View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 586 (2012) 3385–3390

FEBS Letters
journal homepage: www.FEBSLetters.org

The strength of the miR398-Csd2-CCS1 regulon is subject to natural variation in *Arabidopsis thaliana*

Ilona Juszczak, Margarete Baier *

Plant Physiology, Freie Universität Berlin, Königin-Luise-Straße 12-16, 14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 20 May 2012
Revised 29 June 2012
Accepted 10 July 2012
Available online 25 July 2012

Edited by Tamas Dalmay

Keywords:
Chloroplast
Cold
Cu/Zn-superoxide dismutase
miR398
Photooxidative stress

ABSTRACT

miR398 links expression of the three major chloroplast copper proteins, plastocyanin, CCS1 and Csd2, to copper availability. miR398 abundance was stronger plastocyanin-controlled in accessions from cold and continental habitats (*Kas-1*, *Ms-0*, *WS*) than in *Cvi-0* and *Col-0*. Target gene regulation was broken for Csd2 in *Cvi-0* upon cold-treatment. Comparison of miR398 levels, target gene regulation as well as Ago1 and miR168 expression demonstrated that the miR398 regulon can be overwritten by accession specific transcriptional regulation in *Cvi-0*. It is concluded that the escape from the miRNA control of copper homeostasis is linked to adaptation of *Cvi-0* to its harsh natural habitat.
© 2012 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Plastocyanin (PetE), Cu/Zn-superoxide dismutase 2 (Csd2) and the copper chaperon of Csd2 (CCS1) are the most abundant copper-binding proteins in chloroplasts [1]. In parallel to being copper sinks, they serve key functions in photosynthesis and photoprotection. PetE transfers electrons from the cytochrome b_6f complex to photosystem I. Csd2 is the major superoxide detoxifying enzyme inside chloroplasts catalyzing dismutation of superoxide into oxygen and H_2O_2 [2]. CCS1 is the chaperon, which delivers copper ions to the Csd2 apo-protein [3].

Expression of Csd2 is adjusted to the photosynthetic and photooxidative demands by light [1,4]. PetE expression is transcriptionally regulated by light, redox signals and sugars [5]. PetE is often the limiting component of the photosynthetic electron transport chain [6] and its expression is crucial for plant energy metabolism. PetE2 expression also depletes the copper pool. In response, miR398 expression is induced [1], miR398 binds to the Csd2 and CCS1 transcripts and catalyzes their degradation [7].

Micro-RNAs are single-stranded, small (21–24 nucleotides) RNA molecules, which are transcribed by their own promoters. The primary transcripts are processed by RNase III to mature miRNAs forming RNA-Induced Silencing Complexes (RISC) with Argonaute (Ago) proteins. A RISC guides cleavage or translational repression of target mRNA and regulates diverse physiological processes in a miRNA-specific manner [8,9].

miR398 is a conserved micro-RNA identified by computational and experimental analyses [9]. miR398 is encoded by three loci in *Arabidopsis thaliana*: *MIR398a*, *MIR398b* and *MIR398c*. miR398b and c are identical in their sequence, whereas miR398a differs from them by a single nucleotide at 3′end [9]. Expression of miR398b/c is much higher than that of miR398a [10] giving miR398b/c a stronger regulatory impact. Four targets of miR398 have been identified, namely chloroplast Cu/Zn-SOD (Csd2), cytosolic Cu/Zn-SOD (Csd1), mitochondrial cytochrome-c oxidase sumbunit 5b (COX5b-1) and the copper chaperone for SOD (CCS1) [7,9]. Via regulation of its target genes, miR398 is involved in abiotic stress responses, such as oxidative stress [11], salt stress [12], ABA signalling [12], as well as biotic stress caused by bacterial pathogen *Pseudomonas syringae* [12].

Recent comparison of the expression regulation of chloroplast antioxidant enzymes between different accessions of *A. thaliana* revealed natural variation in the regulation of Csd2 upon temperature acclimation [13]. Here, we analyzed whether the accession specific variation depends on miR398 regulation, on the regulation of its interaction partner Ago1 or on its upstream regulator

Abbreviations: Ago, agonaute; CCS1, copper chaperon of Csd2; Csd2, chloroplast copper-zinc superoxide dismutase; Cu/Zn-SOD, copper-zinc superoxide dismutase; nt, nucleotides; qPCR, quantitative real-time polymerase chain reaction; r_S, Spearman's correlation coefficient; SOD, superoxide dismutase

* Corresponding author. Fax: +49 (0)30 838 51688.
E-mail address: margarete.baier@fu-berlin.de (M. Baier).

