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MYB59 transcription factor behaves differently in metallicolous and non-metallicolous populations of *Arabidopsis halleri*

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Abstract. In *Arabidopsis thaliana* (L.) Heynh., MYB59 transcription factor participates in regulating Ca homeostasis and signal transduction and is induced by Cd excess. To investigate its role in the facultative metallophyte *Arabidopsis halleri* ssp. *halleri* (L.) O'Kane and Al-Shehbaz, *MYB59* expression was investigated under Cd treatment or Ca depletion in three populations belonging to distinct phylogeographic units (metallicolous PL22 and I16 and non-metallicolous I29), and compared with the expression in *A. thaliana*. In control conditions, *MYB59* transcription in *A. thaliana* and the non-metallicolous population I29 follow a comparable trend with higher expression in roots than shoots, whereas in metallicolous populations I16 and PL22 its expression is similar in roots and shoots, suggesting a convergent evolution associated with adaptation to metalliferous environments. After 6 h of Ca depletion, *MYB59* transcript levels were very high in I16 and PL22 populations, indicating that the adaptation to metalliferous environments requires tightly regulated Ca homeostasis and signalling. Cd treatment caused variability in *MYB59* expression. In I29, *MYB59* expression, as in *A. thaliana*, is likely associated to stress response, whereas its modulation in the two metallicolous populations reflects the different strategies for Cd tolerance and accumulation. In conclusion, *MYB59* regulation in *A. halleri* is part of the network linking mineral nutrition and Cd tolerance/accumulation.

Keywords: MYB59 transcription factor, metal hyperaccumulation, *Arabidopsis halleri*, cadmium, calcium homeostasis, calcium signaling.

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Introduction

The family of MYB transcription factors (TFs) is ubiquitous in all eukaryotic organisms (Rosinski and Atchley 1998), and is characterised by the presence of conserved repeated motifs (R) of ~52 residues in their DNA binding region (Dong *et al*. 2018). While a relatively small number of MYB proteins have been identified in animals and fungi, MYB TFs represent a large family of important regulators in plants where they play key roles in different physiological processes such as plant development (Baumann *et al*. 2007; Mu *et al*. 2009), response to hormones and endogenous signals (Abe *et al*. 2003; Peng 2009), secondary metabolism (Zhong *et al*. 2008) and response to environmental stimuli (Agarwal *et al*. 2006; Jung *et al*. 2008). Based on the number of the R repeats present in the highly conserved DNA-binding domain, four major MYB classes (1R to 4R) have been recognised (Roy 2016).

Since the first discovery of the MYB-like gene *C1* from *Zea mays* L., involved in kernel anthocyanin biosynthesis (Paz-Ares *et al*. 1987), plant genomes have been found to contain many MYB members, most of them belonging to the R2R3-MYB subfamily (138 of the 197 MYB TFs in *Arabidopsis thaliana* (L.) Heynh.; Katiyar *et al*. 2012). Many of them have been characterised, revealing an enormous variety of targets and physiological functions, including key roles in stress response. For instance, the *Arabidopsis* MYB15 is involved in the response to cold stress and in freezing tolerance (Agarwal *et al*. 2006), whereas AtMYB2, AtMYB52 and AtMYB60 participate in drought stress tolerance, the latter by controlling stomatal movement (Hoeren *et al*. 1998; Cominelli *et al*. 2005; Park *et al*. 2011). Similar roles have been found also in non-model species. In rice (*Oryza sativa* L.), MYB2 regulates salt, cold and dehydration tolerance (Yang *et al*. 2012), while the activity of MYB10 is responsible for red colouring in the fruits of apple (*Malus domestica* L.; Espley *et al*. 2007), and the PHAN protein, a MYB TF of *Nicotiana benthamiana* L., both regulates leaf development and plays a role in drought tolerance (Huang *et al*. 2013).

