. The predictions of the subcellular location of the protein and of the location of the cleavage site were performed by the MitoProt II software (https://ihg.gsf.de/ihg/mitoprot.html). Transmembrane helices were predicted using the TMpred software \[17\], whereas hydropathicity levels were identified using the ProtScale program (http://web.expasy.org/protscale/). The NetPhos 3.1 Server \[18\] and the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) were used to identify putative phosphorylation sites (Ser/Thr/Tyr) and putative N-glycosylation sites (Asn-X-Ser/Thr type), respectively. The protein motif analysis was conducted using the program MEME/MAST \[19\]. The maximum number of motifs
was set to 20, the maximum motif length was set to 80 amino acids, the optimum motif width was constrained to between 6 and 300 residues, and the other parameters were used as default.

**Analysis of the promoter regions and chromosomal locations of AOX genes**

To identify the presence of the cis-regulatory elements in the promoter regions of the AOX genes, the 1500 bp upstream region from the translation start site of the genes was analyzed using the plantCARE (sphinx.rug.ac.be:8080/PlantCARE/cgi/index.html) software [20]. The chromosomal locations of the AOX genes were obtained by screening the GFF3 file of each genome (C. clementina and C. sinensis deposited in the Citrus Genome Database) using the AOX sequence ID.

**Phylogeny**

Phylogenetic analysis was performed based on the alignment of the amino acid sequence of the AOX proteins from C. sinensis and C. clementina with alternative oxidase proteins from Arabidopsis thaliana. The sequences were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [21]. The MEGA 5.1 program [22] was used to construct a phylogenetic tree by using the neighbor-joining statistical method [23] reliably established by 1000 bootstrap samples.

**Molecular modeling**

To select the best 3-D template for AOX molecular modeling from resolved 3-D structures, the AOX proteins from C. clementina and C. sinensis were aligned with the Protein Data Bank (Pdb) using the PSI-BLAST program [24]. Target 3-D structures were modeled using templates that presented the highest identity and coverage, starting from a minimum of 25% of identical amino acids in the alignment. Additionally, the minimum template resolution considered was 2.0 Å. The predicted 3-D protein model was obtained using the SWISS-MODEL server (https://swissmodel.expasy.org) and the Swiss-Pdb Viewer program v.3.7 [25]. The α-carbon chain RMSD between targets and their respective templates was calculated using PyMOL V3.0 [26]. The stereochemical quality of both AOX models was calculated by Procheck 3.4 [27] and the Atomic Non-Local Environment Assessment (ANOLEA) program [28]. The validation of the secondary structure was performed using the Protein Structure Prediction Server-PSIPRED program [29].

**Molecular docking of CcAOXd with ubiquinone**

Before performing the docking between the ligand and the target protein, the ubiquinone (UQ) structure (C_{59}H_{90}O_{4}) was downloaded from pubchem database (https://pubchem.ncbi.nlm.nih.gov/) in SMILES format. The UQ structure was converted into 3-D format using MarvinSketch 15.7.13.0 (https://www.chemaxon.com/products/marvin/marvinsketch/) and saved in mol2 format. Furthermore, AutoDockTools V1.5.6 [30] was used to prepare the protein and UQ structure for docking calculations. First, polar hydrogens were added to the UQ structure and all torsions were checked; the ligand structure was then saved in PDBQT format. Based on the alignment between CcAOXd and AOX structures, the amino acids of the active were marked in order to get the grid box coordinates for the docking process. Afterward, the CcAOXd structure was saved in PDBQT format. Calculations for the docking between CcAOXd and UQ were performed using AutoDock Vina software [30] considering 9 different docking poses and based on UQ bond torsions. All docking results were evaluated using
PyMOL V1.7.4 [26] in order to check which UQ poses appeared in the CcAOXd active site and to identify which pose presents the best docking affinity energy. Additionally, Discovery Studio 4.5 was used to generate the 2-D map of the interaction between CcAOXd and UQ.

**In silico C. sinensis AOX gene expression**

CsAOXa, CsAOXb, CsAOXc and CsAOXd gene sequences were blasted on the Citrus sinensis Annotation Project database (CAP; http://citrus.hzau.edu.cn/orange/ [31]) to obtain the CAP accession number of each gene. Using the CAP accession number, the complete data of each gene, including the RNA-seq gene expression values in four tissues (callus, leaf, flower and fruit) was obtained [31].

