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Saponin determination, expression analysis and functional characterization of saponin biosynthetic genes in *Chenopodium quinoa* leaves

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ABSTRACT

Quinoa (*Chenopodium quinoa* Willd.) is a highly nutritious pseudocereal with an outstanding protein, vitamin, mineral and nutraceutical content. The leaves, flowers and seed coat of quinoa contain triterpenoid saponins, which impart bitterness to the grain and make them unpalatable without postharvest removal of the saponins. In this study, we quantified saponin content in quinoa leaves from Ecuadorian sweet and bitter genotypes and assessed the expression of saponin biosynthetic genes in leaf samples elicited with methyl jasmonate. We found saponin accumulation in leaves after MeJA treatment in both ecotypes tested. As no reference genes were available to perform qPCR in quinoa, we mined publicly available RNA-Seq data for orthologs of 22 genes known to be stably expressed in *Arabidopsis thaliana* using geNorm, NormFinder and BestKeeper algorithms. The quinoa ortholog of *At2g28390* (*Monensin Sensitivity 1, MON1*) was stably expressed and chosen as a suitable reference gene for qPCR analysis. Candidate saponin biosynthesis genes were screened in the quinoa RNA-Seq data and subsequent functional characterization in yeast led to the identification of *CqbAS1*, *CqCYP716A78* and *CqCYP716A79*. These genes were found to be induced by MeJA, suggesting this phytohormone might also modulate saponin biosynthesis in quinoa leaves. Knowledge of the saponin biosynthesis and its regulation in quinoa may aid the further development of sweet cultivars that do not require postharvest processing.

1. Introduction

Chenopodium quinoa Willd. (quinoa), a member of the Amaranthaceae (formerly Chenopodiaceae) plant family is an annual herb native to the Andean region, where it is extensively cultivated as a food crop in many South American countries. Quinoa is a pseudocereal with high nutritional value. The seeds have a high protein content (15%) with a good balance of amino acids, minerals, vitamins and other nutraceuticals like polyphenols and flavonoids [1,2]. The outer layers of quinoa seeds accumulate saponins, which represent the major anti-nutritional factor in the grain [3]. The presence of saponins in the seed coat (over 0.11%) makes the quinoa grain extremely bitter, hence the need for postharvest processing of the grain to remove the saponins [3]. Saponins can be removed by either washing the grain or by mechanical dehulling. As the former method requires large amounts of clean water and the latter method requires specialized machinery, quinoa cultivars with

Abbreviations: *βAS*, β-amyrin synthase; AOS, Allene Oxide Synthase; *CqbAS1*, Chenopodium quinoa β- amyrin synthase; OSC, 2,3-oxidosqualene cyclase; MON1, Monensin Sensitivity 1; GC–MS, Gas Chromatography–Mass spectrometry; qPCR, Quatitative Real-Time PCR; RNA-seq, RNA-Sequencing; MeJA, Methyl Jasmonate.

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reduced saponin content in the seed coat are desired [4]. Accumulation of saponins is not restricted to the seeds, but also occurs in leaves and, to a lesser extent, in flowers and fruits of the quinoa plant [5–7]. Previous studies were focused on the identification and quantification of saponins in seeds [7–10]. In terms of transport, saponins have been shown to be synthetized in some plant tissues such as roots and not translocated to others as in the case of *Gypsophila paniculata* [11]. However, it is hypothesized that the accumulation of these metabolites in the quinoa grain is the result of increased concentration and remobilization from aerial source tissues during plant development [12]. Besides, saponin translocation from aerial parts to storage tissues such as rhizomes has been reported in *Paris polyphylla* and in flowers and roots of *Calendula officinalis* [13,14]. Consequently, our research attempts to identify genes involved in the biosynthetic pathway of these metabolites in quinoa leaves, a vegetative tissue where the synthesis of these compounds has been demonstrated in plant species such as *Saponaria vaccaria* and *Medicago truncatula*, well-known saponin sources within the plant kingdom [15,16].

Saponins comprise a large group of structurally diverse bioactive specialized metabolites produced by many plant species. They are amphipathic compounds comprised of a hydrophobic triterpenoid backbone and hydrophilic sugar moieties. They display a wide array of commercial applications in the pharmaceutical, food and cosmetic sectors [17–19]. In plants, the primary mevalonate pathway generates the precursors for the biosynthesis of triterpenoid saponins. The condensation of two units of isopentenyl pyrophosphate (IPP) with one unit of its allylic isomer dimethylallyl pyrophosphate (DMAPP) yields farnesyl pyrophosphate (FPP). The subsequent condensation of two units of FPP results in the triterpene precursor squalene which is composed of 30 carbon atoms [20]. Squalene is then epoxidized to 2,3-oxidosqualene, the dedicated precursor for all triterpenes in higher plants. This reaction catalyzed by the 2,3-oxidosqualene cyclase (OSC) family of proteins typically cyclizes the linear precursor to tetra- or pentacyclic structures of the cycloartane-, oleanane-, ursane-, taraxasterane-, dammarane-, lupane-, cucurbitane-, hopane-, lanostane- or tirucallane-type [21]. The cyclization products therefore are the committed precursors for the synthesis of a specific branch of saponins. For instance, β-amyrin is the dedicated triterpene precursor for the oleanane-type triterpenoid saponins and is formed by the cyclization of 2,3-oxidosqualene by β-amyrin synthase (bAS). The triterpene precursor then undergoes a series of oxidative reactions by one or more cytochrome P450-dependent monooxygenases (P450s) to form highly decorated triterpenoid structures known as sapogenins. The subsequent glycosylation of sapogenins by UDP-dependent glycosyltransferases (UGTs) results in the synthesis of saponins, which may be further modified by acyl-, malonyl- or methyltransferases [22].

C. quinoa accumulates a complex mixture of pentacyclic oleanane-type triterpenoid saponins [7,23,24]. A majority of these saponins are derived from the triterpenoid oleanolic acid that is in turn derived from β-amyrin and that undergoes further P450-mediated oxidations and/or glycosylation by UGTs. However, the saponin biosynthesis pathway and the corresponding enzymes have not yet been completely elucidated from *C. quinoa* [9,10]. In several plant species, the saponin biosynthesis is inducible by the phytohormone jasmonate (JA) [25,26]. Exogenous application of jasmonates (JAs) leads to the elicitation of saponin biosynthesis and could be used as a tool to identify biosynthetic and regulatory genes involved in their biosynthesis. However, the JA responsiveness of saponin biosynthesis in *C. quinoa* is currently unknown.