In *A. thaliana*, evidence suggests that the MYB TF AtMYB59 participates in the response to nutrient deficiency. Indeed, it is differentially modulated by potassium (K) starvation (Nishida *et al*. 2017) and regulates the transport of K^+/NO_3^- in response to low K^+ (Du *et al*. 2019); at the same time, it is strongly induced by calcium (Ca) deficiency and likely targets Ca homeostasis and signalling genes (Fasani *et al*. 2019). Moreover, *AtMYB59* is also upregulated in response to stresses, in particular cadmium (Cd) excess similarly to his orthologue *BjCdR12* in

Brassica juncea L. (Fusco *et al*. 2005; Yanhui *et al*. 2006; Fasani *et al*. 2019). Following brief exposure to $CdSO_4$, a moderate induction was observed in leaves, whereas a significant increase of *AtMYB59* expression occurred in roots after $CdSO_4$ exposure for 4 days (Fasani *et al*. 2019). AtMYB59 involvement in the response to Cd is further confirmed by the higher Cd tolerance of *myb59* mutant in comparison to wild-type (Fasani *et al*. 2019). Furthermore, transcriptomic comparison between *myb59* mutants and wild-type plants revealed that the majority of transcripts upregulated in the *myb59* mutant were annotated as proteins involved in Ca homeostasis, transport and signal transduction. Among these, *CAX1* modulation is likely linked with the response to Cd stress (Fasani *et al*. 2019). Indeed, in the Cd hypertolerant/hyperaccumulator species *Arabidopsis halleri* ssp. *halleri* (L.) O'Kane and Al-Shehbaz, the expression of the vacuolar Ca transporter *CAX1* was observed to positively correlate with Cd tolerance (Baliardini *et al*. 2015). These observations have driven us to test the expression of the *MYB59* gene in *A. halleri* in the presence of Cd or without Ca, conditions in which *MYB59* is significantly modulated in *A. thaliana*. Three *A. halleri* populations (I16, I29 and PL22) were chosen to investigate the expression profiles of *MYB59* TF under Cd excess or Ca deficiency. Individuals belonging to the three populations are characterised by different ability to tolerate and accumulate Cd: PL22 and I16 (from southern Poland and northern Italy, respectively) are metallicolous populations, and have comparable Cd tolerance but different accumulation capacity. PL22 hyperaccumulates Cd, whereas I16 stores significantly lower Cd levels by adopting an excluder strategy (Corso *et al*. 2018). On the contrary, I29 is a non-metallicolous population and does not accumulate Cd. Indeed, the natural variability of Cd tolerance and accumulation within populations (Meyer *et al*. 2015) was found to be associated with marked differences in both ionomic (including Ca) and transcriptomic profiles (Corso *et al*. 2018). In view of this, MYB59 confirms its role in the response to Ca deficiency also in *A. halleri*, whereas its modulation under Cd treatment mirrors the different strategies for Cd tolerance and accumulation.

Materials and methods

Plant material, growth conditions and treatments

Arabidopsis thaliana (L.) Heynh. accession Columbia (Col-0) and three populations of *Arabidopsis halleri* ssp. *halleri* (L.) O'Kane and Al-Shehbaz, characterised by different edaphic behaviours, were used for this work. Populations I16 (Val del Riso, northern Italy, 45°51′34.40N, 9°52′34.94 E) and I29 (Val Camonica, northern Italy, 46°03′29.8 N, 10°15′15.20 E; Godé *et al*. 2012) belong to the south-eastern genetic unit, and population PL22 (Bukowno, southern Poland, 50°16′58.08 N, 19°28′43.38 E; Meyer *et al*. 2015) belongs to the hybrid zone genetic unit (Pauwels *et al*. 2012). *A. halleri* I29 is non-metallicolous (Godé *et al*. 2012), whereas I16 and PL22 are both metallicolous populations (Meyer *et al*. 2015).