0014-5793/$36.00 © 2012 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.febslet.2012.07.049

miR168, as well as whether the variation in Csd2 correlates with accession specific regulation of the transcript levels of the other main chloroplast copper-binding proteins, PetE and CCS1. It will be shown that miR398 regulation can be specifically overwritten.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana accessions *Col-0* (NASC-N1092), *Kas-1* (NASC-N1264), *Cvi-0* (NASC-N1096), *Ms-0* (NASC-N1376), *WS* (NASC-N1602), *C24* (NASC-N906) and *Van-0* (NASC-N1584), were germinated and grown in potting soil containing 30 mg/kg copper and composed of 1 volume of Terreau Professionell Gepac Einheitserde Typ P (Einheitserde, Sinntal-Altengronau, Germany), 1 volume of Terreau Professionell Gepac Einheitserde Typ T (Einheitserde, Sinntal-Altengronau, Germany) and 1 volume of Perligran G (Knauf Perlite, Dortmund, Germany) for 2 weeks in a climate-controlled chamber (CU-41L4X; Percival Scientific Inc., Perry, IA, United States) at day/night temperature of 20 °C/18 °C and 120 $\mu mol\ m^{-2}\ s^{-1}$ light with 10 h light/14 h dark photoperiod. After two weeks, the plants were transferred for one week to 10, 20, 30 °C and 120 $\mu mol\ m^{-2}\ s^{-1}$ light in a 10 h light/14 h dark photoperiod.

2.2. RNA isolation

Total RNA was extracted from rosette leaves using the GeneMatrix Universal RNA Purification Kit (EURx, Gdansk, Poland). To remove genomic DNA contaminations, on-column digestion with RNase-free DNaseI (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions was performed. The purity of extracted RNA was assessed by measuring the $A_{260/280}$ and $A_{260/230}$ ratios. The integrity of the samples was checked on formaldehyde-agarose gels.

2.3. qPCR analyses

cDNA was synthesized from 500 ng DNaseI-treated total RNA and analyzed according to the MIQE standards [14] as described previously [13] with gene-specific primers (Table 1). Micro-RNAs were produced according to [15] using stem-loop primers (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGG-3′, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGGT-3′ and 5′-GTCGTATCCAGTTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCC-3′ for miR398a, miR398b/c and miR168a, respectively). qPCR analyses were performed with miRNA-specific forward primers and a common reverse primer (Table 1). The specificity was analyzed from the melting curves (CFX96 thermocycler, Bio-rad, Munich, Germany). The efficiency of amplification was tested with tenfold dilution series of respective cDNA sample. All qPCR reactions were performed for two biological replicates, each analyzed in three technical repeats.

2.4. Sequence analysis

All sequences were obtained from the websites of Salk Institute (http://signal.salk.edu/atg1001/download.php) and 1001 Genome Project (http://www.1001genomes.org/accessions.html) and compared by CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results

3.1. The transcript levels encoding the main chloroplast copper proteins in A. thaliana accessions

The analyzed *A. thaliana* accessions originate from habitats characterized by different average temperatures. Due to distinct adaptation, temperature variation can be expected to differently affect gene expression and, therefore, increase the variability between accessions. Here, the steady state mRNA levels of genes encoding superoxide dismutase 2 (Csd2), the copper chaperone for SOD (CCS1) and plastocyanin (PetE2) were analyzed in parallel in RNA samples isolated out of 3-week-old rosettes of *Col-0*, *Kas-1*, *Cvi-0*, *Ms-0*, *WS*, *C24* and *Van-0* using quantitative real-time PCR (qPCR). Plants used in the studies were cultivated for 2 weeks at day/night temperature of 20 °C/18 °C and then transferred for one additional week to 10, 20 or 30 °C. These temperatures span the temperature range at the natural habitats of all selected accessions. In case of plastocyanin the focus was set on PetE2, which is more prominently expressed than PetE1, responds to copper availability and affects regulation of Csd2 and CCS1 [1]. The transcript abundances were normalized on actin transcript levels (At3g18780) and compared to the expression of the same gene in the reference accession *Col-0*.

The steady state mRNA levels varied for all analyzed genes between the accessions cultivated at 20 °C (Fig. 1). All tested accessions, except *C24* (1.19 ± 0.19 in comparison with *Col-0*), accumulated at 20 °C less Csd2 transcripts than *Col-0*, consistent with previous reports with different plant sets [13]. The strongest differences to *Col-0* were detected for *Cvi-0* (0.48 ± 0.07) and *Ms-0* (0.56 ± 0.1), whose steady state Csd2 transcript levels were only half of that observed in *Col-0* (Fig. 1).

The relative transcript levels of all studied genes responded to temperature acclimation (Fig. 1). Such a responsiveness is prerequisite for transcript level correlation studies. For Csd2 and CCS1, the RNA level gradually increased with the growth

Table 1
Primers used for RNA quantification by real-time PCR.

Gene	Gene code	Forward primer Reverse primer	Annealing temperature [°C]
Act2	At3g18780	AATCACAGCACTTGCACCAAGC CCTTGGAGATCCACATCTGCTG	60
Csd2	At2g28190	ATGACACACGGAGCTCCAGAA ATTGTTGTTTCTGCCACGCCA	60
CCS1	At1g12520	GAGCCATGCCTCAGCTTCTTAC TCACAGCATTAACACAACCCTCAC	60
Ago1	At1g48410	GCACACGCTCAGTTTCAATTGTTC ATGCTCCCACTAGCCATTGAGC	60
PetE2	At1g20340	TCACCGGCCTTAAAGCCTCAAC TGATGCAACAGCAGCAGTTTGG	60
miR398	At2g03445	CTCGACGTGTGTTCTCAGGTCAC CCAGTGCAGGGTCCGAGGT	60
miR168a	At4g19395	GCTATCGCTTGGTGCAGGTC CCAGTGCAGGGTCCGAGGT	60

