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ORIGINAL ARTICLE

Type-B cytokinin response regulators link hormonal stimuli and molecular responses during the transition from endo- to ecodormancy in apple buds

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Abstract

Key Message **Cytokinin together with *MdoBRR1*, *MdoBRR8* and *MdoBRR10* genes participate in the downregulation of *MdoDAM1*, contributing to the transition from endo- to ecodormancy in apple buds.**

Abstract The final step of cytokinin (CK) signaling pathway culminates in the activation of type-B response regulators (BRRs), important transcriptional factors in the modulation of CK-responsive genes. In this study, we performed a genome-wide analysis aiming to identify apple *BRR* family members and understand their involvement in bud dormancy control. The investigation identified ten *MdoBRR* protein-coding genes. A higher expression of three *MdoBRR* (*MdoBRR1*, *MdoBRR9* and *MdoBRR10*) was observed in dormant buds in comparison to other developmental stages. Interestingly, in ecodormant buds these three *MdoBRR* genes were upregulated in a CK-dependent manner. Transcription profiles, determined during dormancy cycle under field and artificially controlled conditions, revealed that *MdoBRR1* and *MdoBRR8* played important roles in the transition from endo- to ecodormancy, probably mediated by endogenous CK stimuli. The expression of *MdoBRR7*, *MdoBRR9,* and *MdoBRR10* was induced in ecodormant buds exposed to warm temperatures, indicating a putative role in growth resumption after chilling requirement fulfillment. Contrasting expression patternsin vivo between *MdoBRRs* and *MdoDAM1*, an essential dormancy establishment regulator, were observed during dormancy cycle and in CK-treated buds. Thereafter, in vivo transactivation assays showed that CK stimuli combined with transient overexpression of *MdoBRR1*, *MdoBRR8,* and *MdoBRR10* resulted in downregulation of the reporter gene *gusA* driven by the *MdoDAM1* promoter. These pieces of evidences point to the integration of CK-triggered responses through MdoBRRs that are able to downregulate *MdoDAM1*, contributing to dormancy release in apple.

Keywords *MdoBRR* · Cytokinin · Bud dormancy · *MdoDAM1* · Apple

Introduction

The multiple steps of cytokinin (CK) signal transduction lead to the activation of type-B response regulators (BRRs), transcriptional factors, that once activated are able to modulate the CK-responsive genes. (To and Kieber 2008; Hill

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et al. 2013). This protein family is characterized by the presence of two essential domains: a signal receiver (REC), involved in phosphorylation-mediated switches of response regulators, and a Myb-like DNA binding domain (Imamura et al. 1999; Hosoda et al. 2002). The REC domain core consists of a quintet of highly conserved amino acid residues comprising three adjacent Asp, one Ser/Thr and one Lys residue. In the Asp triplet, the first one is associated with the phosphorylation site and the other two with metal ion binding (Mg^{2+}), important for phosphoryl group changes. The conserved Thr/Ser interacts with the phosphoryl group and together with the conserved Lys enables phosphorylation-mediated conformational changes. The Myb-like output of BRRs shares a B-motif of approximately 60 amino acids and is responsible for DNA binding (Hosoda et al. 2002). Commonly, they are associated with transcriptional activation and the mechanisms involved in the negative regulation mediated by BRRs remains unclear. In poplar, for example, the constitutive expression of one *BRR* (*PtRR13*) disrupted the normal development of adventitious roots through the regulation of many genes, including the downregulation of *TINY-like* transcriptional factors, important in the stress/ethylene-inducible response (Ramírez-Carvajal et al. 2009). BRRs play a pivotal role in the early CK plant responses, being involved in shoot cell division, seed and root development and light responses (Argyros et al. 2008).

CKs are generally considered as positive regulators of shoot apical meristems through stimulating cell division and negative regulators of root apical meristem through promoting cell differentiation (Kieber and Schaller 2018). Besides that, studies have suggested that CKs are important regulators of bud dormancy release, acting upstream of the gibberellic and abscisic acid response pathways by stimulating meristematic activity (Cutting et al. 1991; Liu and Sherif 2019).

Bud dormancy is characterized by the growth inability of shoot apical meristem even under favorable environment conditions (Rohde and Bhalerao 2007). Perception of external signals like photoperiodic changes and chilling exposure as well as internal stimuli such as hormonal balance, genetic and epigenetic regulation are key factors modulating the main physiological aspects of dormancy (Beauvieux et al. 2018; Lloret et al. 2018; Cattani et al. 2018; Miotto et al. 2019). Bud dormancy cycle is divided into endo-, eco- and paradormancy stages (Lang et al. 1987). Endodormant buds are not capable of resuming growth even when exposed to permissive conditions. The growth arrest is mainly determined by endogenous plant signals. At the ecodormancy stage, growth inhibition is associated with adverse external stimuli, and once environmental conditions become favorable, the vegetative growth restart. Paradormancy is related to growth suppression by distal organs signaling, and is usually referred as apical dominance (Lang et al. 1987).

The dormancy mechanism in *Malus* species is cyclic and the same perception of low temperatures during the autumn that induces bud set is necessary for budburst in spring, as a result of continuous chilling exposure over the winter. The chilling requirement for dormancy release is genotype dependent and guided by a complex molecular network. Castel Gala cultivar, for example, which is a natural mutant of 'Gala' (Kidd's Orange × Golden Delicious) requires 50% less chilling to bud break and is capable of reach ecodormancy under natural field conditions (Denardi and Seccon 2005). Differently, another spontaneous mutation from 'Gala' called 'Royal Gala' is not able to accumulate the enough chilling to reach ecodormancy without the usage of chemical inducers, such as hydrogen cyanamide.

The first important dormancy regulator genes were identified in the *evergrowing* (*evg*) peach mutant, which phenotype is associated with dormancy settlement failure (Bielenberg et al. 2008). The *evg* mutant is related to the disruption of six tandemly repeated $MIKC^c$-type MADS-box genes that were further called *Dormancy-associated MADS-box* (*DAM*; Bielenberg et al. 2008). The *DAM* genes are phylogenetic close to the *SHORT VEGETATIVE PHASE* (*SVP*) genes and have been widely associated with dormancy cycle control in several tree species (reviewed in Falavigna et al. 2019). In Arabidopsis, SVP plays important roles in the response to ambient temperature changes and in the modulation of hormonal responses, delaying flowering by directly repressing flowering-time genes (Lee et al. 2007; Andrés et al. 2014). Similarly, environmental signals, hormonal pathways and epigenetic changes modulate the transcriptional regulation of *DAM* genes during bud dormancy (Falavigna et al. 2019). However, the transcriptional regulators identified so far do not fully explain the expression dynamics of the *DAM* genes during the dormancy cycle.

In apple (*Malus* × *domestica* Borkh.), one of the most cultivated perennial species worldwide, ectopic expression of *MdoDAMb* and *MdoSVPa* resulted in delayed bud break and changes in plant architecture due to constrained lateral shoot outgrowth (Wu et al. 2017). Moreover, the promoter analysis of *MdoDAM1*, an important regulator of endodormancy, revealed the presence of BRRs binding sites, suggesting that the CK pathway may act upstream of the *DAM* genes (Porto et al. 2016). However, the functional relevance of these sites has not yet been evaluated. Therefore, considering the importance of CK in plant growth and the lack of knowledge about transcriptional regulators of the *DAM* genes, this study aimed to understand the roles of MdoBRRs factors during dormancy and investigate whether they could be involved in the modulation of *DAM* genes expression, a key regulator of dormancy cycle.

Materials and methods

Identification, sequencing and phylogenetic analysis of *MdoBRRs*

The identification of the predicted gene models coding for *BRRs* was performed by BLASTP (Altschul et al. 1990) using Apple Genome (GDDH13_v1.1) proteins database (https://www.rosaceae.org/; Daccord et al. 2017) with REC (Imamura et al. 1999) and Myb-like DNA binding domains (Hosoda et al. 2002) as queries. Only predicted gene models that exhibited both domains and were annotated as response regulators were selected for further analysis. The protein families were assigned by InterPro v.70.0 online search tool (Mitchell et al. 2019) and illustrated by IBS v.10.3 software (Liu et al. 2015).

The CDS regions of *MdoBRR*s were amplified by PCR using specific primers (Table S1) and cDNA from pool of tissues samples of 'Royal Gala' as template (Table S1). Amplicons were cloned into the pENTR™/D-TOPO™ vector (Invitrogen, USA) as instructed by manufacturers. The full-length sequence confirmation for the coding regions of the ten *MdoBRR* candidates was performed through Sanger sequencing using M13 and walking primers (Table S1). In vitro sequencing results were aligned to the reference genome described by Daccord et al. (2017) using MEGA7 v.7.0 software (Kumar et al. 2016).

For phylogenetic analysis, full-length deduced-protein sequences of 68 type-B response regulators from *Arabidopsis thaliana*, *Malus* × *domestica*, *Oryza sativa* sub. japonica, *Populus trichocarpa*, *Prunus persica* and *Pyrus bretschneideri* were aligned using MUSCLE (Edgar 2004). References and accession codes from all sequences are listed in Table S2. The phylogenetic tree was inferred using MRBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001) employing the mixed amino acid substitution model. Ten million generations were run, sampled every 100 generations and the first 25% of the trees were discarded as burn-in. The remaining ones were summarized in a consensus tree, which was visualized and edited using FIGTREE v.1.4.4 (https://tree.bio.ed.ac.uk/software/Figtree/).