A. thaliana and *A. halleri* seeds were washed in 70% ethanol for 1 min, followed by sterilisation (10% sodium hypochlorite and 0.03% TritonX-100) for 15 min and three rinses with sterile water. Sterile seeds were sown on solid MS medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and vernalised for two days at 4°C. Plantlets were grown *in vitro* with a 16 h light/8 h dark photoperiod at 23°C. Two weeks post germination, plants were transferred in hydroponic culture in Hoagland solution (Hoagland and Arnon 1950) and acclimated for a further 1 week in the growth chamber (16 h light/8 h dark photoperiod, illumination 100–120 µmol m^{-2} s^{-1}, day/night temperature 22°C/18°C). Three-week-old plants (six plants for each genotype and treatment) were then treated as follows: control conditions in standard Hoagland solution, Cd treatment in Hoagland solution supplemented with 10 µM $CdSO_4$, and Ca deficiency in Hoagland solution without $Ca(NO_3)_2$. Leaves and roots were collected in triplicate before treatment and after 6 h and 24 h.

AhMYB59 cloning and sequencing

AhMYB59 genomic and transcript sequences were amplified from genomic DNA and RNA of *A. halleri* I16 plants grown in control conditions. Genomic DNA was extracted using the Qiagen Genomic DNA Extraction Kit (Qiagen, Redwood City, CA, USA). Total RNA was purified with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA); after DNase treatment, first-strand cDNA was synthesised using the Superscript III Reverse Transcriptase Kit (Thermo Fisher Scientific). PCR amplification of *AhMYB59* was performed with Platinum Pfx DNA polymerase (Thermo Fisher Scientific), using primers designed on the 5′ and 3′ untranslated regions (Table 1). The amplified fragments were cloned in the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced. All *AhMYB59*

Table 1. List of primers used in this work

Application	Sequence
AhMYB59 cloning	Fw: 5′-AGAGAAAGAGATGAAACTTGTGC-3′Rev: 5′-AGTTCTGACTTGTATTACACATC-3′
Real-time RT–PCR on *MYB59*	Fw: 5′-ATTTCCCTCCTCTGGCTTCT-3′Rev: 5′-TAGAGGAAACTGATCAATAGCA-3′
Real-time RT–PCR on *MYB59.1*	Fw: 5′-ATAGGTATAGGTTTGTTTTGGAA-3′Rev: 5′-AACCTACAACCAAAACCAGGT-3′
Real-time RT–PCR on *MYB59.2*	Fw: 5′-GAAACATAAGAATAGGTTTAAACA-3′Rev: 5′-TGGAGTCATCTTACCACGTTT-3′
Real-time RT–PCR on *MYB59.3*	Fw: 5′-ACTTGTGCAAGAAGAATACCG-3′Rev: 5′-CTGTTCTGTTTAAACCTGAAAC-3′
Real-time RT–PCR on *CAX1*	Fw: 5′-ATCGGAGTATGTTGTAGCCAC-3′Rev: 5′-AACGGCTCCAGCATGTTCAG-3′
Real-time RT–PCR on *CNGC12*	Fw: 5′-GCTCTTCTTCGTAGTCTTCCA-3′Rev: 5′-GGTCGCACACAGCTTCTAGT-3′
Real-time RT–PCR on *GSTF10*	Fw: 5′-CAGAGAAGTATAGATCACAAGG-3′Rev: 5′-AGACAATGTTGAGCGTTAAAGC-3′
Real-time RT–PCR on β-*actin*	Fw: 5′-GAACTACGAGCTACCTGATG-3′Rev: 5′-CTTCCATTCCGATGAGCGAT-3′
Real-time RT–PCR on *UBQ10*	Fw: 5′-AGGACAAGGAAGGTATTCCTC-3′Rev: 5′-CTCCTTCTGGATGTTGTAGTC-3′

nucleotide sequences obtained have been deposited in GenBank (accession numbers: *AhMYB59* genomic sequence, MT993851; *AhMYB59.1*, MT993852; *AhMYB59.2*, MT993853; *AhMYB59.3*, MT993854; *AhMYB59.5*, MT993855; *AhMYB59.6*, MT993856). The sequence obtained was considered to design primers for real-time PCR on *MYB59* (reported in Table 1). The primers have been tested by PCR in *A. halleri* I29 and PL22, using genomic DNA and cDNA produced as described as templates. Obtained amplicons were also cloned in the pGEM-T easy Vector and submitted to sequencing, confirming the specificity of primer recognition and amplification.