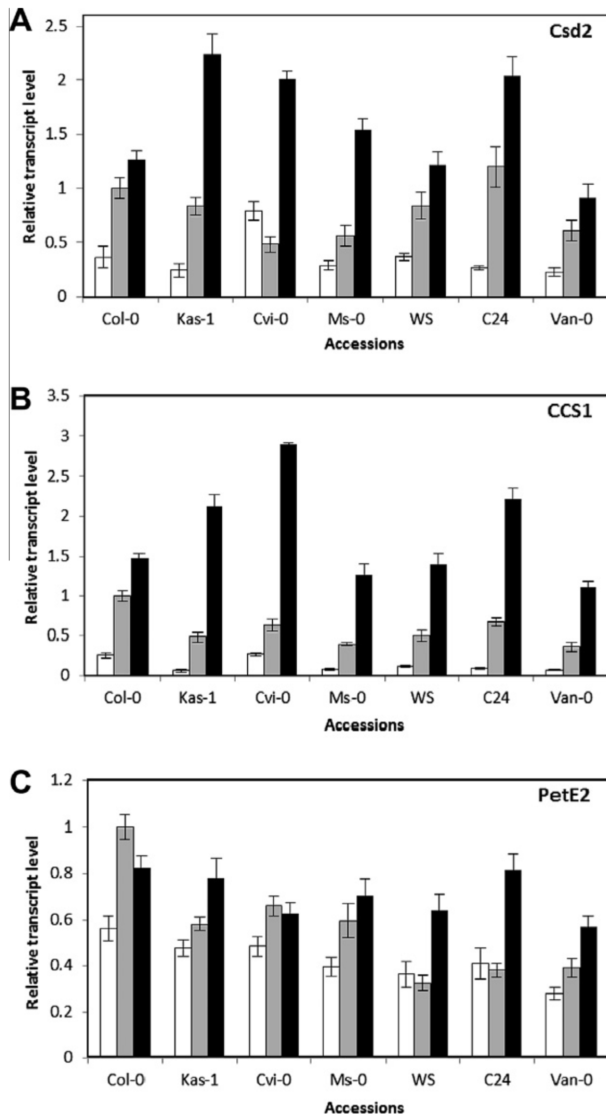

Fig. 1. Transcript level of genes encoding the main chloroplast copper proteins in six accessions of *A. thaliana* relative to *Col-0*: (A) superoxide dismutase (Csd2), (B) copper chaperone for SOD (CCS1), (C) plastocyanin 2 (PetE2). Following two weeks at 20 °C the plants were transferred for one week to 10 °C (white bars), 20 °C (grey bars) or 30 °C (black bars), respectively. Data are means ± SD from two independent RNA isolation, each representing 3 technical replicates.

temperature in almost all tested accessions, except Csd2 in *Cvi-0*. In *Cvi-0*, the Csd2 transcript level was higher at 10 °C than at 20 °C.

The highest Csd2 levels were observed in *Kas-1* (followed by *C24* and *Cvi-0*) at 30 °C, the lowest in *Kas-1* and *Van-0* at 10 °C. The CCS1 transcript levels were the highest in *Cvi-0* followed by *Kas-1* and *C24*. The lowest CCS1 levels were detected in *Kas-1*, *Ms-0* and *Van-0*. In general, *Cvi-0* showed the highest overall induction potential of Csd2 and CCS1, while *Kas-1* had the highest regulatory dynamic.

Transcript abundance regulation of PetE2 was the most variable between the tested accessions. Gradual variation of transcript abundance with growth temperature was observed for accessions originating from colder and continental habitats, such as *Kas-1*, *Ms-0* and *Van-0*. In accessions coming from moderate and warm climates (*Col-0* and *Cvi-0*), the transcript levels of PetE2 increased or decreased in parallel upon elevated and decreased growth temperature. The lowest PetE2 levels were observed in *Van-0* and *WS* at 10 °C coming from continental cold habitats. The 10 °C-PetE2-levels were highest in *Col-0* and *Cvi-0*. Generally, the PetE2 transcript abundance data showed the closest dependency to the temperature conditions at the natural habitats from all transcripts tested. PetE2 transcript levels positively correlated with Csd2 and CCS1 transcript levels in all accessions, but the correlation coefficients were below 0.5 for both genes in *Cvi-0* (Table 2).

3.2. Transcript levels of Csd2 and CCS1 differentially correlate with miR398

Sunkar et al. [11] and Beauclair et al. [16] showed that expression of Csd2 and CCS1 is regulated post-transcriptionally by miR398. However, such regulation has been studied so far only in *Col-0* and *WS* backgrounds. In our comparison, the transcript levels of Csd2 and CCS1 were strongly correlated ($r_s > 0.95$) at 20 °C between all tested accessions indicating that post-transcriptional co-regulation is widely conserved.