Plant material, RNA extraction and RT-qPCR

Plant material used in this study were collected in the experimental orchard at the Temperate Fruit Tree Experimental Station of Embrapa Uva e Vinho in Vacaria, RS, Brazil (28° 30′50″ S, 50° 54′41″ W, 972 m altitude). The apple tissues for the analysis of gene expression among different developmental stages were harvested from Gala Baigent® cultivar as described in Perini et al. (2014). The samples from the entire growth cycle (from dormancy to fruit maturation) were classified according to the Fleckinger´s phenological scale (EPPO 1984) and their complete description is reported in Perini et al. (2014) and exemplified in Fig. S1.

RNA extraction was performed as described by Zeng and Yang (2002) and scaled to micro-centrifuge tubes. DNA contaminants were removed by TURBO DNAse (Applied Biosystems, USA) and cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following the manufacturer instructions. RT-qPCR was performed as described in Falavigna et al. (2014) with specific primers (Table S1). Relative expression of mRNA was calculated by the Pfaffl method (Pfaffl 2001). Primer efficiency was determined by LingRegPCR v2017.1 software (Ruijter et al. 2009) and threshold cycle (CT) values were normalized by the reference genes *MDH* (*malate dehydrogenase*), *TMp1* (*type 1 membrane protein-like*) and *WD40* (*transcription factor WD40-like repeat domain*) when samples of all tissues were analyzed. Specifically for bud samples calculations, *TMp1* was replaced by *ARC5* as described in Perini et al. (2014).

Dormancy cycle evaluation

For dormancy cycle evaluation, the environmental temperatures were recorded by an automated meteorological station (National Meteorological Institute – INMET, Brazil) located inside the orchard site. One chilling hour (CH) was defined as a period of one full hour below 7.2 °C (Labuschagné et al. 2002). Apical dormant buds of 'Castel Gala' were harvested under field conditions in four distinct dates during the year of 2016 (Table S3a) with different chilling accumulation (118, 325, 707 and 778 CH) in order to cover endo- and ecodormancy stages.

The assay conducted under artificially controlled conditions was performed with 'Royal Gala' twigs (20 cm long) containing the apical buds collected from the field with 315 CH (winter 2016). The twigs were decontaminated, wrapped in black plastic bags and placed in dark chambers with controlled temperature set to 3 °C in upright position as described by Falavigna et al. (2015). The apical buds were sampled every 7 days (168 CH) from 315 to 1403 CH. After 1403 CH, twigs were accommodated into wet floral foam under growth permissive conditions (25 ± 1.5 °C, 16 h photoperiod and 70% relative humidity) from 24 to 360 h for evaluation of gene expression during bud break. For all RT-qPCR assays, samples were divided into three biological replicates (15 buds each).

In order to evaluate the dormancy depth (endo- or ecodormancy) and bud break competence of the apical buds, a batch of 40 twigs at each sampled time-point was placed in a

growth chamber under forcing conditions (25 ± 1.5 °C, 16 h photoperiod and 70% relative humidity) for 35 days. In this study, we defined that the transition from endo- to ecodormancy was reached when more than 50% of the apical buds were at the green tip stage.

For 'Royal Gala' annual growing cycle evaluation, buds were harvested during a full year at six different dates to cover all seasons (Table S3b). Exceptionally, this sampling was done in an experimental area belonged to a commercial orchard in Papanduva, SC, Brazil (26° 26′68″ S, 50° 05′47″ W, at 788 m altitude), during the 2009/2010 cycle.

t-zeatin quantification

The hormone extraction was performed by adding 4.0 mL of extraction solution (methanol:water:formic acid, 75:20:5, *v*:*v*:*v*) in a tube containing 500 mg of plant material. Samples were incubated at − 20 °C for 3 h. After that, they were conditioned in an ultrasound bath (40 kHz frequency) for 25 min at 4 °C and supernatants were recovered by centrifugation at 1750*g* at 4 °C for 30 min. The extraction step was repeated twice. Then, the supernatants were transferred into a new 1.5 micro-centrifuge tube and dried in a benchtop vacuum centrifuge (Eppendorf, DE) at 1400 rpm at 30 °C under 20 mbar vacuum pressure until reaching the mark of 100 µL of residual liquid. To the remaining solution, 1.0 mL of water was added followed by vortex homogenization. The samples were then transferred to Oasis MCX columns (Waters Corporation, USA). The column elution was conducted using a gradient of ammonium hydroxide from 0.004 to 0.4 M. To the eluted solution, 1.7% *w*/*v* of PVPP was added and the samples were homogenized in a vortex. The sample supernatants were recovered by centrifugation at 10,000*g* for 45 min at 4 ºC. The supernatant was then transferred into a new 1.5 micro-centrifuge tube and dried in the vacuum centrifuge as described above. After drying, samples were ressuspended in 75 µL of methanol, filtered through a 0.22-µm polytetrafluoroethylene (PTFE) membrane and used in the quantification of *t*-zeatin by UPLC-ESI-MS/MS (Waters Corporation, USA). Results are represented in ng of *t*-zeatin/g of dry mass.

CK treatments

CK treatments were performed by two different ways: (1) the TOP method consisted in dipping the apical bud of each 'Royal Gala' twig (10 cm long) in 10 mL of the tested CK solution for 10 min. Subsequently, twigs were accommodated in wet floral foams inside a growth chamber (25 ± 1.5 °C, 16 h photoperiod and 70% relative humidity) for 16 h. (2) The BASE method consisted in submerging the basal portion of the twigs (10 cm long and containing the apical bud) in 10 mL of the tested CK solution for 16 or 48 h. During this period, the twigs were conditioned in the growth chamber as described above. After each time-point, apical buds were harvested, frozen in liquid nitrogen and stored at − 80 °C until RNA extraction. Two types of synthetic CK were used: 6-benzylaminopurine (BAP; Sigma, cat. no B3408) and thidiazuron (TDZ; Sigma, cat. no 45686) in three different concentrations (1, 2 and 4 mM). All CK solutions were diluted in 0.5% Tween 20 (Sigma, cat. no P7949). Treatments were performed in three biological replicates (15 buds each) and a solution of 0.5% Tween 20 was used as control. All CK treatments were performed on both endodormant (254 CH—9% bud break) and ecodormant (1614 CH—100% bud break) buds.

Vector constructions for transactivation assays

The promoter region (from − 614 to − 111 bp) of *MdoDAM1* (MDP0000322567; GenBank accession: KT582786) was amplified by PCR using specific primers and gDNA from a pool of buds samples of 'Royal Gala' as template (Table S1). Amplicon was cloned into the entry vector pGEM®-T Easy (Promega, USA) as instructed by manufactures. The directional cloning of *MdoDAM1* promoter into the multiple cloning site 1 (MCS1) of pGUSXX-90 vector (Pasquali et al. 1994) was done by double-digest with BamHI and HindIII restriction enzymes. The mutated *MdoDAM1* promoter for the two BRR biding sites was synthetized and cloned into pMK-RQ vector by GeneArt® Gene Synthesis (Thermo Fisher Scientific, USA). Four nucleotides substitutions on each BRR biding site were introduced (−518A > C, −517G > C, −516A > T, −513 T > G) and (−179A > C, −178G > C, −177A > T, −176 T > G). The mutated *MdoDAM1* promoter was digested from pMK_RQ entry vector and cloned into pGUSXX-90 by double-digest with the same restriction enzymes used in the native version. The final construction consists of the promoter region of the respective native or mutated version of *MdoDAM1* promoter fused to CaMV 35S − 90 minimal promoter region, that together guide the expression of *gusA* reporter gene into pGUSXX-90 vector, creating the vectors constructions *MdoDAM1promo*::*gusA* (native promoter) and *MdoDAM1promo*^{mut}::*gusA* (mutated promoter). The cloning confirmation of both native and mutated *MdoDAM1* promoter regions as well as the verification of the BRR biding sites was performed by Sanger sequencing. The transient overexpression of *MdoBRR1*, *MdoBRR8* or *MdoBRR10* was achieved by cloning each CDS region into the pART7 vector (Gleave 1992) using Gateway® technology (Thermo Fisher Scientific, USA). The *Renilla Luciferase (rLuc)* gene cloned into p2rL7 (De Sutter et al. 2005) was used as an internal control of the transfection process.

Transactivation assay

Five-week-old *A. thaliana* plants were used for protoplast isolation by the Tape-Arabidopsis Sandwich method (Wu et al. 2009). Two genotypes of *A. thaliana* were used in the experiments: Col-0 (wild type) and the loss of function triple mutant *arr1-3/arr10-5/arr12-1*, purchased from the Arabidopsis Biological Resource Center (ABRC; Stock number: CS39992). *A. thaliana* mutant plants were genotyped by PCR using specific primers to T-DNA insertion sites (Table S1). PEG–calcium mediated transfection method was used to deliver plasmid DNA into protoplasts, followed by 16 h incubation to allow gene expression (Yoo et al. 2007). Three independent plasmids were transfected to 1×10^5 protoplast suspension: p2rL7::*rLuc,* *MdoDAM1promo*::*gusA* (native or mutated version) and the respective pART7::*MdoBRR* tested.

In the assay using CK treatments, protoplasts were transfected just with p2rL7::*rLuc,* and *MdoDAM1promo*::*gusA* without MdoBRRs effectors. After overnight incubation, three different concentrations of TDZ (1.0, 0.1 and 0.0001 µM—diluted in 1% of DMSO) were added to protoplasts for 4 h. DMSO 1% was used as control. For each transactivation experiment, four biological and three technical replicates were analyzed. Fluorescence and luminescence were evaluated as described in Yoo et al. (2007) using a SpectraMax® i3 Multi-Mode Detection Platform (Molecular Devices, USA).