**Results and discussion**

**AOX gene family in the sweet orange and tangerine genomes**

Existing annotation in the Citrus Genome Database allowed for the identification of a total of 8 AOX genes, with 4 belonging to *C. clementina* (named CcAOXa, CcAOXb, CcAOXc and CcAOXd) and 4 to *C. sinensis* (named CsAOXa, CsAOXb, CsAOXc and CsAOXd; Table 1). The CcAOX genes were distributed in chromosomes 2, 5 and 8 (Table 1). The gene ORFs ranged from 927 to 1150 bp, and the number of exons ranged from 4 to 9 (Table 1; Fig 1; S1 Fig). The CsAOX genes were located in chromosomes 2, 3 and 8 (Table 1). The gene ORFs ranged from 852 to 1050 bp, and the number of exons ranged from 4 to 9 (Table 1; Fig 1; S1 Fig). For most of the genes, the 5’ end of the introns presented the GT sequence as a splicing donation site, whereas the 3’ end presented the AG sequence as a splicing acceptor site (S1 Fig). The number of AOX genes found in *C. clementina* and *C. sinensis* is small, which is similar to what has been observed in other species such as *Arabidopsis thaliana*, whose AOX family is represented by five genes [7]; *Glycine max* [32], *Oryza sativa* [33] and *Zea mays* [34], each represented by three genes; and *Nicotiana tabacum* [2], *Triticum aestivum* [35] and *Hypericum perforatum* [36], each represented by two genes. Most of the AOX genes in this study have structures with 4 exons and 3 introns, which has also been observed in other species such as *A. thaliana*, *G. max*, *Theobroma cacao*, *Citrus sinensis*, *Gossypium hirsutum*, *O. sativa*, *T. aestivum*, *Vigna unguiculata*, *Vitis vinifera* and *Z. mays* [36, 37]. In contrast to the 4-exon structure reported for most of the organisms, the CcAOXa and CsAOXa genes presented 9 exons and 8 introns.

Genes that are readily adjustable—for example, those that respond to stress—generally exhibit a smaller number of introns, which results in a slower response time for the production of the protein and gives to these genes them a selective advantage [38]. The presence of introns may

| Species       | Gene name | Gene ID            | Location     | ORF size (bp) | Quantity of introns | Quantity of exons |
|---------------|-----------|--------------------|--------------|---------------|---------------------|-------------------|
| *C. clementina* | CcAOXa   | clementine0.9_012574m | Chromosome 2 | 1150          | 8                   | 9                 |
|               | CcAOXb   | clementine0.9_034013m | Chromosome 5 | 927           | 3                   | 4                 |
|               | CcAOXc   | clementine0.9_015158m | Chromosome 5 | 1011          | 3                   | 4                 |
|               | CcAOXd   | clementine0.9_015716m | Chromosome 8 | 978           | 3                   | 4                 |
| *C. sinensis* | CsAOXa   | orange1.1g018664m   | Chromosome 2 | 1050          | 8                   | 9                 |
|               | CsAOXa*  | orange1.1g022654m   |              | 8             | 7                   | 8                 |
|               | CsAOXb   | orange1.1g037339m   | Chromosome 3 | 852           | 3                   | 4                 |
|               | CsAOXc   | orange1.1g019765m   | Chromosome 3 | 1008          | 3                   | 4                 |
|               | CsAOXd   | orange1.1g020532m   | Chromosome 8 | 960           | 3                   | 4                 |

https://doi.org/10.1371/journal.pone.0176878.t001
result in production delays due to the steps required for splicing and transcription, as well as
an additional energy costs caused by the additional length of the nascent transcript [38].
Colinearity analysis was performed for the AOX genes in the C. clementina and C. sinensis
genomes using the MCScanX toolkit, and the analysis showed that the citrus AOX genes did
not come from duplication events (data not shown).