Here, we analyzed saponin content in leaves treated with methyl jasmonate (MeJA) using a spectrophotometric methodology. In addition, candidate enzymes involved in saponin biosynthesis and reference genes used for qPCR were identified using publicly available *C. quinoa* RNA-Seq data. Subsequently, three candidate enzymes were functionally characterized using a heterologous yeast expression system. qPCR analysis revealed that the expression of *bAS*, encoding the first committed enzyme for saponin biosynthesis in quinoa, is JA-responsive, as well as two P450s involved in further oxidations of triterpenoid saponins.

2. Materials and methods

2.1. Chemicals

Methyl β-cyclodextrin (MβCD) was purchased from CAVASOL (Wacker, Germany), β-amyrin, erythrodiol and oleanolic acid from Extrasynthese (Lyon, France). MeJA 95% was obtained from Sigma-Aldrich (CAS Number: 39924-52-2).

2.2. Plant material

Seeds of sweet (ECU-225) and bitter (ECU-229) cultivars of *Chenopodium quinoa* Willd. (quinoa) were obtained from a germplasm collection of INIAP (National Institute of Agricultural Research) of Ecuador (MTA, 26-10-2012) [27]. Both ecotypes were collected at the Province of Pichincha, town of Quito, 00°21′S; 78°33′W, altitude 3050 m above sea level. Quinoa seeds were planted in an equal mixture of sterile soil and peat. The plants were watered with Hoagland's plant nutrient solution every week and grown under greenhouse conditions with an average temperature of 23 °C with a 12 h light/12 h dark regime for 2 months.

2.3. MeJA treatment and sampling

For saponin content determination and qPCR (detached leaf method), leaves were collected and dipped for 30 s in a solution containing 100 μM of MeJA (dissolved in ethanol) as described previously [28,29]. Solutions were prepared with 0.01% Tween-20 and control solution included ethanol proportional to MeJA treatment. After elicitation, leaves were placed in glass petri dishes placing paper at the bottom to avoid humidity accumulation. Samples were taken at different time intervals (8, 24 and 48 h) after MeJA treatment. Each measurement consisted of five biological replicates.

For qPCR analysis and gene cloning (entire plants), quinoa plants that reached the principal growth stage 4, according to the extended BBCH-scale [30], were drenched with a single dose of 100 μM MeJA solution (dissolved in ethanol). Control plants were drenched with control solution (distilled water and ethanol) instead of MeJA. In total 14 plants were selected for each quinoa ecotype, of which 7 were used for MeJA-treatment and the remaining plants were used as control. Three holes were made at the bottom of the pots and transferred to separate plastic trays containing 100 μM MeJA or distilled water/ethanol to facilitate solution absorption. Both groups of plants were separately enclosed in plastic containers for the treatment. Three leaves from MeJA-treated and control plants were collected and pooled at three time points 0, 8 and 24 h after drenching. The harvested leaves were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Three independent biological replicates were performed for each experiment.

2.4. Saponin extraction and quantification

Leaves were collected from MeJA treated and non-treated sweet and bitter quinoa plants at different time points upon MeJA elicitation. After elicitation, leaves were placed in glass petri dishes placing paper at the bottom to avoid humidity accumulation. Samples were taken at different time intervals (8, 24 and 48 h)

after MeJA treatment. Each measurement consisted of five biological replicates. One gram of dried sample was ground to a fine powder and dissolved in 20 ml of 20% isopropanol. The mixture was heated at 86 °C during 20 min for saponin extraction using microwave-assisted method and filtered (using Whatman paper) for further quantification [31,32]. Saponin levels were measured through spectrophotometry as described by Gianna with minor modifications [32]. The Liebermann-Burchards (LB) reagent was used for saponin quantification as it is capable of producing a light brown staining if these compounds are present in a sample. The LB reagent consisted of a 1:5 mixture of acetic acid and sulfuric acid, respectively. After mixing 1 ml sample solution with 3.5 ml of LB reagent, absorbance at 580 nm was measured in all samples after 10 min. A calibration curve based on pure quinoa saponins was used to determine the final concentration of saponins (mg/ml) in each solution on the basis of absorbance measurements (Absorbance = 4.5725 × Concentration of saponins + 0.0164) [32]. The % saponin content was calculated on the basis of fresh weight.

2.5. RNA extraction and cDNA synthesis

The frozen leaf tissue collected from MeJA treated and non-treated sweet and bitter-types was ground to a fine powder in liquid nitrogen and total RNA was isolated according to the protocol described by van Wees et al. [33]. Double stranded cDNA was synthesized from 2 μg of total RNA using an oligo-dT_{25} primer and purified using the Purelink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen).

2.6. Transcriptome assembly

The FASTQ files with the read sequences and quality scores were extracted from the NCBI Short Read Archive (accessions SRR799899 and SRR799901) using the NCBI SRA Toolkit version 2.1.7. For each accession, a *de novo* assembly of the reads was generated using the CLC genomics workbench 5.0.1 software with default settings (CLC bio, Aarhus, Denmark).

2.7. Selection of reference genes and qPCR analysis

qPCR primers were designed using Beacon Designer version 4.0 (Premier Biosoft International, Palo Alto, CA, USA) and qPCR was carried out in triplicate with a Lightcycler 480 (Roche) and SYBR Green QPCR Master Mix (Stratagene). For selection of reference genes, quinoa sweet and bitter types treated with or not with MeJA in different time points after application, were used at this stage to proof stability in different conditions. Stable reference genes were selected using the geNorm [34], NormFinder [35] and BestKeeper [36] algorithms, and relative gene expression quantification with MON1 and PTBP as reference genes was performed with qBase [37].

For *Allene Oxide Synthase* (*AOS*), *CqbAS1*, *CqCYP716A78* and *CqCYP716A79*, qPCR primers were designed using Primer3 software version 4.0.0 [38]. All reactions were performed in triplicate on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The reaction mix contained 1 μl of cDNA template, 0.3 μM of each primer, 1X SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) and PCR water to reach a final volume of 10 μl. Amplification specificity was assessed through melting curve analysis. MON1 was used as the reference gene and the Second Derivative Maximum ($2^{-\Delta\Delta Ct}$) was applied for data normalization [39].