Statistical analysis

All datasets were compared using one-way ANOVA followed by a multiple comparison test (Tukey or Dunnet) with statistical significance set at 0.05 using GraphPad Prism version 6.01 for Windows, GraphPad Software (La Jolla, California, USA). For transcriptional profiles in different apple developmental stages and transactivation assays, Tukey multiple comparison test was used in order to compare all against all. In the evaluation of *MdoBRRs* and *MdoDAM1* expression in the dormancy cycle, *t*-zeatin quantification and CK treatments experiments, Dunnet multiple comparison test was the choice, once samples were compared against a specific point. For 'Castel Gala' dormancy cycle evaluation, the relative expression of each *MdoBRR* and *t*-zeatin amounts were compared to 118 CH time-point. In controlled conditions for 'Royal Gala', the time-point chosen was 315 CH. Relative expression of each *MdoBRR* tested in the CK treatments was compared to its respective control.

Results

Identification, structural and phylogenetic analysis of apple *BRR* genes

The in silico identification of apple *BRR*s was performed based on the new haploid genome GDDH13 v1.1 (Daccord et al. 2017) and resulted in 10 predicted gene models (Table 1). In this study, we propose an update on the nomenclature of the members of apple BRR gene family, based on: the identification of two extra genes (*MdoBRR2* and *MdoBRR3*) and the use of a more curated version of the apple genome compared to the previous study conducted by Li et al. (2017). Additionally, the *MdoBRR* gene names were inferred following the guideline developed for Rosaceae family members (Jung et al. 2015).

Full-length coding regions of seven identified *MdoBRRs* (*MdoBRR1; MdoBR5-10*) were confirmed by sequencing. Partial sequences were obtained for two *MdoBRR*s (*MdoBRR2* and *MdoBRR3*), covering 70% and 40% of the coding regions, respectively. For *MdoBRR4*, just the RT-qPCR amplicon could be retrieved under our PCR conditions. With

Table 1 Identification of apple *BRR* genes

Genome accession[a]	Chromosomal localization[a]	Li et al. (2017)	Proposed nomenclature	GenBank accession
MD08G1059000	Chr08:4701285..4705253	*MdRRB3*	*MdoBRR1*	MN590295
MD16G1036100	Chr16:2601907..2605099	–	*MdoBRR2*	MN590296
MD01G1047800	Chr01:15007174..15008926	–	*MdoBRR3*	MN590297
MD02G1229600	Chr02:27354515..27358617	*MdRRB1*	*MdoBRR4*	–
MD16G1108400	Chr16:7572032..7576420	*MdRRB10*	*MdoBRR5*	MN590298
MD13G1108300	Chr13:7783159..7786774	*MdRRB7*	*MdoBRR6*	MN590299
MD16G1159400	Chr16:12890367..12894414	*MdRRB11*	*MdoBRR7*	MN590300
MD13G1159700	Chr13:12567112..12571259	*MdRRB9*	*MdoBRR8*	MN590301
MD13G1019800	Chr13:1246553..1250362	*MdRRB6*	*MdoBRR9*	MN590302
MD16G1017900	Chr16:1305300..1309682	*MdRRB4*	*MdoBRR10*	MN590303

[a]Genome accession codes and chromosomal localization are provided by the '*Malus*×*domestica* Genome' (https://rosaceae.org/) based on GDDH13_v1.1 version

the exception of *MdoBRR4*, all sequences (partial and full-length) were deposited in the GenBank database and accession codes are presented in Table 1. In the case of partial sequences, the genome GDDH13 v1.1 predicted gene models for *MdoBRR2*, *MdoBRR3* and *MdoBRR4* were used as input for further analysis.

The functional annotation of MdoBRR deduced-protein sequences was performed with InterPro database (Mitchell et al. 2019) and resulted in the identification of the REC domain (IPR001789) and the plant Myb-like family (IPR006447) domain (Fig. 1a, b). The motif related to MdoBRR REC domain shows the quintet of highly conserved amino acid residues essential to REC activity (Fig. 1a). The conserved Lys and Ser residues were found in all MdoBRR proteins. The presence of the three-conserved Asp was observed in seven MdoBRRs. The MdoBRR2, MdoBRR3 and MdoBRR4 exhibited an amino acid substitution in the second position of the triplet (Fig. 1a). The B-motif related to Myb-like family in MdoBRRs shares a group of highly conserved amino acid residues in all protein sequences. Exceptionally, MdoBRR2 presents gaps and a good number of substitutions (Fig. 1a).

The plant model Arabidopsis (*A. thaliana*), the monocot rice (*O. sativa* sub. Japonica) and the dicot species poplar (*P. trichocarpa*), peach (*P. persica*) and pear (*P. bretschneideri*) were used in MdoBRR phylogenetic analysis (Table S2). The ten MdoBRRs deduced-protein sequences along with 58 other full-length proteins already described as BRRs were used for phylogenetic inference. The results allowed the identification of six MdoBRRs putative orthologs with pear and peach (Fig. 1c). Pear proteins B-PpRR3, B-PpRR2, B-PpRR8, B-PpRR5 and B-PpRR9 are more closely related to MdoBRR1, MdoBRR2, MdoBRR5, MdoBRR8 and MdoBRR10, respectively. The peach protein PpARR14-b

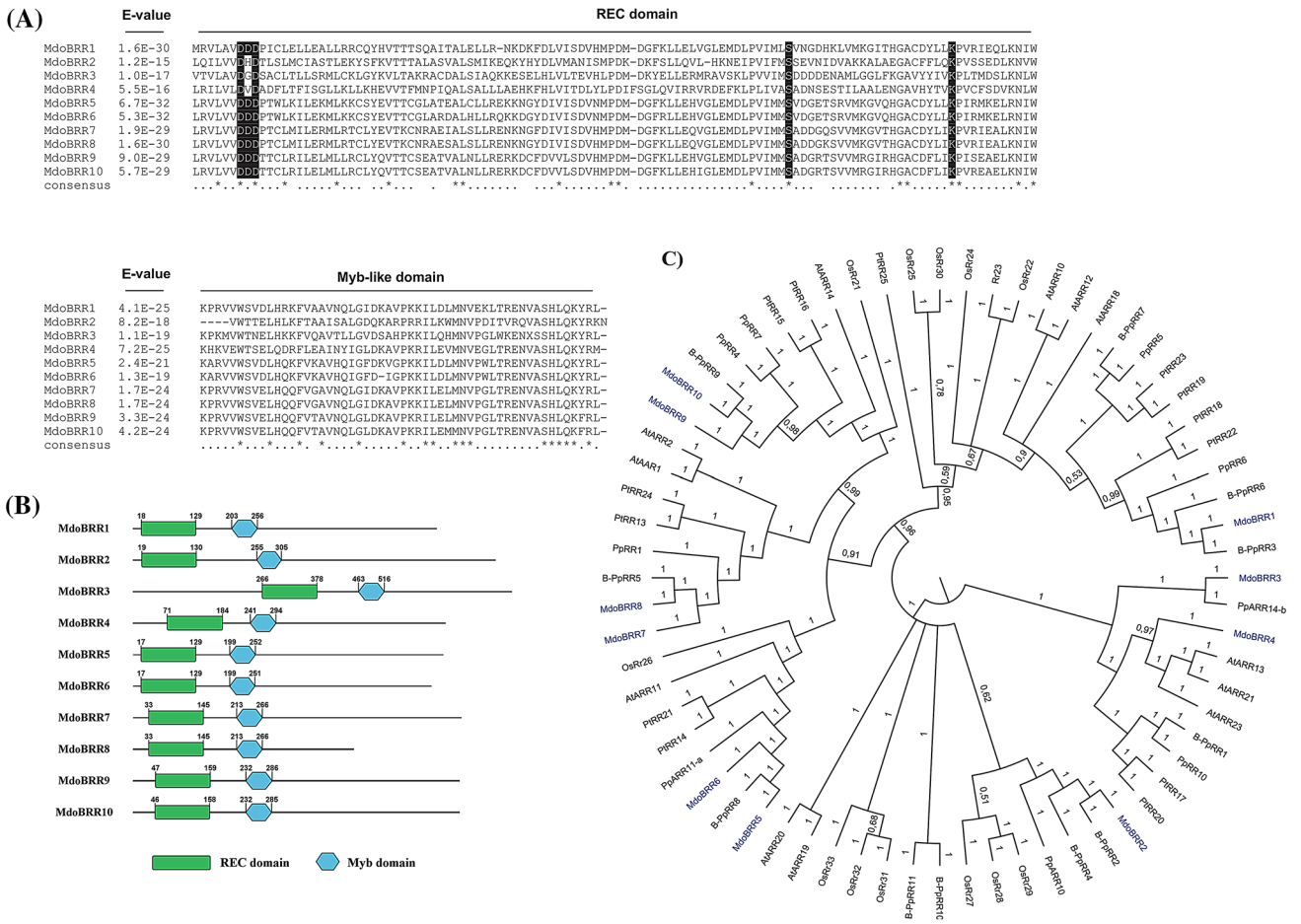


Fig. 1 Identification of conserved domains and phylogenetic analysis of MdoBRRs. **a** Alignment of protein motifs related to REC and Myb-like DNA binding conserved domains found in MdoBRRs. The quintet of essential amino acid residues for REC activity is highlighted in black. **b** Graphical illustration representing the position of the two conserved domains found in MdoBRR proteins. The first Met of each protein is considered as position number 1. **c** The phylogenetic tree was inferred by the Bayesian method using 68 protein sequences annotated as BRR from six different species (*A. thaliana*, *O. sativa* sub. Japonica, *P. trichocarpa*, *P. persica*, *P. bretschneideri* and *M. domestica*). MdoBRRs are colored in blue

clustered with MdoBRR3, separately. The remaining proteins MdoBRR6, MdoBRR7, MdoBRR9 grouped in the same cluster of MdoBRR5, MdoBRR8 and MdoBRR10, respectively. The MdoBRR4 did not group with any other apple, pear or peach protein sequences, but with three Arabidopsis BRRs (AtARR13, AtARR21 and AtARR23).