Promoter sequence analysis of the citrus AOX genes

A fragment belonging to the upstream region of each AOX gene was analyzed to find plant-
specific cis-elements using the PlantCARE database. Except for the CsAOXd gene, for which
the only fragment available in the Citrus Genome Database was 353 bp in length, the promoter
fragment size used was 1500 bp (S2 Fig). The TATA and CAAT-box elements were found in
all citrus AOX promoter regions (S3 Fig); the other cis-elements varied between sequence pro-
moters (Fig 2, S3 Fig). Most of the cis-elements (quantity of 4 to 21, according to the promoter)
were involved in the response to light (Fig 2). In smaller proportions, cis-elements were found
that were responsive to i) hormones or inducers such as methyl jasmonate (MeJA), gibberellin,
ethylene, auxin, abscisic acid and salicylic acid; and ii) biotic, abiotic or mechanical stresses
such as drought, wounds, heat, low temperature, fungal elicitors and anaerobiosis. Others cis-
elements related to plant development such as zein metabolism, endosperm expression, differ-
etiation of palisade mesophyll cells, meristem expression, circadian control and leaf morphology
were also present in the promoters of the citrus AOX genes (Fig 2). This analysis revealed a
large number of motifs responding to different external or endogen inductions, suggesting a
complex regulation of AOX gene expression. Under stress conditions, it is common to observe
the accumulation of reactive oxygen species and/or of molecules or ion such as salicylic acid,
jasmonate, calcium and ethylene in the organism [39]. All these signaling molecules have the
ability to induce AOX gene expression [40–42]. Indeed, the overexpression of AOX genes has
already been reported in response to a number of biotic and abiotic stresses [5, 43, 44]. In Ara-
bidopsis thaliana, the mutants AOX1a-deficient and AOX1b-deficient were more severely
photodamaged by high light intensity when compared with wild-type plants [45]. These results
indicated that in high light intensity conditions, AOX1a and AOX1b genes may favor plant
adaptation. According to Feng et al. [8], light may induce AOX gene expression by increasing
ROS production.
Analysis of the citrus AOX proteins

The number of amino acid residues of the citrus AOX proteins ranged from 284 (CsAOXb) to 349 (CsAOXa) (Table 2). All proteins were predicted to be located in mitochondria (73.00% to 99.65%).

Table 2. Characteristics of the AOX proteins present in the citrus genomes. **GRAVY**: grand average of hydropathicity; **Mw**: molecular weight; **pl**: isoelectric point; **SP**: signal peptide. *Protein resulting from the alternative transcript of the CsAOXa gene.

| Protein   | Protein size (aa) | pl with/without SP | Mw with/without SP (kDa) | Export probability to mitochondria (%) | SP size (aa) | GRAVY   |
|-----------|-------------------|--------------------|--------------------------|----------------------------------------|--------------|---------|
| CcAOXa    | 349               | 6.09 / 5.26        | 39.9 / 34.5              | 99.55                                  | 49           | -0.210  |
| CcAOXb    | 309               | 8.27 / 6.36        | 35.2 / 30.1              | 99.47                                  | 45           | -0.329  |
| CcAOXc    | 336               | 8.81 / 6.68        | 38.1 / 32.8              | 99.36                                  | 49           | -0.384  |
| CcAOXd    | 325               | 8.29 / 6.68        | 37.1 / 33.9              | 73.00                                  | 30           | -0.183  |
| CsAOXa    | 349               | 5.64 / 5.07        | 40.2 / 34.7              | 99.65                                  | 49           | -0.246  |
| CsAOXa*   | 294               | 7.06 / 5.56        | 34.2 / 28.7              | 99.69                                  | 49           | -0.145  |
| CsAOXb    | 284               | 6.60 / 6.07        | 32.2 / 28.8              | 93.36                                  | 31           | -0.310  |
| CsAOXc    | 335               | 8.60 / 6.49        | 37.9 / 32.7              | 98.98                                  | 48           | -0.381  |
| CsAOXd    | 319               | 8.29 / 6.49        | 36.4 / 33.7              | 81.90                                  | 24           | -0.191  |

https://doi.org/10.1371/journal.pone.0176878.t002

Fig 2. **Cis-elements present in the promoter region of citrus AOX genes.** The cis-elements were analyzed in the upstream promoter region of the translation start site using the plantCARE database.

https://doi.org/10.1371/journal.pone.0176878.g002
Table 3. Post-translational modifications of citrus AOX proteins. * Protein resulting from the alternative transcript of the CsAOXa gene.