To test this hypothesis, the seven accessions of *A. thaliana* were analyzed for miR398a and miR398b/c regulation at 10, 20 or 30 °C by qPCR. The levels of all miR398 were highly variable between the accessions and the treatments. In general, the level of miR398a and miR398b/c decreased with an increase in the growth temperature and, as it was observed by Yamasaki et al. [10], miR398b/c was stronger expressed than miR398a. The highest miR398a level was observed in *Cvi-0* acclimated to 10 °C and the lowest in *Ms-0* at 30 °C, whereas the level of miR398b/c was the highest in *Kas-1* acclimated to 10 °C and the lowest in *Cvi-0* at 30 °C (Fig. 2).

To analyze whether miR398 regulates Csd2 and CCS1 transcript abundance, the miR398a and miR398b/c levels were compared with Csd2, CCS1 and PetE2 transcript levels (Table 2). The transcript pairs Csd2/miR398 and CCS1/miR398 showed almost perfect negative correlation for *Ms-0*, *WS* and *C24* ($r_s < -0.95$). In *Kas-1*, the levels of both types of miR398s correlated well with the levels of Csd2 ($r_s = -0.93$), whereas the correlation coefficient for the pair CCS1 and miR398a/miR398b/c were higher than −0.9. In *Van-0* the correlation coefficients between Csd2 and the miR398s were −0.978 and −0.999. CCS1 perfectly correlated just with miR398b/c ($r_s = -0.94$). The correlation coefficient for the pair CCS1 and miR398a was −0.87 (Table 2).

Strongest accession specific regulation of Csd2 was observed for *Cvi-0* (Fig. 1). The Spearman's correlation coefficient was only −0.66 for Csd2/miR398a and −0.91 for Csd2/miR398b/c. Under the same conditions the correlation coefficient for pair CCS1 and miR398a was −0.87 and −0.99 for miR398b/c and CCS1. Perfect and low correlation of miR398a and miR398b/c expression, respectively, with the transcript level of Csd2 and CCS1 was revealed for *Col-0* in the temperature acclimation experiment suggesting that Csd2 and CCS1 expression in *Col-0* is preferentially regulated by miR398a. In *Col-0*, Csd2 and CCS1 transcript levels were co-regulated ($r_s = 0.99$), while they were specifically inversely adjusted in *Cvi-0* at 10 °C relative to the transcript levels at 20 °C (Fig. 1).

3.3. Correlation intensity of PetE2 expression with the miR398 varies in Arabidopsis accessions

miR398 expression in linked to PetE2 expression intensity via sensing of the copper availability [1]. Spearman's correlation analyses done for the transcript pairs PetE2/miR398a and PetE2/miR398b/c showed that the expression of PetE2 and miR398a is weakly correlated in *Col-0* ($r_s = -0.60$), *Cvi-0* ($r_s = -0.85$) and *C24* ($r_s = -0.82$). The correlation between PetE2 and miR398b/c was even lower for *Col-0* ($r_s = -0.23$) and *Cvi-0* ($r_s = -0.56$). Expression of these genes was well to almost perfectly negatively correlated in *Kas-1* ($r_s < -0.95$), *Ms-0* ($r_s < -0.96$), *WS* ($r_s < -0.90$) and *Van-0* ($r_s < -0.92$), which grew on the same, non-copper depleted soil,

Table 2
Spearman's rank correlation coefficients (r_S) for pairs of genes.

Accession	Csd2 miR398a	Csd2 miR398b/c	CCS1 miR398a	CCS1 miR398b/c	PetE2 miR398a	PetE2 miR398b/c	Ago1 miR168a	PetE2 Csd2	PetE2 CCS1	Csd2 CCS1	Ago1 Csd2	Ago1 CCS1	miR168a Csd2	miR168a CCS1
Col-0	−0.971	−0.800	−0.992	−0.863	−0.595	−0.231	0.278	0.769	0.691	0.993	0.354	0.245	−0.800	−0.963
Kas-1	−0.925	−0.931	−0.881	−0.888	−0.946	−0.951	0.849	0.998	0.987	0.995	−0.983	−0.997	−0.931	−0.888
Cvi-0	−0.660	−0.908	−0.862	−0.994	−0.850	−0.564	0.672	0.165	0.467	0.949	−0.299	−0.584	−0.908	−0.994
Ms-0	−0.967	−0.977	−0.978	−0.986	−0.974	−0.963	0.797	0.883	0.904	0.999	−0.649	−0.684	−0.977	−0.986
WS	−0.951	−0.968	−1.000	−0.999	−0.924	−0.900	0.382	0.761	0.919	0.955	−0.603	−0.339	−0.968	−0.999
C24	−1.000	−0.996	−0.960	−0.982	−0.818	−0.869	−0.738	0.818	0.947	0.960	0.672	0.853	−0.996	−0.982
Van-0	−0.978	−0.999	−0.866	−0.937	−0.920	−0.973	0.633	0.982	0.993	0.993	−0.597	−0.323	−0.973	−0.937