Expression patterns of *MdoBRR* genes in different apple developmental stages

The expression of all MdoBRRs was evaluated in apple developmental stages from dormancy to fruit maturation (Fig. S1) based on Fleckinger's phenological scale (EPPO 1984). Results showed that in general, among all tissues, the highest expression levels were observed for *MdoBRR6-10* genes (Fig. 2). Specifically, close terminal buds (A) presented higher expression levels of *MdoBRR1*, *MdoBRR9* and *MdoBRR10*, especially in relation to *MdoBRR10* (Fig. 2), leading us to better investigate their roles during the bud dormancy cycle. The *MdoBRR6* gene was preferentially expressed in seeds of unripe fruit (JS) and in leaves independently of the stage (E2L, IL and JL). *MdoBRR7* transcripts accumulated in apple inflorescences (E2IN). Expression of *MdoBRR8* is higher in flowering (E2IN) and fruit development (J). Transcriptional profile of *MdoBRR5* showed higher transcript levels in skin and pulp of unripe 40 mm apple fruit (J). *MdoBRR2*, *MdoBRR3* and *MdoBRR4* exhibited the lowest transcript levels (Fig. 2). *MdoBRR4* expression was only barely detected in seeds of unripe fruit (JS). Based on these results, these three *BRRs* (*MdoBRR2-4*) were excluded from further analyses.

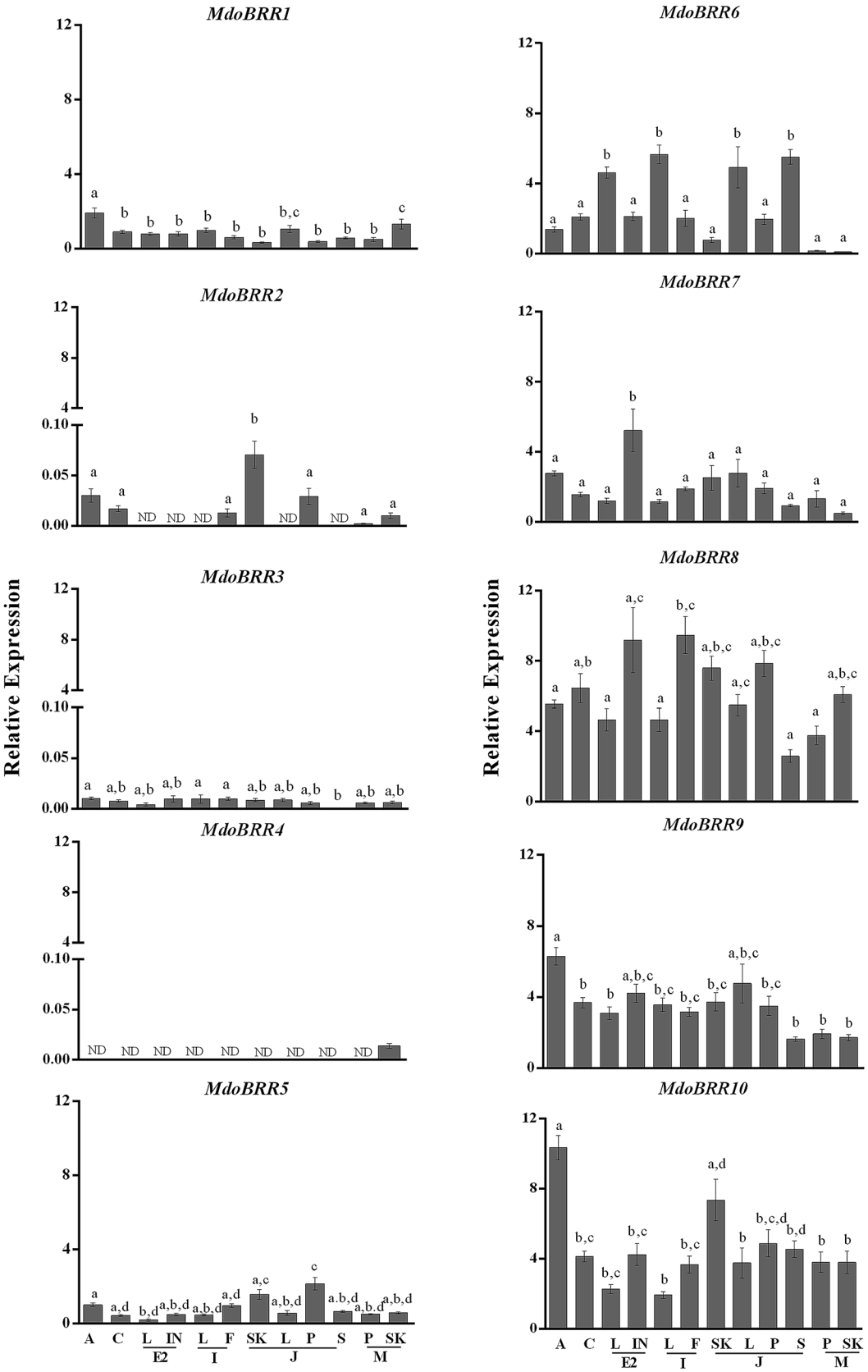
Transcriptional profiles of *MdoBRR* genes during the bud dormancy cycle

In order to monitor all stages of dormancy cycle progression (from establishment to release), an artificially controlled assay was performed to determine the transcriptional profile of *MdoBRRs* and *MdoDAM1* under the perspective of chilling accumulation and growth-promoting conditions. Under these aspects, ecodormancy was reached at 1067 CH and overlapped to a peak expression of *MdoBRR1* and *MdoBRR8* genes (Fig. 3). The transition from endo- to ecodormancy was also characterized by downregulation of *MdoDAM1*. Apical buds exposed to forcing conditions (25 °C) presented high levels of *MdoBRR10* transcripts since the first 24 h and remained constant all over the sampled time-points. The expression of *MdoBRR9* has also increased from 24 to 96 h during 25 °C temperature exposition. In a later response, after 168 h at growth permissive conditions, dormant buds started to accumulate *MdoBRR7* transcripts. The importance of *MdoBRR7*, *MdoBRR9* and *MdoBRR10* during bud growth resettlement was further reinforced in the 'Royal Gala' annual cycle transcriptional profile assay, which demonstrated upregulation of these three *MdoBRRs* during summer (Feb/09) whether compared to autumn and winter (May-Aug/09; Fig. S2). The expression of *MdoBRR5* and *MdoBRR6* did not change along the dormancy cycle (Fig. 3). The measurement of the native CK, *t*-zeatin, in these same samples, revealed peaks of *t*-zeatin accumulation before the ecodormancy stage, decreasing after that, and resuming after prolonged warm temperatures exposure, close to bud break (Fig. 3).

Aiming to investigate the expression pattern of *MdoBRRs* during the transition from endo- to ecodormancy in the context of field cultivation conditions, apical buds of Castel Gala cultivar were evaluated at four time-points from 118 to 778 CH. In this assay, ecodormancy was observed at approximately 700 CH, concomitantly with increased transcript levels of *MdoBRR1*, *MdoBRR8* and *MdoBRR9* and *t*-zeatin amounts (Fig. 4). From 707 to 778 CH, the expression of *MdoBRR1* and *MdoBRR8* kept increasing in the same way as *t*-zeatin, suggesting the induction of *MdoBRRs* by the endogenous CK during ecodormancy stage. In an opposite way, the highest levels of *MdoDAM1* were observed from 118 to 325 CH, during endodormancy, followed by a significant reduction at 700 and 778 CH. The expression of the other four *MdoBRRs* (*MdoBRR5-7*; *MdoBRR10*) remained constant all over the dormancy cycle. Taken together, these results indicate that CK accumulation with consequent *MdoBRR1* and *MdoBRR8* upregulation, followed by *MdoDAM1* downregulation constitute important steps in the transition from endo- to ecodormancy stages.