| Protein | Phosphorylation sites | N-glycosylation sites |
|---------|-----------------------|-----------------------|
| CsAOXa  | T4, T8, T27, T30, T81, T127, T198, T223, T281, T290, T343, S6, T10, S13, S21, S37, S38, S41, S43, S66, S67, S87, S162, S205, S213, S218, S267, S304, S309, S319, S342, Y119, Y126, Y200, Y276 | - |
| CsAOXb  | T3, T7, T29, T88, T119, T144, T188, T241, T273, S2, S13, S27, S36, S48, S50, S51, S52, S53, S54, S55, S56, S57, S58, S65, S67, S68, S69, S81, S161, S168, S221, S330, S351, S277, Y1, Y117, Y240, Y246, Y293 | N22 |
| CsAOXc  | T12, T14, T20, T31, T37, T52, T131, T132, T141, T166, T210, T263, T284, T294, S11, S19, S36, S47, S51, S88, S134, S144, S183, S190, S243, S261, S290, S329, Y116, Y286 | N49, N292 |
| CsAOXd  | T26, T104, T120, T121, T130, T138, T155, T199, T252, T283, T289, S8, S90, S110, S255, S256, S263, V54, Y128 | - |
| CsAOXa  | T4, T8, T27, T30, T81, T127, T198, T223, T281, T290, T343, S6, T10, S13, S21, S37, S38, S41, S43, S66, S67, S87, S162, S205, S213, S218, S267, S304, S309, S319, S342, Y119, Y126, Y200, Y276 | - |
| CsAOXa* | T4, T8, T27, T30, T81, T127, T198, T223, T281, S6, S10, S13, S21, S37, S38, S41, S43, S66, S67, S87, S162, S205, S213, S218, S267, S304, S309, S319, S342, Y119, Y126, Y200, Y276 | - |
| CsAOXb  | T15, T74, T105, T130, T174, T227, T258, S11, S13, S16, S22, S33, S34, S36, S37, S38, S40, S41, S42, S43, S44, S45, S46, S47, S147, S154, S207, S229, S237, S257, Y103, Y226, Y293 | N8 |
| CsAOXc  | T11, T13, T19, T30, T51, T130, T131, T140, T166, T203, T247, T283, T292, S10, S18, S25, S46, S50, S87, S133, S143, S162, S189, S242, S260, S291, S328, Y256, Y134 | N48, N291 |
| CsAOXd  | T20, T98, T114, T115, T124, T149, T193, T246, T277, T283, S14, S46, S47, S49, S50, S51, S52, S54, S71, S86, S104, S166, S173, S226, S244, S254, S256, Y48, Y122 | - |

https://doi.org/10.1371/journal.pone.0176878.t003
The motif analysis of the predicted citrus AOX proteins by the MEME program showed that the mandarin and orange AOX proteins contained the typical LET, NERMHL, LEEEA and RADEV conserved motifs (Fig 3; S4 Fig). These motifs were found in AOX proteins from other plant species [47]. The hydropathicity analysis revealed a profile with two hydrophobic regions for all the citrus AOX proteins (data not shown).

Phylogeny analysis

Phylogenetic analysis of the AOX citrus and A. thaliana sequences showed that the CcAOXb and CsAOXb were closed to the AtAOX1D sequence while CcAOXd and CsAOXd were closed to AtAOX2 (Fig 4). The CcAOXc and CsAOXc sequences were grouped with three A. thaliana sequences AtAOX1A, AtAOX1C e AtAOX1B (Fig 4). The CcAOXa, CsAOXa, CsAOXb* constituted a separated group in the phylogenetic tree, without proximity with the A. thaliana sequences. The comparative analysis of the citrus and A. thaliana sequences did not allow a clear classification of the citrus AOX sequences in relation to A. thaliana ones, mainly in the case of AOXa, AOXa* and AOXc.

| Species         | Motif (E-value) | Consensus sequence |
|-----------------|-----------------|--------------------|
| C. clementina   | 9.2e-087        | FFRR...           |
|                 | 2.4e-071        | KLLEEA...         |
|                 | 1.3e-021        | LVSYW...          |
|                 | 1.2e-008        | PTFD...           |
|                 | 9.9e-004        | FFNA...           |

| C. sinensis     | 1.1e-052        | GYV...            |
|                 | 3.9e-054        | LEKL...           |
|                 | 8.2e-039        | GOWK...           |
|                 | 1.2e-023        | GSJENX...         |
|                 | 1.7e-017        | QGUKL...          |

Fig 3. Conserved motifs in citrus AOX proteins obtained by the MEME program.