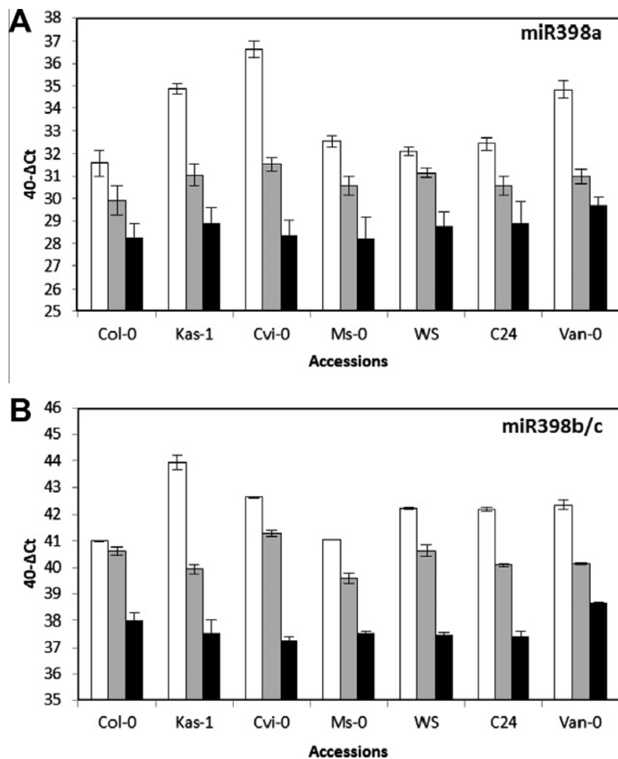

Fig. 2. Mature A. miR398a and B. miR398b/c levels in seven accessions of *Arabidopsis thaliana* cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (white bars), 20 °C (grey bars) or 30 °C (black bars) for low, control or high temperature treatment, respectively. Expression levels are given on a logarithmic scale expressed as 40 – ΔCt, where ΔCt is the difference in qPCR threshold cycle number of the miR398 and the reference gene Act2, wheras 40 is the cycle number when qPCR reaction ends. Data are means ± SD from two biological replicates.

demonstrating an accession specific regulatory impact of PetE2 expression on miR398 abundance.

3.4. Sequence analysis of miR398 and miR398 target sites in Csd2 and CCS1

The transcript abundances of Csd2 and CCS1, respectively, were closely correlated in some accession, but only loosely in others, such as *Cvi-0* and *Kas-1* (Table 2). The variability could be either due to specific individual control of gene expression or results from mutations in the miRNA sequence or in the mRNA target site in the post-transcriptionally controlled transcripts. To distinguish between the two options, the miR398 sequence and the sequences of its target site in Csd2 and CCS1 transcripts were compared. No variation was found (data not shown). Thus, it can be assumed that the binding affinity is the same in all accessions and the regulatory force of the miRNA only depends on its levels and the transcript abundances of the target genes.

3.5. The regulation of Csd2 and CCS1 expression by miR398 is modulated by Ago1 transcript abundance

To analyze further what stabilizes or breaks miR398 controlled regulation, the expression of the miR398 upstream regulators Argonaute1 (Ago1) and miR168a were studied. Ago1 protein forms a RISC with miR398 [17]. Ago1 expression is controlled by miR168a [17].

At 10, 20 and 30 °C, stronger variations were observed in the level of Ago1 and miR168a between the Arabidopsis accessions than for the other tested genes (Fig. 3). The calculated Spearman's correlation coefficients showed a positive correlation between Ago1 and miR168a for all accessions, except for *C24* (r_s = −0.74), in which the Ago1 transcript level was increased at 30 °C growth temperature and the miR168a transcript level was decreased. However, for most accessions (*Col-0*, *Cvi-0*, *Ms-0*, *WS*, *Van-0*) the correlation coefficients were less than 0.8 indicating that the miR168-Ago1 auto-regulon described by Vaucheret et al. [17] is generally weaker than the miR398 controlled regulon.

To analyze the impact of miR168 and Ago1 on the miR398 regulon, the transcript abundances were pairwise compared by

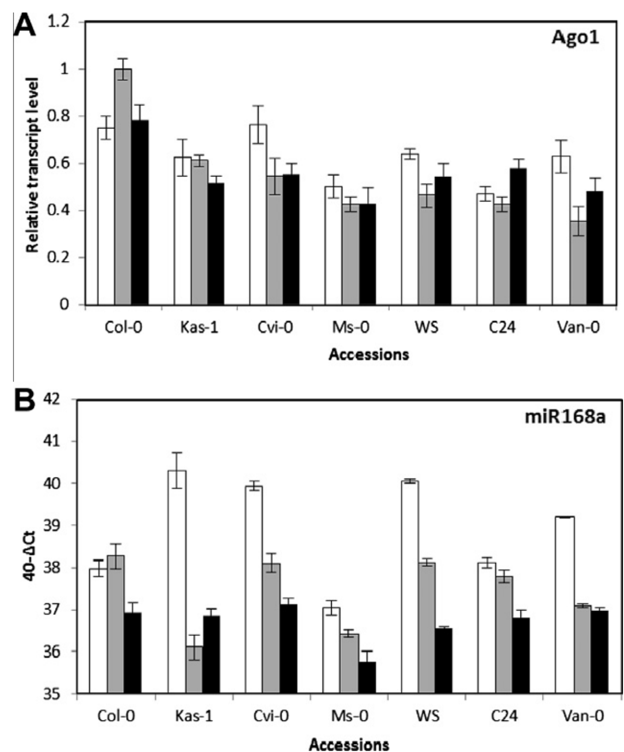

Fig. 3. Transcript level of genes encoding: A. Argonaut 1 (Ago1) and B. miR168a in six accessions of *A. thaliana*. All accessions were cultivated for two weeks at 20 °C and then transferred for one week to 10 °C (white bars), 20 °C (grey bars) or 30 °C (black bars), respectively. Data are means ± SD from two independent replicates.