CK triggers *MdoBRR* gene expression in ecodormant buds

In order to determine if the CK stimulus could affect *MdoBRR* expression and investigate if the dormancy depth could influence on this modulation, exogenous CK was applied in the same way on both endo- and ecodormant apple buds. The most effective treatment in *MdoBRR* transcriptional activation was achieved in ecodormant buds treated by the TOP method (direct CK application, for more details see Material and methods). In this case, *MdoBRR9* and *MdoBRR10* demonstrated almost fourfold more transcript amounts when compared to the control after the treatment with 4 mM of BAP. In these same samples, downregulation of *MdoDAM1*expression was also observed (Fig. 5a). Conversely, TDZ treatments showed a clear tendency of *MdoBRR9* and *MdoBRR10* induction with a significant *MdoDAM1* repression. The CK influx through diffusion via xylem into ecodormant buds (BASE method) resulted in lower responses compared to the direct application of CK (TOP method). In this case, after 16 h *MdoBRR7* expression was induced with 4 mM TDZ at the same time that *MdoDAM1* was repressed,



◂**Fig. 2** Gene expression of *MdoBRRs* in apple developmental stages. The horizontal axis displays the developmental stages according to Fleckinger's phenological scale. *A*-closed dormant buds, *C*-buds in late sprouting stage, *E2 L*-young leaves at E2 stage, *E2 IN*-flower buds at E2 stage, *I L*-leaves at I stage, *I F*-whole set fruits at I stage, *J SK*-fruit skin at J stage, *J L*-mature leaves at J stage, *J P*-fruit pulp at J stage, *J S*-seeds at J stage, *M P*-pulp from mature fruits at M stage, *M SK*-skin from mature fruits at M stage. Relative expression was calculated based on the calibrator represented by the levels of mRNA from *MdoBRR5* gene at stage A. Different letters indicate statistically significant differences between means of three biological replicates using one-way ANOVA followed by Tukey's test ($p \leq 0.05$). Error bars represent the standard error of the mean. *ND* None detected

showing again an opposite modulation of *MdoBRR* and *MdoDAM1* gene expression by the CK stimulus (Fig. S3b). However, the same was not observed with *MdoBRR1* and *MdoBRR7* activation with 1 mM BAP treatment (Fig. S3b). The expression of *MdoBRR5*, *MdoBRR6* and *MdoBRR8* did not change in any of the tested conditions (Fig. S3a–c). After 48 h of treatment with the BASE method, no significant modulation of *MdoBRR* by CK was observed in ecodormant buds (Fig. S3c).

The influence of the dormancy depth in the CK-mediated *MdoBRRs* transcriptional modulation could be clearly seen when exogenous CK was applied to endodormant buds. In this case, the direct application of CK (TOP method) did not induce any *MdoBRR* expression (Fig. 5b), resulting in a completely different response to that obtained in ecodormant buds. Additionally, CK transport in endodormant buds for 16 h (BASE method) resulted mainly in downregulation of *MdoBRR6* (Fig. S4b) and after 48 h, just a subtle induction of *MdoBRR6* and *MdoBRR10* was achieved in the TDZ treatment (Fig. S4c). The little response of *MdoBRR*s observed in endodormant buds focused our investigation on CK-mediated *MdoDAM1* modulation only in ecodormant buds.

In summary, these findings corroborate those found during the dormancy cycle evaluation and emphasize that *MdoBRR* expression is accentuated during the stage of ecodormancy. Furthermore, we could also observe that the CK signal was not just able to increase the level of *MdoBRR9* and *MdoBRR10* transcripts, but also to downregulate *MdoDAM1* expression.

CK and transient overexpression of *MdoBRR*s negatively regulate *MdoDAM1* gene expression

Considering the different putative roles of *MdoBRR*s and *MdoDAM1* during the bud dormancy cycle, and taking into account that the CK stimuli induce *MdoBRR*s expression whilst downregulates *MdoDAM1,* we aimed to evaluate whether MdoBRR could modulate *MdoDAM1* expression through an in vivo transactivation assay using Arabidopsis protoplasts. For this purpose, a segment of 503 bp from the *MdoDAM1* promoter region containing two putative BRR binding sites (Fig. S5a; Porto et al. 2016) was used to guide the expression of *gusA* reporter gene (*MdoDAM1prom::gusA)* in the pGUSXX-90 vector (Pasquali et al. 1994). Based on their consistent expression pattern related to a possible role during bud dormancy regulation, *MdoBRR1*, *MdoBRR8* and *MdoBRR10* were chosen to be tested as effectors on the transactivation assays.

Results revealed that all MdoBRRs tested were able to bind BRR motifs and downregulate *MdoDAM1prom::gusA* activity in wild-type protoplasts (Fig. 6a). Wondering to know if CK signal is also capable of repress *MdoDAM1* expression as observed in CK-treated ecodormant buds (Fig. 5), Arabidopsis wild-type protoplasts cells were transfected only with *MdoDAM1prom::gusA* construct, without any effector (MdoBRR1, MdoBRR8 or MdoBR10). For this group of samples, TDZ was applied in increasing concentrations, leading to a significant reduction in *MdoDAM1prom::gusA* activity even when the lowest concentration was tested, confirming the repressive effect of CK signal on *MdoDAM1* gene regulation (Fig. 6b).

The availability of the Arabidopsis triple loss-of-function mutant (*arr1-3 arr10-5 arr12-1*) allowed us to perform the same assay described above, with the advantage of minimizing the Arabidopsis BRRs (ARRs) endogenous effects and possible technical artifacts, since these plants have lost the ability to respond to external CK stimuli (Argyros et al. 2008). Consistent with the previous observations, the assay employing *arr* triple mutant protoplasts resulted in *MdoDAM1prom::gusA* repression when transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* was tested, confirming that *MdoDAM1* downregulation is due to MdoBRRs (Fig. 6c). Moreover, the comparison between control (DMSO) and TDZ treatments resulted in no significant differences in *MdoDAM1prom::gusA* activity, reinforcing the hypothesis that *MdoDAM1* downregulation occurs by cellular perception of CK stimuli through ARRs (Fig. 6d). In order to confirm the MdoBRRs ability to bind in BRR regulatory motifs, site-specific mutations were introduced into the two BRR biding sites found along the *MdoDAM1* promoter region (Fig. S5b). The results showed that the loss of native BRR biding sites disrupted the ability of MdoBRR1, MdoBRR8 and MdoBRR10 to downregulate *MdoDAM1prom^{mut}::gusA* activity, confirming the hypothesis that the transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* is responsible for the transcriptional repression of *MdoDAM1* (Fig. 6e). These findings suggest a redundant mechanism on which *MdoDAM1* downregulation could be mediated by CK signaling (probably mediated by the activation of already existing pools of BRRs in the cell) followed by de novo transcriptional induction of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* and CK post-translational activation.

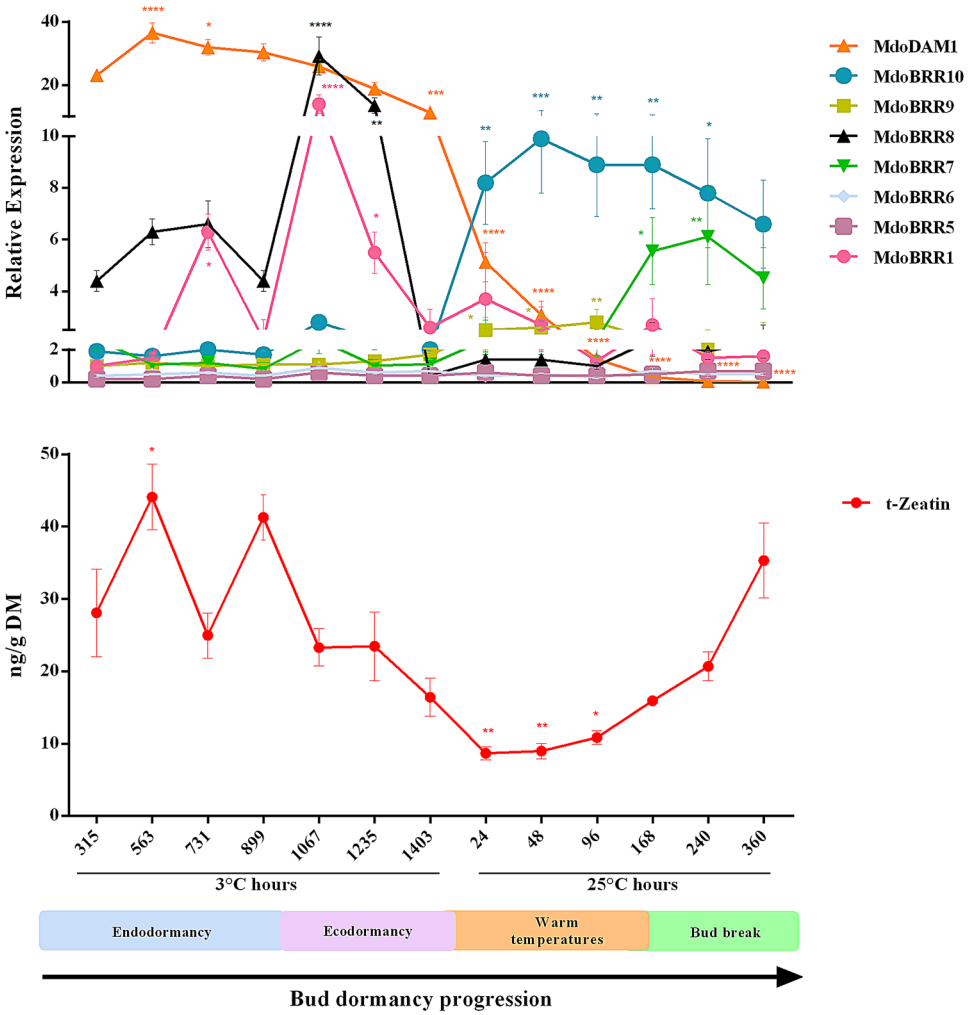


Fig. 3 Expression pattern of *MdoDAM1* and *MdoBRR* genes over 'Royal Gala' bud dormancy progression under artificially controlled conditions. RT-qPCR was performed with apical dormant buds of 'Royal Gala' sampled in the beginning of endodormancy with 315 CH and conditioned into dark chambers (3 °C) until the ecodormancy stage was reached (50% of green tip buds). After 1403 CH, the ecodormant buds were transferred to growth permissive conditions (25 ± 1.5 °C, 16 h photoperiod and 70% relative humidity) for 24–360 h in order to favor bud break. Expression data was calculated in relation to *MdoBRR1* gene at 315 CH point. *t*-zeatin quantification was done using UPLC-ESI-MS/MS. The asterisks indicate statistical differences (*$0.01 < p < 0.05$, **$0.001 < p < 0.01$***) between means of mRNA levels of three biological replicates (15 buds each) for every evaluated gene at 315 CH and the respective time-point sampled. Amounts of *t*-zeatin were compared in the same way. Error bars represent the standard error of the mean. *DM* dry mass