https://doi.org/10.1371/journal.pone.0176878.g003
The best alignment of the citrus AOX proteins with the Pdb was obtained between the CsAOXc and CcAOXc protein orthologues and the AOX protein from *Trypanosoma brucei* (TbAOX, PDB ID: 3VV9, MMDB ID: 108244). The protein CcAOXc was chosen for the molecular modeling and the subsequent docking. The alignment of the amino acid sequences of CcAOXc and TbAOX presented 68% coverage, 45% identity (E-value 7e-55) and an RMSD of 2.85 Å (Fig 5A); these values (identity >25%) indicate that the TbAOX protein is a good model to be used as a template [48]. The validation analysis (Ramachandran plot) of the CcAOXc model showed that 92.9% of residues was in most favored regions and 5.7% was in additional allowed regions, indicating that 98.6% of the amino acid residue was located in favored regions (S6 Fig). In addition, ANOLEA showed good energy values as well (S6 Fig).

The 3-D model of CcAOXc showed a total of six helices, two of them anchored in the inner membrane of the mitochondria, and the other fourth helices–rich in histidine and glutamate–were in contact with the mitochondrial matrix (Fig 5B). The first transmembrane helix has 21
Fig 5. Tridimensional structure of CcAOXd obtained by homology modeling with the T. brucei AOX (Pdb code 3VV9) as a template. A. Alignment of TbAOX and CcAOXd proteins. Gaps introduced to get the best alignment are indicated by (-). Highly conserved domains related to protein structure and activity are indicated in grey. Identical amino acids are indicated by an asterisk (*), conservative substitutions by a colon (:) and semiconserved substitutions by a period (.). B. Representation of the 3-D structure of CcAOXd (in Alternative oxidase family in citrus PLOS ONE | https://doi.org/10.1371/journal.pone.0176878 May 1, 2017
amino acid residues in the positions 150–170, and the second has 20 residues in the positions 112–131 (Fig 5A and 5B). The length of the two transmembrane helices is compatible with the length required to cross the mitochondrial membrane. The largest portion of the CcAOXd protein remained in contact with the mitochondrial matrix, with only few residues anchored in the mitochondria membrane, which explains the negative values of hydropathicity, typical of cytoplasmic proteins (Table 2). CcAOXd presents 4 highly conserved domains (LET, NERMHL, LEEEA and RADE-H; Fig 5A, S4 Fig) that contains histidine and glutamate residues responsible for the interaction with the iron atoms; all these elements constitute the diferric center of the AOX enzyme (Fig 5C) [46, 49]. This association with iron atoms classifies the AOX proteins as belonging to the R2 subunit of ribonucleases [46, 49]. Two cysteine residues [C68(I) and C118(II)] that are conserved in the AOX proteins of different plant species and assumed to be involved in the redox regulation of AOX activity were identified in the CcAOXd structure (C68(I) and C118(II); Fig 5D). C68(I) and C118(II) also play a role in the post-translational regulation of most angiosperm AOX proteins [50]. The CcAOXd structure contains a redox-active Y221 that is highly conserved across other AOX proteins [47, 51] and that could play a key role in the AOX catalytic site (Fig 5F). The active site, which is located in a hydrophobic environment deep inside the CcAOXd molecule, is composed of the diiron center as well as 4 glutamate (E124, E163, E214 and E265) and 2 histidine (H166 and H268) residues, all of which are highly conserved among AOX proteins (Fig 5F). Molecular docking results presented an affinity energy of -7.0 Kcal/Mol and indicated that UQ bound to CcAOXd in a recessed pocket formed between the helices and submerged into the membrane (Fig 5D and 5E); the pocket is formed by Arg105, Asp109, Arg119, Leu123, Glu124, Ala127, Glu163, Leu213, Glu214, Glu216, Ala217 and Glu265 amino acid residues. The 2-D map of the interaction between CcAOXd and UQ showed the van der Waals, carbon hydrogen bonds and alkyl interactions, among others, which related the CcAOXd proteins to UQ (S7 Fig). As in TbAOX, this second cavity connects the diiron active site with the outer mitochondrial membrane and interacts with the inhibitor-binding cavity at the active site [52].