calculating the Spearman's coefficients. Strong negative correlation coefficients (r_S < 0.98) between Csd2 and CCS1 transcript levels and Ago1 transcript levels were only observed for *Kas-1* indicating that Ago1 expression only weakly impacts on the transcript levels of the miR398 targets. miR168a levels were generally negatively correlated with Csd2 and CCC1 transcript levels, but less linked to Ago1 transcript levels.

4. Discussion

PetE2, Csd2 and CCS1 are linked by a miRNA regulon adjusting the copper availability in chloroplasts [7]. Plastocyanin is an important and often limiting electron carrier in the photosynthetic electron transport chain [6]. Consequently higher plants prioritize the delivery of copper to PetE by down-regulation of other copper-containing proteins [1]. The control of copper availability is regulated by miR398 over a wide range of copper concentrations in *Col-0* [1]. In the comparison of Arabidopsis accessions, the levels of miR398a and miR398b/c were negatively correlated with PetE2 expression in all tested genotypes demonstrating that the copper regulons has been conserved over a wide range of species diversification and adaptation to distinct habitats. In all tested accession, miR398 controlled the level of both, Csd2 and CCS1, and the miR398 interaction sites were absolutely conserved (data not shown).

4.1. PetE2 effect on miR398 regulation

The best correlations between PetE2 transcript levels and miR398 availability were observed for accessions originating from colder and continental climates, namely *Kas-1*, *Ms-0* and *WS*, while in accessions coming from moderate and warm habitats (*Col*-0 and *Cvi*-0) the correlations were weaker (Fig. 1; Table 2). Systematic comparison of photosynthetic performance has not been available for all of the tested accession. However, consistent with Adel-Ghany's [1] conclusion, that plastocyanin levels are preferentially adjusted to maintain the photosynthetic performance, comparison of *Col-0* and *WS* [18] demonstrated that the *Arabidopsis* accessions do not differ in the quantum yield of photosystem II and non-photosynthetical quenching upon light induction and over a wide range of tested light intensities. Similarly, *Cvi-0* and *Col-0* stabilized the quantum yield of photosystem II in field experiments in ambient and elevated CO_2 [19].

PetE expression is strongly regulated by photosynthetic parameters, such as light, carbohydrate availability, abscisic acid and the redox state of the plastoquinone pool [5]. Most of these signals vary between accessions and are affected by the growth temperature [20,21]. In the comparison of the Arabidopsis accessions, the negative correlation between PetE2 transcript levels and miR398a and miR398b/c levels were the weaker the stronger PetE2 was expressed at 20 °C (Fig. 1C and 2). Consistent with comparison of *petE2* deficient lines and *Col-0* wild-type plants [1], the data demonstrate that PetE2 transcripts do not directly act on miR398 levels, but trigger a non-linear parameter translating plastocyanin availability into miR398 abundance.

4.2. miR168a and Ago1 regulation

miR168 and Ago1 expression are transcriptionally co-regulated [17]. miR168 is post-transcriptionally stabilized by Ago1 [17] and, vice versa, miR168 controls Ago1 homeostasis via a feedback regulatory loop [22]. In the acclimation response of the Arabidopsis accessions to temperature, the correlation coefficients between Ago1 transcript levels and miR168 levels were low (Fig. 3 and Table 2), demonstrating low co-regulation and, therefore, different adjustment of the transcriptional and post-transcriptional effects.

4.3. miR398. regulation of Csd2 and CCS1

miR398 regulation is conserved in all accessions (Table 2). The negative correlation of miR398a and miR398b/c with Csd2 transcript levels was stronger in *Van-0*, *C24*, *Ms-0* and *WS* than in *Kas-1*, *Cvi-0* and in *Col-0*. Correlation between the miR398 abundances and CCS1 transcript levels was also the strongest ($r_S < -0.90$) for *WS*, *Ms-0*, *C24* and *Van-0*. In summary, these data show that the miRNA regulation dominates in *Van-0*, *C24*, *Ms-0* and *WS* upon temperature acclimation, while it is overlaid (to variable extends) in the other accession.

At 20 °C, Ago1 transcript levels were lower in *Van-0*, *C24*, *Ms-0* and *WS* than in *Col-0*, *Kas-1* and *Cvi-0*. Since Ago1 protein forms a RISC with miR398 [17], which enables cleavage of the Csd2 and CCS1 target transcripts [7], lower Ago1 levels (Fig. 3) cannot explain higher regulatory impacts of miR398 (Table 2). Also, the regulation to increased or decreased growth temperature gave no indication that the Ago1 transcript level controls miR398 function on its target genes (Fig. 1 and 3; Table 2). Since the miRNA interaction sites are fully conserved (data not shown), it is concluded that the difference in the correlation between the abundance of the target transcript and miR398 results from accession specific transcriptional regulation of Csd1 and CCS1.