Discussion

BRR genes were firstly reported in Arabidopsis (D'Agostino et al. 2000) followed by rice (Ito and Kurata 2006); poplar (Ramírez-Carvajal et al. 2008), peach (Immanen et al. 2013), pear (Ni et al. 2017) and apple (Li et al. 2017). It is already known that activated BRRs are responsible for transcription modulation in a CK-dependent manner of many downstream genes involved in plant development (reviewed by Kieber and Schaller 2018). However, their potential regulatory role during the bud dormancy cycle has not been well explored. Our investigation based on the GDDH13_v1.1 apple genome dataset (Daccord et al. 2017) rendered the identification of ten *MdoBRRs*, including two additional members for the family (*MdoBRR2* and *MdoBRR3*; Table 1) when compared to the previous report (Li et al. 2017) that explored the apple draft genome described by Velasco et al. (2010). It is important to consider that the GDDH13_v1.1genome has a higher accuracy and a better contig assembly with a consequent more precise gene annotation. An example of the problems of the first apple genome draft assembly could be seen in the three predicted gene models MDP0000607144, MDP0000307383 and MDP0000124301 considered as true *MdoBRRs* by Li et al. (2017) that actually, are miss-predicted versions of *MdoBRR1*, *MdoBRR6* and *MdoBRR9*, respectively, and are not even assigned in GDDH13_v1.1 dataset.

The ten MdoBRR deduced proteins (Table 1) contain the two typical REC and Myb-like domains necessary for activation and DNA binding, respectively (Fig. 1a, b). Detailed analysis of all MdoBRR REC motifs revealed the lack of one of the three highly conserved Asp residues in MdoBRR2, MdoBRR3 and MdoBRR4 deduced-proteins (Fig. 1a). Another class of response regulators is characterized by atypical receiver domains and is related to the absence of one or more residues in the core region of the REC domain. This class is called pseudo-response regulators and also lacks the invariant phospho-accepting Asp residue, often replaced by a Glu residue (Mizuno and Nakamichi 2005; Bourret 2010). Although MdoBRR2, MdoBRR3 and MdoBRR4 deduced-proteins do not have the Asp triplet, they present the phospho-accepting Asp residue (Fig. 1a)

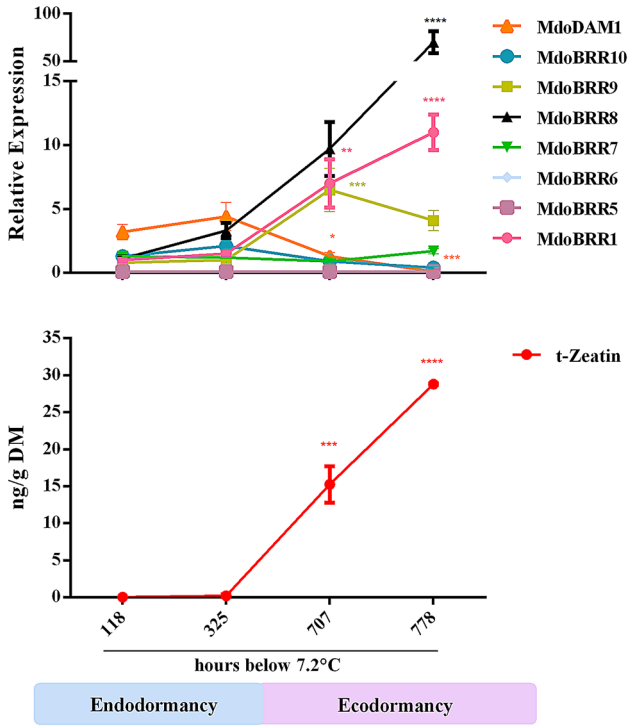


Fig. 4 Transcriptional profile of *MdoDAM1* and *MdoBRR* genes during 'Castel Gala' bud dormancy transition at field. Relative expression of *MdoDAM1* and seven *MdoBRRs* was evaluated by RT-qPCR in dormant buds of 'Castel Gala' sampled in two different time-points of endo- (118 and 325 CH) and ecodormancy (707 and 778 CH) stages. Calculation of gene expression data and statistical analysis was performed as described in Fig. 3 considering point 118 CH as reference; *$0.01 < p < 0.05$, **$0.001 < p < 0.01$***. Error bars represent the standard error of the mean

and consequently do not fit this definition, remaining as true members of the apple BRR family. These genes are also located in a genomic region rich in repeated sequences that may explain the difficulty in obtaining full-length coding region amplification products. Exactly these three *MdoBRR*s showed very low expression levels in all apple tissues tested (Fig. 2), which could be associated with a potential pseudogenization process through loss-of-function mutations, once the recent duplication of the apple genome created two copies of most genes, resulting in gene function diversification (Panchy et al. 2016).

The wide distribution of *BRR* expression in all development stages of the plant was already reported in Arabidopsis and rice (Mason et al. 2004; Ito and Kurata 2006). We have also observed higher amounts of *MdoBRR* transcripts during developing stages rather than in ripened fruit (Fig. 2). These data are in agreement with peach *BRR* expression profiles that present higher transcript amounts in rapidly growing tissues like semi-opened flowers, fruits at expansion stage and young leaves (Zeng et al. 2017). Similar results were also found in pear in the early stage of fruit growth (Ni et al. 2017) and in Arabidopsis where *BRR* expression was observed at shoot apical meristematic cells and young developing leaves (Mason et al. 2004). In general, our data could not be related to the results obtained by Li et al. (2017), probably by the distinct tissue sampling strategy used and apple cultivars tested ('Gala Baigent' versus 'Nagafu').

In apple trees, chilling accumulation stimulates removal of the endodormancy physiological blocks that inhibit growth, culminating in ecodormancy transition. Subsequently, buds are able to resume growth (bud break) after a certain amount of warm temperatures. Our particular interest in studying MdoBRRs was to gather evidences to understand their involvement in bud dormancy regulation. Within this context, we found three *MdoBRRs* (*MdoBRR1*, *MdoBRR9* and *MdoBRR10*) that showed significant levels of expression in bud tissues (Fig. 2). The evaluation of *MdoBRR* expression during dormancy cycle demonstrated that the transition from endo- to ecodormancy in 'Castel Gala' buds is associated with *MdoBRR1*, *MdoBRR8* and *MdoBRR9* transcriptional upregulation, *t*-zeatin accumulation and *MdoDAM1* gene repression (Fig. 4). In agreement, 'Royal Gala' ecodormancy stage was achieved concomitantly with *MdoBRR1* and *MdoBRR8* transcriptional activation and *MdoDAM1* downregulation. Moreover, when ecodormant buds were transferred to permissive growth conditions, we could observe the negative regulation of *MdoBRR1*, *MdoBRR8 and MdoDAM1* and the activation of three *MdoBRR* in a continuous (*MdoBRR10*), early (*MdoBRR9)* and late (*MdoBRR7*) response to warm temperatures (Fig. 3). Accordingly, the evaluation of 'Royal Gala' buds during a full year growing cycle showed higher transcriptional activity of *MdoBRR7*, *MdoBRR9* and *MdoBRR10* during the summer (Feb/10; Fig. S2) and a remarkable reduction of *MdoDAM1* expression in the same period (Porto et al. 2016). The potential regulatory role of *BRR* genes in the transition from endo- to ecodormancy was also reported in pear RNA-Seq analysis that showed increased transcript levels of *B-PpRR9/6* and *B-PpRR5/3* genes in 'Suli', and *B-PpRR7* gene in 'Kosui' after endodormancy release (Ni et al. 2017). Complementing these findings, our phylogenetic studies revealed putative orthology between MdoBRR1 and B-PpRR3; MdoBRR8 and B-PpRR5; MdoBRR10 and B-PpRR9 proteins (Fig. 1c), the same *MdoBRRs* involved in apple bud dormancy cycle transition.