2.8. Cloning of full length genes

Full-length coding sequences of *CqbAS1*, *CYP716A78*, *CYP716A79* and *CYP716AB1* were amplified from cDNA of sweet type quinoa (leaves) treated with MeJA using the primer pairs described in Table S1. The amplicons were Gateway® recombined into pDONR221 to generate entry clones which were sequence verified. Full DNA sequences of genes found in this study can be view at NCBI/Genbank under the accession numbers: *CqbAS1* (KX343074), *CYP716A78* (KX343075), *CYP716A79* (KX343076), and *CYP716AB1* (KX343077).

2.9. Phylogenetic analysis

The protein sequences (Genbank) were aligned with ClustalW and the phylogenetic tree generated using MEGA 5.10 software [40] with the Maximum-Likelihood method and bootstrapping for 1000 replicates. The evolutionary distances were computed with the Poisson correction method and all positions containing gaps and missing data were eliminated from the dataset using the complete deletion option.

2.10. Construction of yeast destination vector pESC-URA-tHMG1-DEST

The Gateway cassette was amplified from pDEST14 (Invitrogen) using primers P21 and P22, and cloned into pJET1.2 for sequence verification. The cassette was subsequently excised by digestion with *Bcl*I and *Nhe*I. Simultaneously, the pESC-URA[*GAL10/tHMG1*] [41] was linearized using *Bam*HI and *Nhe*I. Both the insert and linearized vector were gel purified and ligated using T4 DNA ligase (Invitrogen) according to manufacturer's protocol. The resulting destination vector pESC-URA-tHMG1-DEST was sequence verified before use.

2.11. Generation and cultivation of yeast strains

The yeast strains TM102 and TM103 were generated from strain TM1 and cultivated as described [41]. The yeast cultures were first grown in synthetic defined (SD) medium containing glucose with appropriate dropout (DO) supplements (Clontech) for 20 h at 30 °C with agitation. To induce heterologous gene expression, the cultures were washed and inoculated in SD Gal/Raf medium containing galactose and raffinose with appropriate DO supplements (Clontech) to a starting optical density of 0.25 on day 1. The induced cultures were incubated for 24 h and on day 2, methionine and MβCD were added to 1 mM and 5 mM, respectively. After further 24 h incubation, MβCD was added once again to 5 mM on day 3, and on day 4 all cultures were extracted with hexane for metabolite analysis.

2.12. GC–MS analysis

GC–MS analysis was performed using a GC model 6890 and MS model 5973 (Agilent). A VF-5 ms capillary column (Varian CP9013, Agilent) was operated at a constant helium flow of 1 ml/min and 1 μl of the sample was injected in splitless mode. The oven was initially held at 80 °C for 1 min, ramped to 280 °C at a rate of 20 °C/min, held at 280 °C for 45 min, ramped to 320 °C at a rate of 20 °C/min, held at 320 °C for 1 min, and at the end of the run cooled to 80 °C at a rate of 50 °C/min. Throughout the analysis, the injector was set to 280 °C, the MS transfer line to 250 °C, the MS ion source to 230 °C, and the quadrupole to 150 °C. A full EI mass spectrum was generated by scanning the *m*/*z* range of 60–800 with a solvent delay of 7.8 min.

2.13. Statistical analysis

The statistical significance of gene expression levels was assessed using t statistics. GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA) was used to perform the analysis. A *p*-value

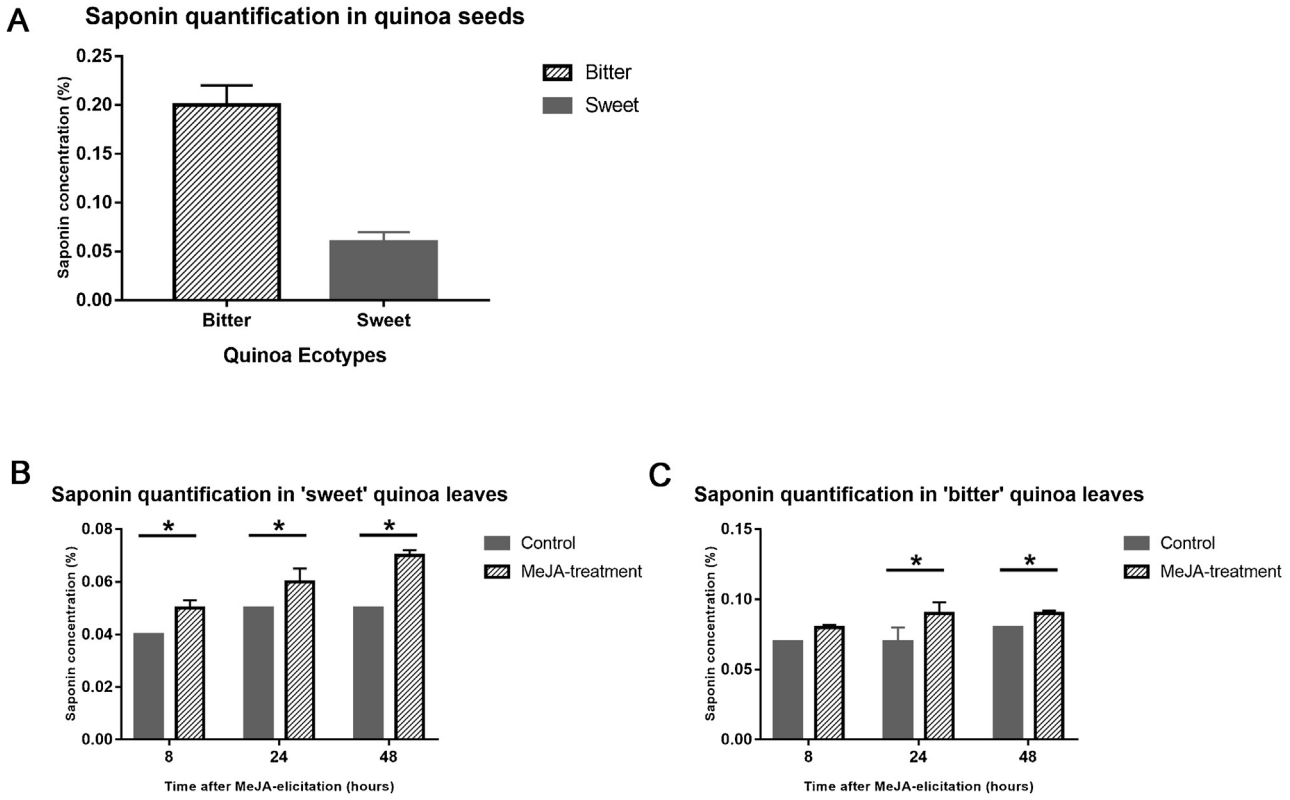

Fig. 1. Saponin content in quinoa organs. (A) Saponin content (%) in seeds in 'bitter' and 'sweet' ecotype. (B and C) Saponin content (%) in leaves of non-treated and MeJA-treated samples measured at three time intervals (8, 24 and 48 h) after MeJA application in ísweetí (B) and 'bitter' (C). Error bars designate standard deviation of the mean (n = 5). Statistical significance was determined by Student's *t*-test (**p-value* <0.05).

less than 0.05 was considered as statistically significant. Biological replicates were mentioned in each performed experiment.