In silico CsAOX gene expression

The expression of the CsAOX genes was previously obtained and was available in the CAP database [31]. Four tissues were analyzed: callus, flower, leaf and fruit (Fig 6). The CsAOXa, CsAOXc and CsAOXd showed high expression levels (>3 Reads Per Kilobase Million/RPKM excepted for CsAOXc in leaf) while the CsAOXb was lowly expressed (<1 RPKM) (Fig 6). The CsAOXa gene was highly expressed in the fruit (17.5 RPKM) but also showed significant expression levels in callus, flower and leaf (6.6, 5.9 and 4.3 RPKM, respectively; Fig 6). The CsAOXc gene showed the highest expression level in callus (78.5 RPKM) and significant expression levels in fruit and flower (7.68 and 3.37, respectively; Fig 6). The CsAOXd gene presented similar expression in callus and fruit (about 15 RPKM) and also close values of expression in flower and leaf (8.4 and 7.2, respectively; Fig 6). These results showed that the CsAOX...
family members were spatially differentially expressed among citrus organs; some similar results were previously described in A. thaliana [53, 54]. The very high expression of the CsAOXc in callus could be correlated with high expression level of AtAOX1A and AtAOX1C – both phylogenetically closed to CsAOXc (Fig 4) – in chilling-stressed callus [53]. The relatively high expression of CsAOXa and CSAOXd in fruits (>15 RPKM; Fig 6) may be related to the expression of AOX genes from other species producing fruits such as tomato, papaya or mango [55–58]. Some AOX genes were related to fruit maturation, ripening and post-harvest ripening in association with ethylene peak emission (climacteric fruits) [56, 57], while other

Fig 6. Expression of CsAOX genes in different C. sinensis tissues.

https://doi.org/10.1371/journal.pone.0176878.g006
AOX genes were associated to gametophyte development [58]. Some AOX genes related to climacteric fruit ripening presented elements responsive to ethylene in their promoter sequences [57]. Here, the CsAOXa and CSAOXd genes did not present any elements responsive to ethylene in their promoter sequences; this could be related to the fact that citrus are non-climacteric fruits, or may suggest an involvement of these CsAOX genes in fruit formation more than in fruit ripening (Figs 2 and 6).

Conclusion

To the best of our knowledge, this is the first characterization of the AOX gene family in C. clementina and C. sinensis. Four AOX genes were identified in each species; the C. clementina genes were orthologues of the C. sinensis genes. Phylogenetic analysis of the AOX citrus and A. thaliana sequences showed that the CcAOXb and CsAOXb were closed to the AtAOX1D sequence while CcAOXd and CsAOXd were closed to AtAOX2. According to the cis-element present in the citrus AOX promoters, the gene expression may be regulated by several external or internal factors. Expression of CsAOX genes revealed that CsAOXc was highly expressed in callus while CsAOXa and CsAOXd were highly expressed in fruits. Other regulation levels were also predicted, such as alternative splicing and post-translational modifications. The corresponding proteins were predicted to be directed to the mitochondria, and the analysis of the 3-D structure of one the C. clementina AOX isoforms showed the presence of two hydrophobic helices that may be involved in the anchoring of the protein in the inner mitochondrial membrane. The active site of the protein is located in a hydrophobic environment deep inside the AOX structure and contains a diiron center. The molecular docking of CcAOXd with UQ showed that the binding site is a recessed pocket formed by the helices and submerged into the membrane. These data are important for future functional studies of citrus AOX genes and/or proteins, as well as for biotechnological approaches leading to AOX inhibition using UQ homologs.

Supporting information

S1 Fig. Nucleotide sequences of AOXs from C. clementina and C. sinensis from the Citrus Genome Database. (DOCX)

S2 Fig. Promoter sequence of the citrus AOX genes (1500 bp upstream, except for CsAOXd). (DOCX)

S3 Fig. List of the cis-elements found in the promoter regions of the citrus AOX genes. (DOCX)

S4 Fig. Amino acid sequences of AOX from C. clementina and C. sinensis. (DOCX)

S5 Fig. Amino acid sequence identity of CcAOXs and CsAOXs. (DOCX)

S6 Fig. Modeling validation of the CcAOX structure using the Ramachandran plot and the ANOLEA analysis. (DOCX)

S7 Fig. 2-D map of the interaction between CcAOXd and UQ. (DOCX)
Acknowledgments

We thank Dr. Sara Pereira Menezes (UESC) for advice on the bioinformatics analysis, and Dr. Marcio Costa (UESC) and Tahise Magalhães de Oliveira (UESC) for their contribution to the organization of the manuscript. We thank the Núcleo de Biologia Computacional e Gestão de Informações Biotecnológicas (NBCIGB) from UESC for providing the infrastructure for the bioinformatics analysis. This work was made in the frame of the Consortium International in Advanced Biology (CIBA).

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