Although the expression of *MdoBRR1* and *MdoBRR8* showed similar patterns in the transition from endo- to ecodormancy between field ('Castel Gala') and artificially controlled ('Royal Gala') conditions, the same was not observed for *t*-zeatin (Figs. 3, 4). In this case, it is important to consider that the CK source is different in these two distinct dormancy datasets. The origin of endogenous CKs that control shoot branching is still controversial. Some studies associate it with root-derived CK and others with local

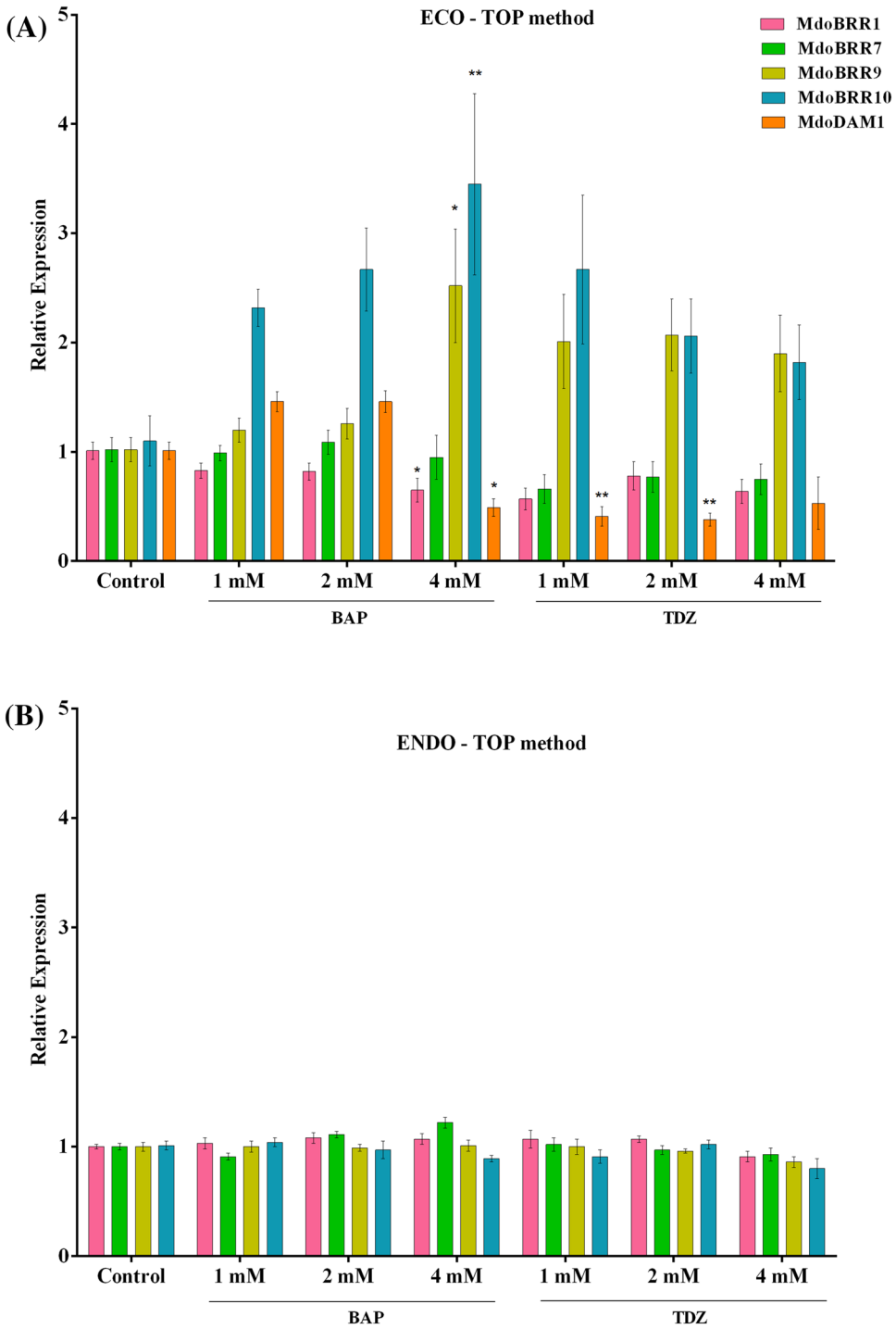


Fig. 5 The transcriptional modulation of *MdoBRR*s in endo- and ecodormant buds treated with CK by the TOP method. **a** The relative expression of *MdoBRRs* and *MdoDAM1* genes were measured in ecodormant buds treated for 16 h with 1, 2 or 4 mM of BAP or TDZ by the TOP method. **b** The expression of *MdoBRRs* was evaluated in endodormant buds under the same conditions. The expression level of each gene in the different treatments was calibrated and compared to its respective control. Asterisks indicate statistically significant differences between means of three biological replicates (15 buds each) using one-way ANOVA followed by Dunnet's test (*$0.01 < p < 0.05$, **$0.001 < p < 0.01$***). Error bars represent the standard error of the mean

production (Muller and Leyser 2011). 'Castel Gala' apical buds were sampled from plants under field conditions, and the two sources of CK (root-derived and locally synthesized) were available. On the other hand, the progression of 'Royal Gala' dormancy was evaluated under controlled artificial conditions where independent twigs containing apical buds were sampled from apple trees, being in this case, the CK local biosynthesis, the only source.

Little is known about the consequences of exogenous CK treatments in buds at dormant stage. The vast majority of reported studies tested CK in newly flowered buds (Chen et al. 2014; Fogelman et al. 2015; Li et al. 2016). Therefore, in order to investigate if *MdoBRRs* transcriptional regulation could be mediated by CK stimuli in endo- and ecodormant buds, we envisaged to test different concentrations, types of synthetic CKs and treatments. The most relevant effect of

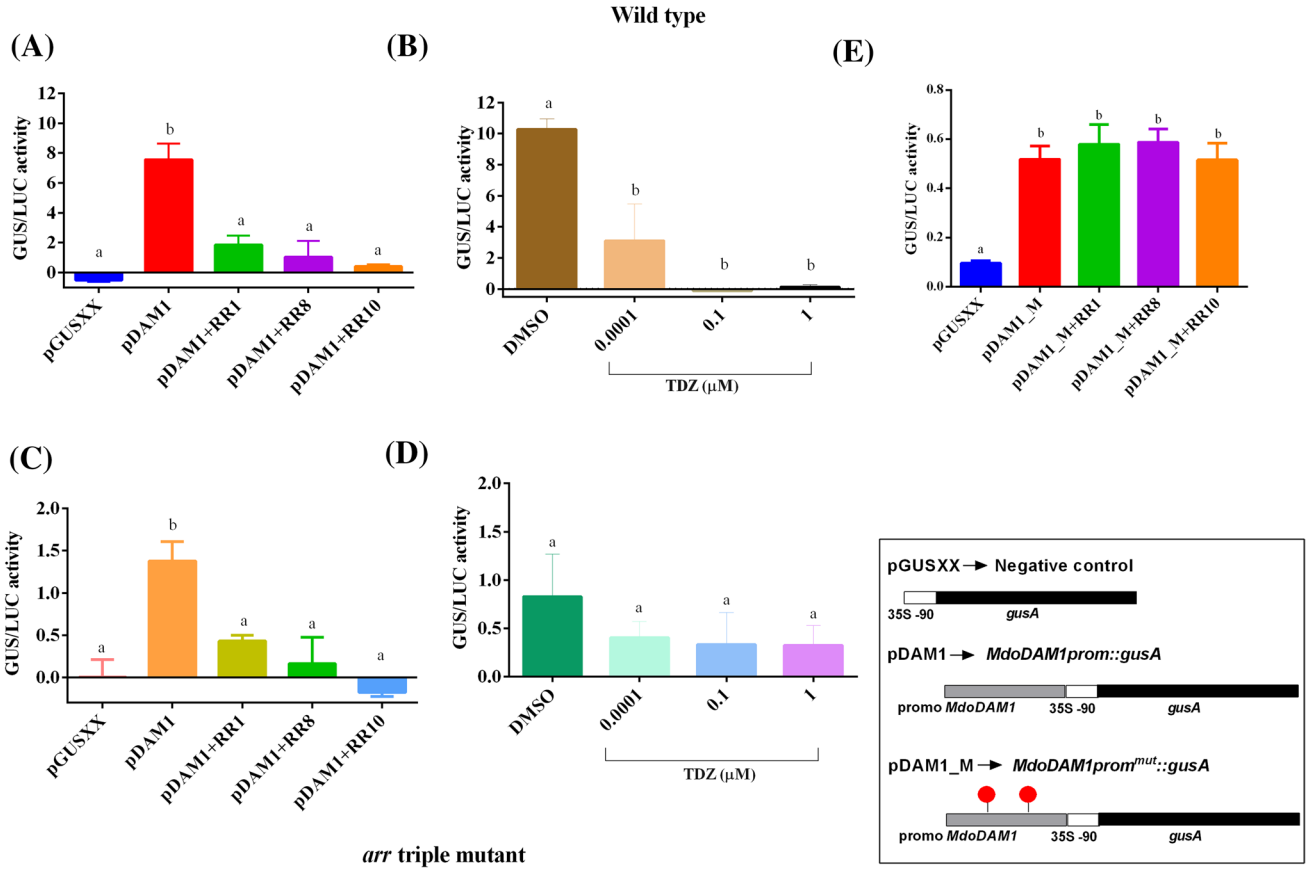


Fig. 6 *MdoDAM1* downregulation mediated by MdoBRRs and CK application. The modulation of *gusA* driven by the *MdoDAM1* promoter through *MdoBRR1*, *MdoBRR8* and *MdoBRR10* was evaluated in Arabidopsis wild-type (**a**) and *arr* triple loss of function mutant protoplasts (**c**). The effect of exogenous application of TDZ (from 0.0001 to 1.0 µM) and DMSO (control) was also tested for both wild-type (**b**) and *arr* backgrounds (**d**). Site-specific mutations were introduced into the *MdoDAM1* promoter and the binding ability of MdoBRRs effectors was tested in wild-type protoplasts (**e**). The GUS/LUC activity was calculated through dividing the enzymatic GUS activity by rLUC luminescence. RR1, RR8 and RR10 represent *MdoBRRs* respective coding regions cloned into the pART7 vector for protoplast transient overexpression. Different letters indicate statistically significant differences between means of four biological replicates using one-way ANOVA followed by Tukey's test ($p \leq 0.05$). Error bars represent the standard error of the mean

the treatments was the transcriptional activation observed for *MdoBRR9* and *MdoBRR10* in ecodormant buds treated with 4 mM BAP (Fig. 5). In agreement with our results, the activation of Arabidopsis *BRR* (*ARR10*) in a CK-dependent manner has been already reported (Hill et al. 2013; Zubo et al. 2017). The transcriptome analysis of rice plants treated with CK showed that the *BRR* gene *OsRR22* was downregulated in roots and upregulated in shoots. In addition, *OsRR26* was downregulated in roots and unaffected in shoots (Raines et al. 2016). The lack of a widespread *BRR* upregulation in response to CK stimulus could be related to its mode of action. BRR proteins are present independently of CK signaling and their activation is primarily related to the Asp phosphorylation in the REC domain rather than protein turnover (Sakai et al. 2001). This is in contrast with other hormone-regulated transcriptional activators like EIN3, involved in ethylene signaling, which is just stabilized in the presence of ethylene (Guo and Ecker 2003).