3. Results and discussion

3.1. Saponin content assessment in leaves of 'sweet' and 'bitter' quinoa

Based on the saponin content of the seeds, quinoa varieties are often classified as 'sweet' when they have no or less than 0.11% saponins, or as 'bitter' when the saponin content is higher than 0.11% [3]. Fig. 1A shows saponin quantification in seeds to prove the contrasting saponin content between 'sweet' and 'bitter' ecotypes used in this study. For the purpose of our analysis, we quantified the saponin content of leaves of 'sweet' and 'bitter' quinoa ecotypes using spectrophotometric methods. Saponin concentration in the leaves was lower than in the seeds in 'bitter' type compared to 'sweet' type which did not have major difference (Fig. 1A–C). Moreover, saponin concentration significantly increased in response to MeJA in leaves of both cultivars with respect to the control (8, 24 and 48 h after elicitation) (Fig. 1B and C). In order to check JA responsiveness upon MeJA treatment, Allene oxide synthase (AOS) a key gene in the JA biosynthesis was analyzed by qPCR. As a result, gene expression analysis revealed the upregulation of *AOS* in both cultivars, confirming that MeJA elicitation was successfully accomplished [42] (Fig. S1A and B). In previous studies, it was demonstrated that saponin content in plant tissue could change upon environmental conditions and stress such as drought, salinity or cold [10,43]. In this study, the results at the transcriptome and metabolome level support the role of the phytohormone JA, a well-known activator of defense responses, as an elicitor capable of boosting saponin accumulation and biosynthesis in quinoa leaves [44–46].

3.2. Identification of housekeeping genes in C. quinoa needed for qPCR analysis

A commonly used technique for gene expression analysis is Quantitative Real-Time PCR (qPCR). For qPCR analysis, the expression of a gene is normalized using the expression data of stable reference genes. As knowledge of such stable reference genes is lacking for quinoa, we first screened and validated reference or housekeeping genes for qPCR analysis. Despite its importance as a food crop, only limited genomic and transcriptomic information is available for quinoa. Through RNA-Sequencing (RNA-Seq), currently the most performing transcript profiling method, large amounts of transcriptome data typically consisting of millions of short sequence reads are being generated [47]. For quinoa, a few of these RNA-Seq libraries are publicly available through the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/). Two of these libraries SRR799899 and SRR799901 containing 189,606,614 and 184,251,795 single-end reads, respectively, of 20–50 base pairs were downloaded and used for *de novo* assembly of two quinoa transcriptome databases (Supplementary datasets 1 and 2). The first assembly (SRR799899, Supplementary dataset 1) contains a total of 88,877 contigs with an average length of 474 base pairs and maximum contig length of 8991 base pairs. The second assembly (SRR799901, Supplementary dataset 2) contains a total of 88,141 contigs with an average length of 477 base pairs and a maximum contig length of 8822 base pairs.

These quinoa transcriptome assemblies were screened for candidate reference genes by TBLASTX searches using the nucleotide sequences of 22 stably expressed housekeeping genes reported in

Arabidopsis thaliana [48]. Nine genes with only one ortholog in the quinoa transcriptome assemblies were retained as candidate reference genes (Table 1). For the remaining genes, multiple orthologs with high sequence similarity to each other were identified. To avoid non-specific qPCR amplification these genes were not considered for further analysis. qPCR primers were designed for the nine candidate reference genes (Table 1) and used to generate quantification cycle values for each primer set using cDNA samples of sweet and bitter types of quinoa treated or not with MeJA. Subsequently, stable reference genes were selected using the geNorm [34], NormFinder [35], and BestKeeper [36] algorithms.

In the geNorm algorithm, an *M* value is calculated for all the candidate reference genes. The *M* value for a particular gene reflects the variation in expression of that gene compared to all other tested genes. As it is assumed that the expression ratio of two perfect reference genes is constant, the gene with the highest *M* value is eliminated from the gene set. This process is repeated until there are only two genes left, which are considered the optimal reference genes. For the candidate quinoa reference genes, geNorm analysis showed that *MON1* and *PTBP* were the most stable, followed by *F17M5* (Table 1). The second algorithm, NormFinder, uses a mathematical model of gene expression to identify the most stable reference genes. The NormFinder algorithm ranked the candidate reference genes in the same order as the geNorm analysis, with *MON1* and *PTBP* being the most stable (Table 1). The third algorithm, BestKeeper calculates a BestKeeper index of the quantification cycle values and subsequently a coefficient of correlation (R) of each reference gene to the BestKeeper index. Also this analysis revealed that *MON1* was the most stable reference gene, followed by *F17M5* and *PTBP*, respectively (Table 1). In conclusion, *MON1* was the most stable reference gene in all the three analyses, with *PTBP* and *F17M5* being the second and third most stable reference genes (Fig. S2).