Expression analysis of MYB59 *and genes involved in Ca signalling and stress response*

Total RNA of untreated and treated leaves and roots was extracted with TRIzol Reagent (Thermo Fisher Scientific) and reverse-transcribed as described above. Real-time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the Platinum SYBR Green qPCR SuperMix-UDG kit (Thermo Fisher Scientific), for 40 amplification cycles. Three technical repeats were produced for each sample; melting curve analysis was used to confirm the amplification of specific targets. Data were normalised on the endogenous reference genes β-*actin* (At5g09810) and *ubiquitin 10* (*UBQ10*, At4g05320). Primers are listed in Table 1. Relative expression was assessed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), after confirming amplification efficiency at ~2 for each primer pair using LinRegPCR ver. 7.5 software (Ramakers *et al*. 2003). *MYB59* gene expression under control conditions at each time point was used as the standard to exclude the contribution of circadian regulation. Relative expression was determined referring to *MYB59* expression in *A. thaliana* leaves. Expression of *AhCAX1*, *AhCNGC12* and *AhGSTF10* was referred against I29 expression either in leaves or in roots.

Statistical analysis

Data in histograms are represented as mean ± s.e. of three replicates. Statistical significance was determined by the one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test using GraphPad Prism 7 (GraphPad Software). Statistically significant differences, when $P < 0.05$, are marked with different letters.

Results and discussion

Among hyperaccumulators, i.e. metallophytes that have evolved the peculiar ability to accumulate high levels of metal(oid)s in their aboveground tissues (van der Ent and Mulligan 2015), *A. halleri* together with *Noccaea caerulescens* (J.Presl and C.Presl) F.K.Mey. are the model species for studying the evolution and adaptation as well as the molecular mechanisms underlying metal hypertolerance and hyperaccumulation (Verbruggen *et al*. 2009; Krämer 2010). In particular, *A. halleri* is a facultative metallophyte including populations adapted to both metalliferous and non-metalliferous soils, distributed in Europe and eastern Asia (Pauwels *et al*. 2012). All populations display Zn hypertolerance and hyperaccumulation, although with some intraspecific variability (Stein *et al*. 2017; Babst-Kostecka *et al*. 2018), whereas, within the species, Cd tolerance and accumulation are highly variable traits (Meyer *et al*. 2015; Stein *et al*. 2017). Metallicolous populations of *A. halleri* in this study, I16 and PL22, belong to distinct genetic units, are both Cd-hypertolerant (Meyer *et al*. 2015) but show contrasting Cd accumulation. PL22 is a Cd hyperaccumulator, whereas I16 has a significantly lower capacity to accumulate Cd (Corso *et al*. 2018). Variations in Cd accumulation are also reflected in differences in nutrient homeostasis, including Ca, as highlighted by ionomic profiles (Stein *et al*. 2017; Corso *et al*. 2018). In particular, Ca root-to-shoot translocation was significantly higher in both control and Cd-treated PL22 plants than in I16; moreover, I16 showed a significant decrease in Ca shoot content upon Cd treatment (Corso *et al*. 2018). On the other hand, the population I29 grows in a non-metalliferous soil (Godé *et al*. 2012; Stein *et al*. 2017) and has only a moderate ability to tolerate Cd, similarly to nearby non-metallicolous populations I28 and I30 (Meyer *et al*. 2015).

Morphological features of *A. halleri* plants belonging to the three populations, compared with *A. thaliana*, are reported (see Fig. S1). In addition to the above-mentioned differences in edaphic behaviour, the three populations also show distinct traits with respects to leaf morphology.