Regulation of Csd2 in *Cvi-0* at 10 °C was the most accession specific variation (Fig. 1). Compared to 20 °C growth temperature, the Csd2 transcript level was increased in *Cvi-0*, while it was decreased in all other accession. Since CCS1, which is also under control of miR398, was decreased under the same condition, the regulation is specific for the Csd2 gene. The atypical *Cvi-0* regulation of Csd2 demonstrates that post-transcriptional regulation by miR398 can be overwritten up to effect inversion.

The comparison of *Cvi-0* and *Kas-1* provides further insight into the regulation of the miR398 and Csd2 balance: In *Kas-1* miR398b/c levels were higher at 10 °C growth temperature than in *Cvi-0* (Fig. 2). The elevated miRNA levels resulted in the lowest Csd2 and CCS1 levels in *Kas-1* observed in the comparison of the accession. In contrast, Csd2 expression was regulated in a gene specific manner in *Cvi-0* and impacted on miR398b/c and CCS1 levels (Fig. 1). miR398b/c is complementary in 16 of 21 nucleotides with CCS1 and Csd2 [7], but a single nucleotide insertion in the miRNA binding site of Csd2 after 12 matching nucleotides weakens the miRNA-target interaction. By average CCS1 transcript levels are only about 1/7 of the Csd2 transcript levels in rosette leaves (as calculated from signal intensities provided on: http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Stronger miRNA binding plus lower steady state transcript levels makes CCS1 transcripts more accessible to miR398 regulation than Csd2 transcripts. Vice versa, Csd2 transcripts can more easily accumulate if Csd2 transcription is specifically induced.

Cvi-0 is less sensitive to drought, heat, high irradiation and to paraquat [23]. The tolerance was linked to a *Cvi-0* specific and more active Csd2 allele (Csd2-2) [23]. Based on Csd2 transcript abundance regulation and correlation analysis (Fig. 1, Table 2) we propose that the higher stress tolerance of *Cvi-0* results not primarily from the two amino acid variation of Csd2-2 [23], but is linked to specific expression regulation and its capability to overwrite the miR398b/c-driven copper regulon.

In the first description of Csd2-2, *Cvi-0* was only compared with *A. thaliana* var. *Landsberg erecta* (Ler) [23]. The wider comparison of Arabidopsis accessions presented here (Fig. 1) demonstrated that in *Cvi-0* the Csd2 transcript levels increase in the cold. The induction can stabilize the activity increase reported by Abarca et al. [23] over time.

Cvi-0 is slightly less cold-tolerant than *C24* [24], which did not activate of Csd2 expression upon cold (Fig. 1). Consequently, induction of Csd2 is not essential for cold-tolerance, but is specific for

Cvi-0. Csd2 induction enables the escape from the otherwise conserved miR398b/c regulation for the sake of improved Csd2 responsibility to stress conditions. *Cvi-0* originates from a rocky habitat 1100–1200 m above sea level on the dark, volcanic mountains of the Cape Verdi Islands. Low amounts of rain are restricted to the second half of the year which limits the vegetation period to late summer and autumn. *Cvi-0* has to develop quickly in an environment with high irradiation. In addition, on a dark rock, light can strongly heat up the temperature around the plants during the day. Specific regulation of Csd2 in *Cvi-0*, might be an adaptation to the specifically harsh environment.

In accessions from colder and less dry habitats with shorter vegetation periods, such as *Kas-1*, *WS-0* and *Van-0* Csd2 and CCS1 expression is much stronger linked to PetE2 expression in a miR398b/c-dependent way (Fig. 1, Table 2). The most common interpretation of the copper homeostasis regulons, proposes it as a mechanism to control the copper pool to maintain PetE expression and defines PetE as essential and indispensable [7]. Limiting CCS1 and Csd2 expression for the sake of stabilization of PetE expression is advantageous if the risk for photooxidative superoxide formation is low. In excess light (combined with drought) Mehler reaction activity is supported [25]. As a consequence, plants face a strain on superoxide detoxification. Here, the uncoupling of Csd2 expression from miR398 regulation was specifically observed in the cold at 120 μmol quanta m^{-2} s^{-1}. The light intensity was much lower than at the natural habitat, but low temperature limited regeneration of the electron acceptors of photosystem I, supported photooxidative stress and induced Csd2 expression.

5. Conclusion

In summary, this analysis demonstrated that the miR398 regulon is active in all tested accession and responds to PetE2 regulation. Conservation of the regulons over such a wide radiation and adaptation demonstrates the strong selective pressure of coordination of the copper supply for stabilization of the most limiting step in the photosynthetic electron transport efficiency, namely plastocyanin expression [6], and avoiding copper-catalyzed Fenton chemistry. The conserved mechanism can be overwritten by transcriptional regulation of Csd2 in *Cvi-0*. The escape from the copper-homeostasis dominated regulation for the sake of superoxide detoxification explains the exceptional high tolerance of *Cvi-0* to a wide range of stresses and to the extremely harsh conditions at the natural habitat.