3.3. *Cloning and functional characterization of* CqbAS1

The bAS enzyme is expected to catalyze the first committed step in the biosynthesis of pentacyclic triterpenoid saponins known to accumulate in quinoa [7]. We performed a TBLASTX analysis of the transcriptome assemblies (Supplementary datasets 1 and 2) using the nucleotide sequence of the characterized *Maesa lanceolata* β-amyrin synthase (GenBank accession KF425519; [49]) to identify putative *bAS* genes in quinoa. Only one full-length OSC that clustered together with other specific bAS enzymes (Fig. 2A) was recovered. The MeJA-responsiveness of this quinoa ortholog of the *bAS* gene that we annotated as *CqbAS1*, was assessed in sweet and bitter quinoa plants (Fig. 2B and C) as well as in detached leaf material of both cultivars (Fig. S3A and B). The qPCR analysis demonstrated higher levels of *CqbAS1* transcripts in both ecotypes upon MeJA-treatment, in the whole-plant analysis as well as in detached leaf material, further supporting the existence of MeJA-dependent induction of saponin biosynthesis in *C. quinoa* leaves. Nevertheless, sweet and bitter-types displayed different expression patterns of *CqbAS1*. A significant increase in the expression of *CqbAS1* was observed 24 h after MeJA elicitation in the bitter ecotype, whereas strong upregulation occurred already after 8 h in the sweet ecotype as a result of MeJA treatment. Sweet quinoa type seems to be rapidly responsive to MeJA whereas in bitter quinoa the effect of the elicitor is delayed.

To further investigate its role in the triterpenoid saponin biosynthesis pathway, we cloned the 2292 bp full-length *CqbAS1* gene that encodes a predicted protein of 763 amino acids. A multiple alignment of CqbAS1 with characterized bAS proteins identified the MWCYCR motif and the Lys449, which are absolutely conserved in all specific β-amyrin synthases [50,51]. Additionally, the highly conserved repetitive R/K-G/A-X_{2-3}-Y/F/W-L-X_3-Q-(X_{2-5}/X_3-G-X)-W or QW motifs and the SDCTAE motif involved in the binding

Table 1
Candidate reference genes, primer sequences, qPCR efficiency and expression stability values.

At-code	Gene description	Name	Primer Sequence (5′ to 3′)	Product (bp)	PCR Efficiency	Stability values		
						geNorm[a]	NormFinder[b]	BestKeeper[c]
At2g28390	Monensin sensitivity 1	MON1	Fw-GTATTGGTGGTCCTTGTG Rv-CTGCTGTGGTGTATTGATT	104	1.958	0.45	0.18	1.00
At2g32170	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	Exp1	Fw-GACATTGGCATTGGACAT Rv-GATTGAGAGTGGTTGAGAATA	105	1.899	1.59	1.50	0.88
At3g01150	Polypyrimidine tract-binding protein	PTBP	Fw-ACTATACCAATCCTTACC Rv-AAGAACATCAACAGTAAC	155	1.921	0.45	0.18	0.97
At3g53090	Ubiquitin-protein ligase 7	UPL7	Fw-GAACGTGATGTTAAGGAA Rv-CCATAGCATGAATGTATTG	142	1.899	1.20	1.15	0.57
At4g26410	Uncharacterized conserved protein	N2227	Fw-TTCTTGCTTCGCCATACT Rv-CCTGCTCTCCTTCTTCATTA	117	1.810	1.38	1.25	0.59
At4g33380	Unknown protein	F17M5	Fw-GAATGTTACCTTGACGATT Rv-GTATCCACAGTCCAATTAC	101	1.920	0.50	0.30	0.98
At4g34270	TIP41-like family protein	TIP41	Fw-AAGCATCATCAGCGAGAG Rv-CAGGTTGGTAGTCTTGGTATT	79	1.984	0.68	0.32	0.95
At5g15710	Galactose oxidase/kelch repeat superfamily protein	Fbox	Fw-GCATTGTAATCAGCAGAG Rv-TGTTCATCTTGGAATCATAC	131	1.900	0.80	0.52	0.88
At5g25760	Ubiquitin-conjugating enzyme	UBC	Fw-TTAATGGCGAACAGTAAT Rv-TAAGGAGATTGAGATGGA	149	1.575	1.00	0.92	0.80

[a] Average expression stability (*M*): the lower the *M* value, the more stable the expression.
[b] Stability value: the lower the stability value, the more stable the expression.
[c] Coefficient of correlation (R): the higher the R coefficient, the more stable the expression.