In view of the differences in Cd accumulation and nutrient homeostasis, MYB59 TF is a particularly interesting regulator to investigate. Indeed, *A. thaliana* MYB59 has been proposed to regulate both Ca signalling under Ca deficiency and to respond to Cd stress (Fasani *et al*. 2019). In *A. thaliana*, four different transcripts were identified for *AtMYB59* by PCR amplification (Yanhui *et al*. 2006), showing an mRNA abundance of ~4:1:1 for the first three respectively, whereas the fourth transcript had very low expression (Li *et al*. 2006). Evidence of alternative splicing was therefore also searched in *A. halleri*. The *AhMYB59* genomic sequence amplified in *A. halleri* population I16 has 93.8% identity with the *A. thaliana* orthologue (aligned in Fig. S2). Amplification of *AhMYB59* sequence from *A. halleri* I16 cDNA revealed the existence of three transcripts exactly corresponding to *AtMYB59.1*,*.2* and.*3* isoforms. In addition, two other transcripts (named *AhMYB59.5* and.*6*) were identified, having very low abundance; in these sequences, the predicted open reading frame does not include any R repeat for DNA binding (see Fig. S3). The sequence information obtained by the amplification of *MYB59* in *A. halleri* population I16 was exploited to design primers for specific amplification of *MYB59* in both *A. thaliana* and *A. halleri*. The sequences obtained upon amplification on genomic DNA and cDNA from populations I16, I29 and PL22 were cloned and sequenced to ensure recognition of *MYB59* in all genotypes.

As previously mentioned, *A. thaliana MYB59* is induced by Cd excess and Ca deficiency and it has a role in regulating Ca transport and signal transduction (Fasani *et al*. 2019). Therefore, *MYB59* expression was measured by real-time PCR under either Ca absence or in the presence of 10 μM $CdSO_4$, to determine the impact of these treatments on the

regulation of this TF in *A. halleri*. Furthermore, the analysis compared transcript levels in non-tolerant *A. thaliana* and in the three populations of *A. halleri* mentioned above. Concurrently, the expression of putative target *CAX1* was considered in *A. halleri* under the same conditions. Differences in *MYB59* expression are noticeable already in plants grown under control conditions (Fig. 1). Indeed, transcript levels in *A. thaliana* and in the non-metallicolous *A. halleri* population I29 follow a similar trend, distinct from metallicolous populations I16 and PL22. In detail, *MYB59* expression was higher in roots than in leaves of *A. thaliana* and I29, analogously to what was observed in previous works (Mu *et al.* 2009; Fasani *et al*. 2019). Interestingly, the behaviour of *MYB59* was different in metallicolous *A. halleri* populations I16 and PL22, grown in the same control conditions. Indeed, *MYB59* expression in these populations is similar in shoots and roots, and about four times higher than what was measured in *A. thaliana* leaves. MYB59 has been reported to negatively regulate root growth by directing the cell cycle (Mu *et al.* 2009); at the same time, this TF is involved in nutrient homeostasis, controlling both root-to-shoot redistribution and subcellular storage (Du *et al*. 2019; Fasani *et al*. 2019). The link between nutrient availability and root development and architecture has been widely demonstrated (reviewed by Aibara and Miwa 2014; Giehl *et al*. 2014). Although genetic variability in *A. halleri* correlates mostly with the phylogeographic group (Pauwels *et al*. 2005) and ionomic profiles are associated with both soil composition and geographic origin (Stein *et al*. 2017), the strategies for root development and architecture seem to be linked mainly to the edaphic context (Dietrich *et al*. 2019). This evidence supports the possible convergent evolution of a specific *MYB59* regulation in the two metallicolous populations I16 and PL22, belonging to distinct geographic units.

When *MYB59* expression was measured under Ca depletion, a significant increase in transcript levels was observed at 6 h of treatment in leaves of all the genotypes considered, followed by a decrease at 24 h (Fig. 2*a*). This result resembles what was previously observed in *A. thaliana* (Fasani *et al*. 2019), supporting the specific role of this TF in the response to Ca deficiency. It should be pointed out that very high transcript levels were detected in the I16 population and, more moderately, in PL22 after 6 h of Ca deprivation, followed by the incomplete recovery of basal expression levels at 24 h. It must be considered that, in hypertolerant plants such