Even though our results demonstrated that *MdoBRRs* could be activated by the CK stimulus in ecodormant buds, the same response was not observed in endodormant buds (Fig. 5). This can be attributed to callose accumulation in the plasmodesmatas that closes the symplastic transport and interrupts intercellular communication during endodormancy (Wu et al. 2018). Throughout the process of dormancy overcoming, callose is degraded and the flow of water, nutrients and signaling molecules is reestablished (Portrat et al. 1995). Moreover, studies have demonstrated that TDZ is not effective to induce growth during apple endodormancy and it only exerts effect when a substantial part of the chilling requirement is reached (Faust et al. 1991). Additionally, the phenological stage also reflected in the different results found by CK treatment methods. Direct application (TOP method) was more effective during ecodormancy where bud tissues become more permeable and responsive to external stimuli. In endodormancy stage, buds

are externally protected by hard scales, which reduce permeability, decreasing the effect of CK direct treatment. Thus, in this case, the transport of CK by influx (BASE method) was responsible for a discreet *MdoBRR* activation. This small activation may be also the reflection of adaptive cell wall and membrane modifications, such as the deposition of callose in the plasmodesmata structures in the apical meristem tissues of the dormant bud (Sun et al. 2019).

Since 2008, with their initial characterization in the *evg* peach mutant (Bielenberg et al. 2008), *DAM* genes have been associated with dormancy establishment and maintenance in many species. The current evaluated dormancy cycles demonstrated higher levels of transcripts of *MdoDAM1* during endodormancy and a drastic reduction towards transition to ecodormancy (Figs. 3, 4). Accordingly, apple *DAM* genes (*MdoDAM1*, *MdoDAM3*, and *MdoDAM4)* are characterized by a seasonal oscillating transcript accumulation pattern, with a remarkable repression at ecodormancy stage (Mimida et al. 2015; Porto et al. 2016). Based on the potential different roles of *MdoDAM1* and *MdoBRR1*, *MdoBRR8* and *MdoBRR10* during the dormancy cycle regulation, in vivo transactivation assays were performed in order to evaluate the MdoBRR ability to modulate *MdoDAM1* expression. Our results demonstrated an interesting regulatory mechanism on which transient overexpression of *MdoBRR1*, *MdoBRR8* and *MdoBRR10* showed a repressive effect on the reporter gene guided by *MdoDAM1* promoter (Fig. 6a). Additionally, the CK signal led to downregulation of *MdoDAM1prom::gusA* (Fig. 6b), agreeing with results found in CK-treated ecodormant buds, where *MdoDAM1* was also repressed (Fig. 5).

The use of Arabidopsis *arr* triple loss-of-function mutant confirmed the consistency of the previously data obtained using wild-type plants in two aspects: minimizing the effect of endogenous BRRs (Fig. 6c) and discarding possible technique artifacts, because the mutant does not respond to exogenous CK stimuli (Fig. 6d; Argyros et al. 2008). The *arr* triple mutant plants display several developmental abnormalities when compared with wild type plants, including reduced leaves size and fewer cells per leaf (Argyros et al. 2008), which might explain the fivefold reduced activity of GUS/LUC found in the mutant background. Moreover, the regulatory region of *MdoDAM1* shares the same DNA motif [AGAT(T/C)] found in already described CK-responsive genes (Hosoda et al. 2002; Zubo et al. 2017) and the introduction of site-specific mutations in native BRR biding sites (Fig. S5B) disrupted *MdoDAM1prom*^{mut}*::gusA* modulation by MdoBRR1, MdoBRR8 and MdoBRR10 (Fig. 6e). Therefore, these data strongly suggest that MdoBRR proteins recognize these *cis*-elements and act as negative regulators of *MdoDAM1* expression.

The regulatory mechanism that involves *MdoDAM1* modulation by MdoBRRs might be associated with a physical barrier, created by MdoBRR protein binding. This barrier prevents the establishment of the correct DNA structural conformation necessary to *MdoDAM1* activation by other MADS-box factors that recognize the CArG-box elements found along the promoter region (Fig. S5; Kaufmann et al. 2005). Thus, in a very simple way, our proposed hypothetical model is based on accumulation of CK in dormant buds towards the transition from endo- to ecodormancy, which triggers MdoBRRs transcriptional activity and post-translational modifications. Once activated, MdoBRRs bind to *MdoDAM1* promoter, repressing it. Turning off *MdoDAM1* expression, dormancy release is favored (Fig. 7).

In conclusion, throughout the dormancy cycle, our results indicate that *MdoBRR1* and *MdoBRR8* apparently exhibit an important regulatory role towards the transition from endo- to ecodormancy, while *MdoBRR7*, *MdoBRR9* and *MdoBBR10* genes were upregulated only after ecodormancy was achieved and favorable environmental conditions were restored, indicating their potential roles in growth resumption. Besides that, in the ecodormancy stage, *MdoBRR1*, *MdoBRR9* and *MdoBBR10* were activated in a CK-dependent manner, leading to *MdoDAM1* downregulation. In vivo transactivation assays showed that the transient overexpression of *MdoBRR1, MdoBRR8* and *MdoBRR10* genes and CK stimuli have negative regulatory effects on *MdoDAM1* expression. Finally, these findings link CK hormonal stimulus with molecular responses through a regulatory mechanism of *MdoDAM1* repression mediated by CK-activated MdoBRRs that within the complex regulation of dormancy in perennials represents a contribution to the understanding of dormancy release in apple.

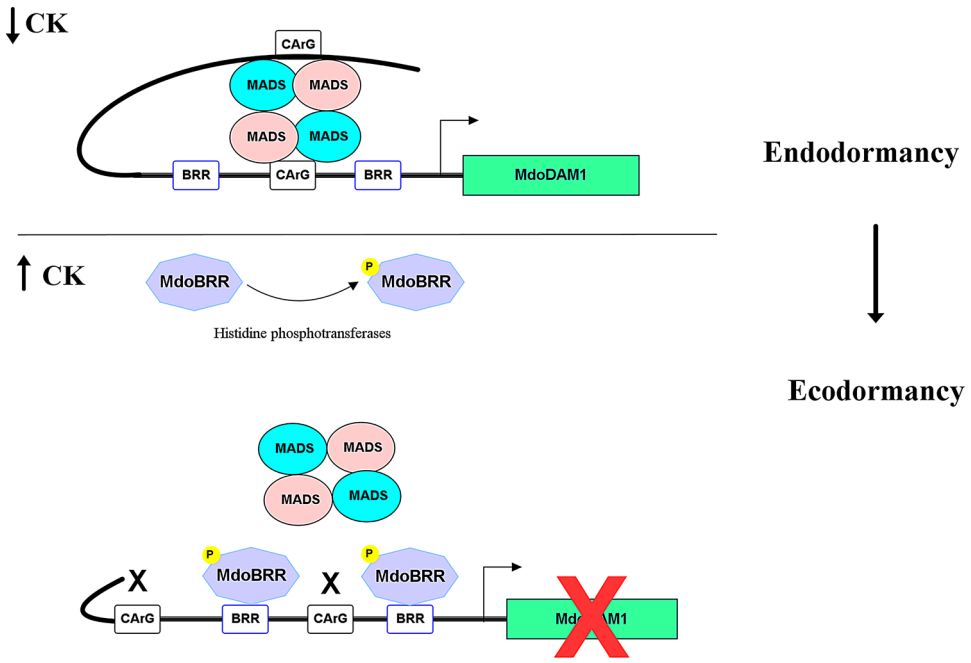


Fig. 7 Hypothetical model of *MdoDAM1* modulation through CK signal and consequent *MdoBRRs* transcriptional activation towards the transition from endo- to ecodormancy in apple buds. The model is based on accumulation of CK in dormant buds during the transition from endo- to ecodormancy, which activate MdoBRRs transcriptional activity and post-translational modifications (highlighted in yellow). Once activated, MdoBRRs bind to *MdoDAM1* promoter on their BRRs motifs, preventing that transcriptional activators, like MADS-box factors, bind to their *cis*-elements (CArG boxes), creating a physical barrier that does not enable DNA correct conformation, culminating in the repression of *MdoDAM1* gene. *P* phosphate group. Designed using IBS v.10.3 software

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Author contribution statement AMC, VSF, CPS, GP and LRF conceived and planned the experiments. AMC, CPS, VB carried out the experiments. AMC, VSF, FSM, GP and LRF contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Data availability Sequence data that support the findings of this study have been deposited in the GenBank database with the primary accession code from MN590295 to MN590303.

Code availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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