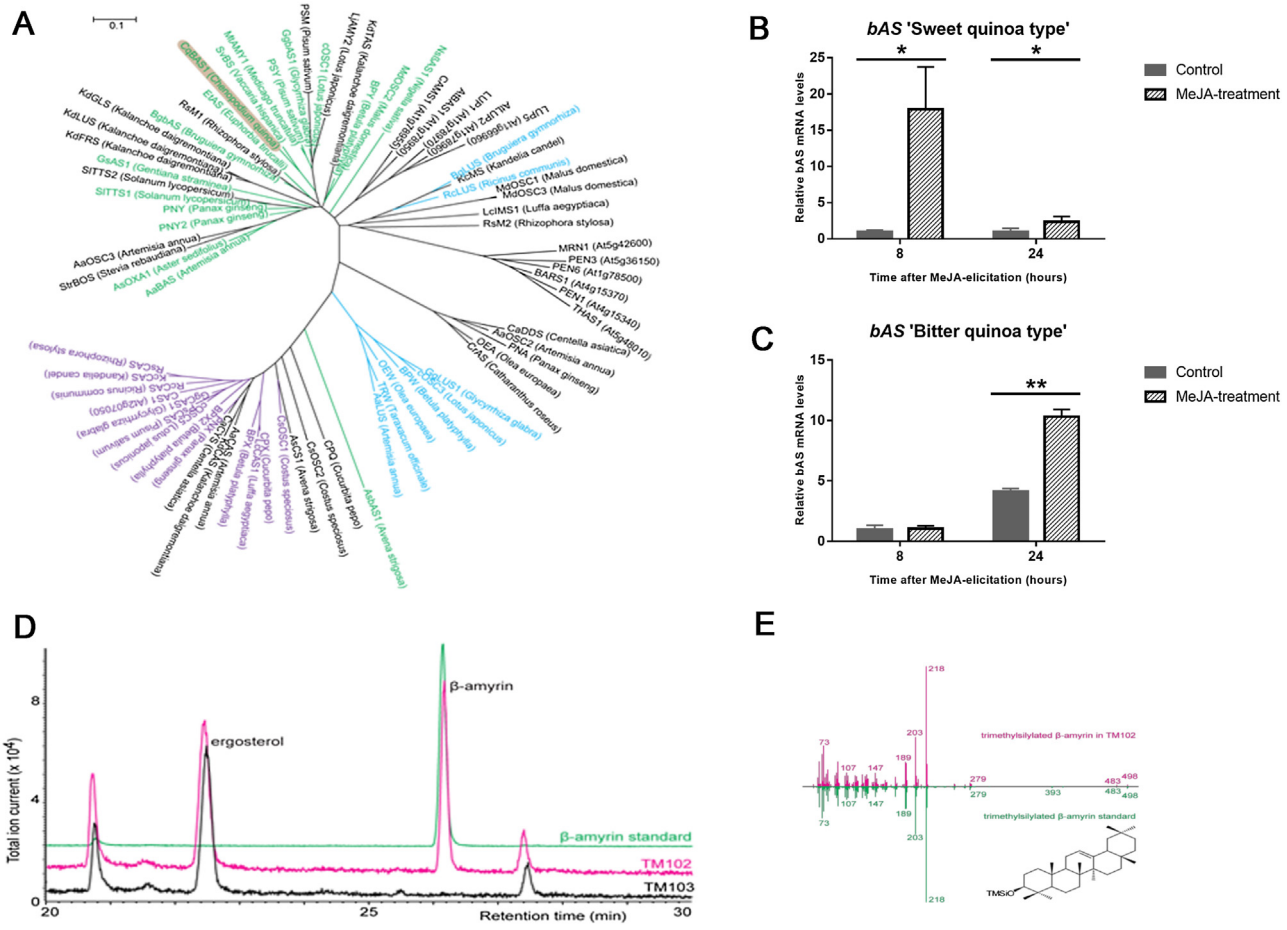

Fig. 2. Phylogenetic and functional analysis of CqbAS1. (A) Maximum-Likelihood analysis of CqbAS1 with other functionally characterized OSCs involved in triterpene biosynthesis. The scale bar shows the number of amino acid substitutions per site and the enzymatic activity of the OSCs is color coded; green, specific β-amyrin synthase; blue, specific lupeol synthase; violet, specific cycloartenol synthase; black, multifunctional synthase. CqbAS1 is highlighted in brown. The sequences of the OSCs were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank). (B) qPCR analysis of MeJA-responsiveness of the *CqbAS1* gene in the sweet (B) and bitter (C) quinoa types. Error bars designate standard deviation of the mean (n = 3). Statistical significance was determined by Student's *t*-test (**p-value* < 0.05). (D) Overlay of GC chromatograms of yeast strain TM102 expressing CqbAS1 (pink) with control strain TM103 (black) and β-amyrin standard (green). β-amyrin eluted at 26.2 min in strain TM102 and the standard. (E) EI-MS spectrum of trimethylsilylated β-amyrin extracted from strain TM102 (top) and the standard (bottom). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and/or catalysis of squalene and 2,3-oxidosqualene during the polycyclization reaction were identified in CqbAS1 [52,53] (Fig. S4).

The sequence-based *in silico* analysis suggested that CqbAS1 is a specific β-amyrin synthase involved in the cyclization of 2,3-oxidosqualene to β-amyrin. To functionally characterize CqbAS1, its coding sequence was cloned into the in-house created yeast destination vector pESC-URA-tHMG1-DEST, to obtain an expression clone which was transformed into *Saccharomyces cerevisiae* strain TM1 to generate strain TM102 (Table 2), using the lithium acetate-mediated transformation method [41]. Simultaneously, a control strain TM103 harboring the empty destination vector in strain TM1 was also generated. The two yeast strains TM102 and TM103 were cultured for 72 h in the presence of methyl-β-cyclodextrin (MβCD) and culture extracts were analyzed by gas chromatography–mass spectrometry (GC–MS) as described previously [41]. The GC chromatogram of strain TM102 expressing *CqbAS1* showed the accumulation of a unique peak at 26.2 min which was absent in the control strain TM103 (Fig. 2D and E). The retention time and electron ionisation (EI) pattern of this peak corresponded to that of an authentic β-amyrin standard, confirming the functional activity of CqbAS1 as a specific β-amyrin synthase, the committed enzyme for the synthesis of oleanane-type pentacyclic triterpenoid saponins in quinoa.

3.4. Identification and functional characterization of P450s involved in triterpenoid saponin biosynthesis

Following the cyclization of 2,3-oxidosqualene to specific triterpene backbones, such as β-amyrin, the next step in triterpenoid saponin biosynthesis involves P450-mediated modifications of the basic backbone [54]. To identify P450s able to modify β-amyrin in *C. quinoa*, we screened for genes encoding CYP716 family proteins in the assembled quinoa transcriptome databases. The CYP716 family of P450s represents the class of P450s with the most characterized enzymes shown to be involved in triterpenoid saponin biosynthesis [55]. To date 14 CYP716 family proteins have been shown to have oxidase activity on α-amyrin, β-amyrin, lupeol and/or dammarenediol or tirucalla-7,24-dien-3β-ol. These include the C-28 oxidases CYP716A12 from *Medicago truncatula* [56], CYP716A15 and CYP716A17 from *Vitis vinifera* [57], CYP716AL1 from *Catharanthus roseus* [58], CYP716A52v2 from *Panax ginseng* [59], CYP716A75 from *Maesa lanceolata* [49] and CYP716A80 and CYP716A81 from *Barbarea vulgaris* [60], CYP716Y1 the C-16α oxidase of α-amyrin and β-amyrin from *Bupleurum falcatum* [41], CYP716A47 and CYP716A53v2, the C-12 and C-6 oxidases of dammaranes, respectively from *P. ginseng* [59,61], CYP716A14v2 from *Artemisia annua* which is a C-3 oxidase [55], and CYP716A1 and CYP716A2 from

Table 2
Yeast strains used in this study.