References

[1] Abdel-Ghany, S.E. (2009) Contribution of plastocyanin isoforms to photosynthesis and copper homeostasis in *Arabidopsis thaliana* grown at different copper regimes. Planta 229, 767–779.
[2] Rizhsky, L., Liang, H. and Mittler, R. (2003) The water-water cycle is essential for chloroplast protection in the absence of stress. J. Biol. Chem. 278, 38921–38925.
[3] Abdel-Ghany, S.E., Burkhead, J.L., Gogolin, K.A., ndres-Colas, N., Bodecker, J.R., Puig, S., Penarrubia, L. and Pilon, M. (2005) AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. FEBS Lett. 579, 2307–2312.
[4] Kliebenstein, D.J., Monde, R.A. and Last, R.L. (1998) Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol. 118, 637–650.
[5] Oswald, O., Martin, T., Dominy, P.J. and Graham, I.A. (2001) Plastid redox state and sugars: interactive regulators of nuclear-encoded photosynthetic gene expression. Proc. Natl. Acad. Sci. U S A 98, 2047–2052.
[6] Schottler, M.A., Kirchhoff, H. and Weis, E. (2004) The role of plastocyanin in the adjustment of the photosynthetic electron transport to the carbon metabolism in tobacco. Plant Physiol. 136, 4265–4274.
[7] Bouché, N. (2012) New insight into miR398 functions in Arabidopsis. Plant Signal. Behav. 5, 684–686.
[8] Chiou, T.J. (2007) The role of microRNAs in sensing nutrient stress. Plant, Cell Environ. 30, 323–332.
[9] Sunkar, R. and Zhu, J.K. (2004) Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 16, 2001–2019.
[10] Yamasaki, H., Abdel-Ghany, S.E., Cohu, C.M., Kobayashi, Y., Shikanai, T. and Pilon, M. (2007) Regulation of copper homeostasis by micro-RNA in Arabidopsis. J. Biol. Chem. 282, 16369–16378.
[11] Sunkar, R., Kapoor, A. and Zhu, J.K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 18, 2051–2065.
[12] Jagadeeswaran, G., Saini, A. and Sunkar, R. (2009) Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis. Planta 229, 1009–1014.
[13] Juszczak, I., Rudnik, R., Pietzenuk, B. and Baier, M. (2012) Natural genetic variation in the expression regulation of the chloroplast antioxidant system among *Arabidopsis thaliana* accessions. Physiol. Plantarum.
[14] Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.
[15] Chen, C.F., Ridzon, D.A., Broomer, A.J., Zhou, Z.H., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., Lao, K.Q., Livak, K.J. and Guegler, K.J. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33.
[16] Beauclair, L., Yu, A. and Bouche, N. (2010) MicroRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. Plant J. 62, 454–462.
[17] Vaucheret, H., Mallory, A.C. and Bartel, D.P. (2006) AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. Mol. Cell 22, 129–136.
[18] Jung, H.S. and Niyogi, K.K. (2009) Quantitative genetic analysis of thermal dissipation in Arabidopsis. Plant Physiol. 150, 977–986.
[19] Li, P.H., Ainsworth, E.A., Leakey, A.D.B., Ulanov, A., Lozovaya, V., Ort, D.R. and Bohnert, H.J. (2008) Arabidopsis transcript and metabolite profiles: ecotype-specific responses to open-air elevated [CO(2)]. Plant, Cell Environ. 31, 1673–1687.
[20] Cross, J.M., von Korff, M., Altmann, T., Bartzetko, L., Sulpice, R., Gibon, Y., Palacios, N. and Stitt, M. (2006) Variation of enzyme activities and metabolite levels in 24 arabidopsis accessions growing in carbon-limited conditions. Plant Physiol. 142, 1574–1588.
[21] Davey, M.P., Woodward, F.I. and Quick, W.P. (2009) Intraspecfic variation in cold-temperature metabolic phenotypes of Arabidopsis lyrata ssp petraea. Metabolomics 5, 138–149.
[22] Li, W., Cui, X., Meng, Z.L., Huang, X.H., Xie, Q., Wu, H., Jin, H.L., Zhang, D.B. and Liang, W.Q. (2012) Transcriptional regulation of Arabidopsis MIR168a and ARGONAUTE1 homeostasis in abscisic acid and abiotic stress responses. Plant Physiol. 158, 1279–1292.
[23] Abarca, D., Roldan, M., Martin, M. and Sabater, B. (2001) Arabidopsis thaliana ecotype Cvi shows an increased tolerance to photo-oxidative stress and contains a new chloroplastic copper/zinc superoxide dismutase isoenzyme. J. Exp. Bot. 52, 1417–1425.
[24] Zuther, E., Schulz, E., Childs, L.H. and Hincha, D.K. (2012) Clinal variation in the nonacclimated and cold acclimated freezing tolerance of Arabidopsis thaliana accessions. Plant, Cell Environ..
[25] Schoner, S. and Krause, G.H. (1990) Protective systems against active oxygen species in spinach – response to cold-acclimation in excess light. Planta 180, 383–389.