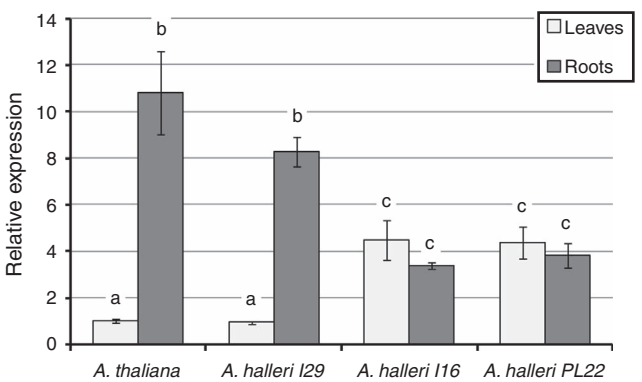

Fig. 1 *MYB59* expression in leaves and roots of plants in control conditions. Two-week-old plants of *Arabidopsis thaliana* and *A. halleri* populations I29, I16 and PL22 were cultivated in hydroponics for 1 week in standard Hoagland solution. Relative expression levels were determined by real-time PCR, setting *MYB59* expression in *A. thaliana* leaves as reference.

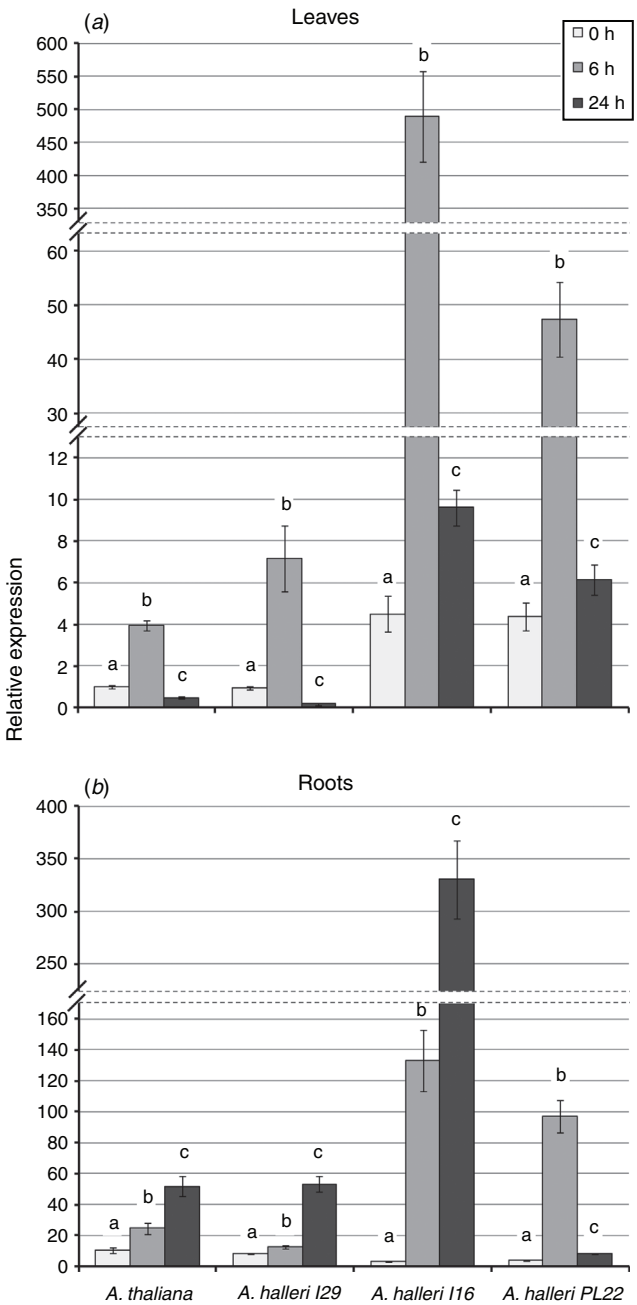

Fig. 2 *MYB59* expression in (*a*) leaves and (*b*) roots of plants under Ca deficiency. Two-week-old plants of *Arabidopsis thaliana* and *A. halleri* populations I29, I16 and PL22 were cultivated in hydroponics for 1 week in standard Hoagland solution, then transferred in Hoagland solution without $Ca(NO_3)_2$. Relative expression levels were determined by real-time PCR, setting *MYB59* expression in *A. thaliana* leaves under control conditions as reference.