Strain	Genotype
S288c BY4742	MATa *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*
TM1	S288c BY4742; P_{erg7}:P_{MET3}-*ERG7*
TM102	TM1; pESC-URA-tHMG1-DEST[*GAL1/CqbAS1*]
TM103	TM1; pESC-URA-tHMG1-DEST
TM112	TM102; pAG415[*GAL1/AtATR1*]; pAG423
TM113	TM102; pAG415[*GAL1/AtATR1*]; pAG423[*GAL1/CYP716A78*]
TM114	TM102; pAG415[*GAL1/AtATR1*]; pAG423[*GAL1/CYP716A79*]
TM115	TM102; pAG415[*GAL1/AtATR1*]; pAG423[*GAL1/CYP716AB1*]

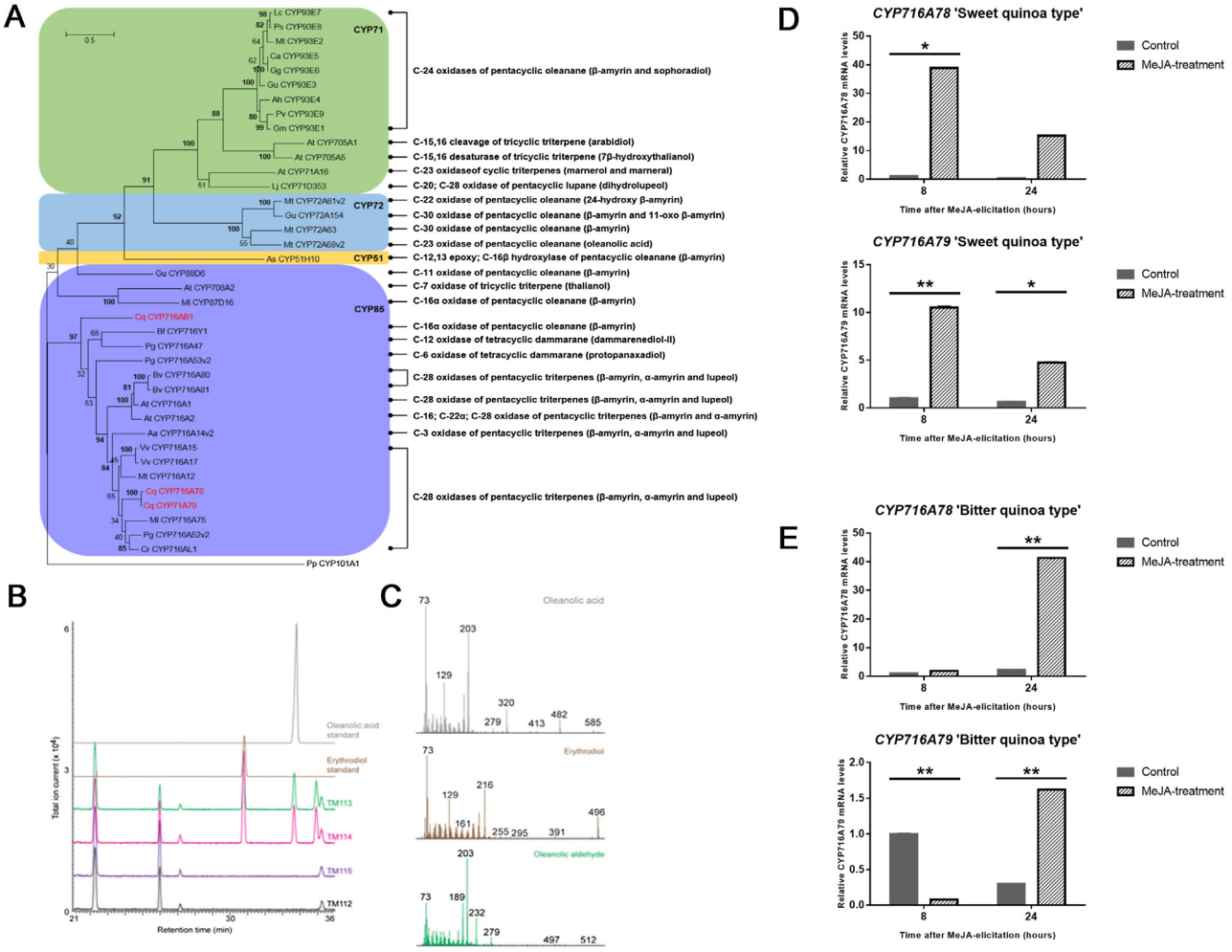

Fig. 3. Phylogenetic and functional analysis of CYP716A78, CYP716A79 and CYP716AB1. (A) Maximum-Likelihood analysis of quinoa P450s (red) with other functionally characterized P450s involved in triterpenoid biosynthesis. The scale bar shows the number of amino acid substitutions per site and the enzymatic activity of the P450s is indicated on the right. The P450 clans are color coded; green, CYP71 clan; blue, CYP72 clan; yellow, CYP51 clan; violet, CYP85 clan. The *Pseudomonas putida* CYP101A1 was used as outgroup. High scoring bootstrap values (≥75%) are highlighted in bold. The sequences of the P450s were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank). (B) Overlay of GC chromatograms of yeast strains TM112-TM115 expressing *CqbAS1* with *AtATR1* and no P450 (black), with *CYP716A78* (green), with *CYP716A79* (pink), or with *CYP716AB1* (violet). Erythrodiol standard (brown) and oleanolic acid standard (grey). Erythrodiol eluted at 30.8 min, oleanolic acid at 33.7 min and oleanolic aldehyde at 34.9 min in the strains TM113 and TM114. (C) EI-MS spectrum of trimethylsilylated erythrodiol (brown), oleanolic acid (grey) and oleanolic aldehyde (green) extracted from strain TM113. qPCR analysis of MeJA-responsiveness of *CYP716A78* and *CYP716A79* genes in the sweet (D) and bitter (E) quinoa types. Error bars designate standard deviation of the mean (n = 3). Statistical significance was determined by Student's *t*-test (**p-value* <0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Arabidopsis thaliana that have been shown to oxidize the C-28 and C-22 positions, respectively, of α-amyrin and β-amyrin [62]. Furthermore, CYP716A1 was also shown to catalyze the oxidation of tirucalla-7,24-dien-3β-ol [63]. Using the nucleotide sequence of the *M. truncatula* CYP716A12 protein (Genbank accession FN995113; [56]) as query, a TBLASTX search was performed in the assembled quinoa transcriptome datasets, resulting in the identification of three candidate full-length CYP716 family P450s. The first two candidates, named CYP716A78 and CYP716A79, showed a sequence similarity of 70% to CYP716A75 from *M. lanceolata* and of 97% to each other at the amino acid level. The third candidate P450 named CYP716AB1 shows 43% sequence similarity to CYP716A75 and is 45% similar to the other two candidate P450s. As expected, a phylogenetic analysis grouped the candidate P450s with the other CYP716 family proteins characterized to be involved in specialized triterpenoid biosynthesis (Fig. 3A).

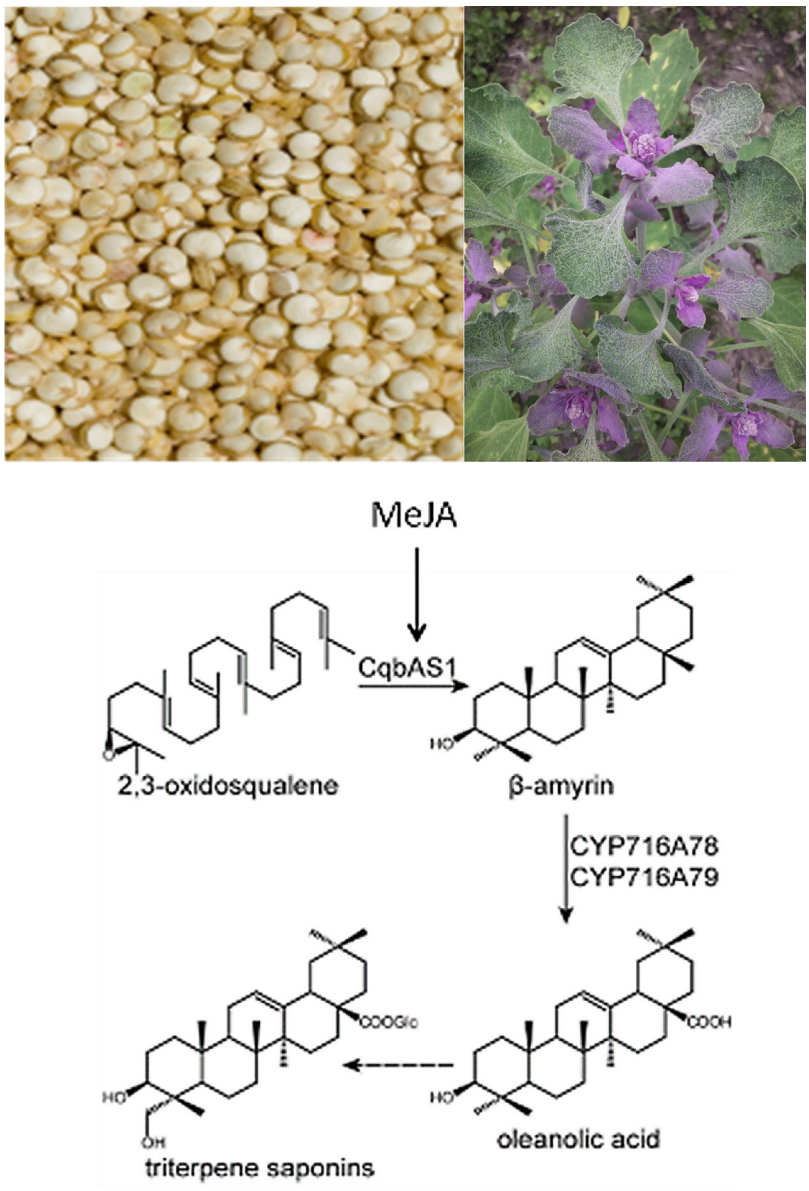

Fig. 4. Molecular identification and functional characterization of saponin biosynthetic genes of quinoa. On the top, quinoa seeds (left) and plants (right) used in this study. The bottom panel shows the biosynthesis of quinoa triterpene saponins from 2,3-oxidosqualene. This pathway includes the characterized quinoa genes that are MeJA inducible in leaf tissue: β-amyrin synthase (CqbAS1), and the two P450s, CYP716A78 and CYP716A79.

The full-length coding sequences of *CYP716A78*, *CYP716A79* and *CYP716AB1* were PCR amplified from the sweet type quinoa and cloned in yeast expression vectors. Yeast strains TM113, TM114 and TM115 expressing the *Arabidopsis thaliana* P450 reductase *AtATR1* and the P450 candidates *CYP716A78*, *CYP716A79* or *CYP716AB1*, respectively, were generated from TM102. Simultaneously, a control strain TM112 expressing *AtATR1*, but no P450 was also generated (Table 2). All yeast strains were cultured as described previously in the presence of MβCD and organic extracts from the cultures were analyzed by GC–MS. Compared to the GC chromatogram from strain TM112, strains TM113 and TM114 showed the presence of three unique peaks. The GC retention time and EI-MS pattern of two of these peaks matched authentic standards of erythrodiol and oleanolic acid, whereas the third peak was identified as oleanolic aldehyde based on our previous studies [49] (Fig. 3B and C). No new peaks could be observed in strain TM115 expressing *CYP716AB1*, therefore it was excluded from further qPCR analysis (Fig. 2B).

The expression of *CYP716A78* and *CYP716A79* was evaluated through qPCR analysis of leaves from entire sweet and bitter quinoa plants (Fig. 3D and E). Again, a distinct pattern was observed between both ecotypes, albeit that in both cases upregulation was observed upon MeJA application. A significant rise of *CYP716A78* and *CYP716A79* transcript levels was observed 8 and 24 h after MeJA elicitation in the sweet-type quinoa whereas such an induction was only visible after 24 h in the bitter-type quinoa. Taken together, the qPCR and saponin quantification results provide consistent evidence of the role of MeJA as an elicitor of triterpenoid saponin biosynthesis in quinoa.

3.5. Conclusion

The seeds of quinoa naturally contain an outer coating of bitter-tasting saponins. Despite its unpalatability, the bitterness of quinoa is beneficial for the cultivation of the plants as saponins prevent herbivory. Over the years, through selective breeding the saponin

content of quinoa has been lowered to obtain more palatable sweeter varieties. Hence, we performed saponin content analysis of leaves treated with MeJA and we found increased accumulation of saponins (Figs. 1 and 4). We found that committed genes for saponin biosynthesis were upregulated by JA, pointing to JA-inducible saponin biosynthesis in quinoa, most likely in the frame of a response to biotic and/or abiotic stress, a finding that has not been reported previously. This was further supported by the identification and functional characterization of three enzymes involved in the synthesis of oleanane-type pentacyclic triterpenoids in quinoa (Fig. 4). Identification of the subsequent enzymes involved in quinoa saponin biosynthesis pathway will need further gene discovery and the transcriptome assemblies generated in this study might prove to be a valuable resource. Finally, here we also describe a set of stably expressed housekeeping genes whose expression was confirmed to be constant in different JA treated and untreated quinoa ecotypes. This set of reference genes further adds to the toolkit for future phytogenomics studies in this highly nutritious pseudocereal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.05.015.

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