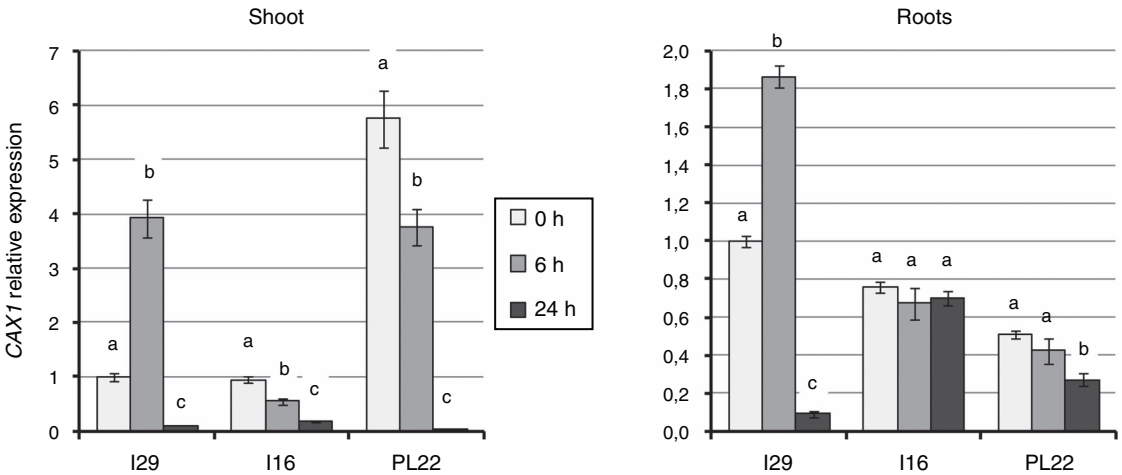

Fig. 3 *CAX1* expression in leaves and roots of plants under Ca deficiency. Two-week-old plants of *Arabidopsis halleri* populations I29, I16 and PL22 were cultivated in hydroponics for 1 week in standard Hoagland solution, then transferred in Hoagland solution without $Ca(NO_3)_2$. Relative expression levels were determined by real-time PCR, setting *CAX1* expression in *A. halleri* I29 under control conditions as reference.

as the *A. halleri* populations I16 and PL22, the adaptation to extreme metalliferous environments has led to a tightly regulated elemental homeostasis. Therefore, the rapid and massive increase of *MYB59*, imposed by its role in Ca homeostasis and signalling, is likely exacerbated by the altered nutritional requirements associated with a different control of ion uptake and translocation. Indeed, transcriptomic analysis revealed that *A. thaliana* MYB59 negatively regulates the expression of *CAX1* and *ACA1*, two Ca transporters localised in the tonoplast and the endoplasmic reticulum respectively, as well as of several calmodulin-like Ca-binding proteins; this evidence had suggested a role in the regulation of Ca signalling and distribution within the cell in response to variations of Ca availability (Fasani *et al*. 2019). Consistently with this result, *CAX1* expression is downregulated after 6 h and 24 h of Ca deficiency in the leaves of I16 and PL22 and after 24 h in I29 (Fig. 3). Therefore, the higher induction of *MYB59* observed in metallicolous populations in the absence of Ca may be imposed by the need to promptly control Ca signalling and subcellular compartmentalisation, that is associated with redox response and Cd tolerance in hypertolerant genotypes (Baliardini *et al*. 2015; Ahmadi *et al*. 2018). In roots, *MYB59* showed gradual induction under Ca deprivation. Its expression peaks at 24 h in *A. thaliana* and *A. halleri* populations I16 and I29, whereas it reaches its maximum at 6 h in PL22, which is the only population able to consistently hyperaccumulate Cd (Fig. 2*b*).

MYB59 expression levels were also measured when Cd was added to the nutrient medium (Fig. 4). In leaves, *A. thaliana* and the non-metallicolous population I29 showed a similar profile with induction at 6 h of treatment, analogously to what was previously observed (Fasani *et al*. 2019). In contrast, population I16 showed an upregulation at 24 h, whereas the Cd hyperaccumulator PL22 did not show variation in *MYB59* expression (Fig. 4*a*). This evidence indicates that, at least in leaves, Cd hyperaccumulation does not require a modulation

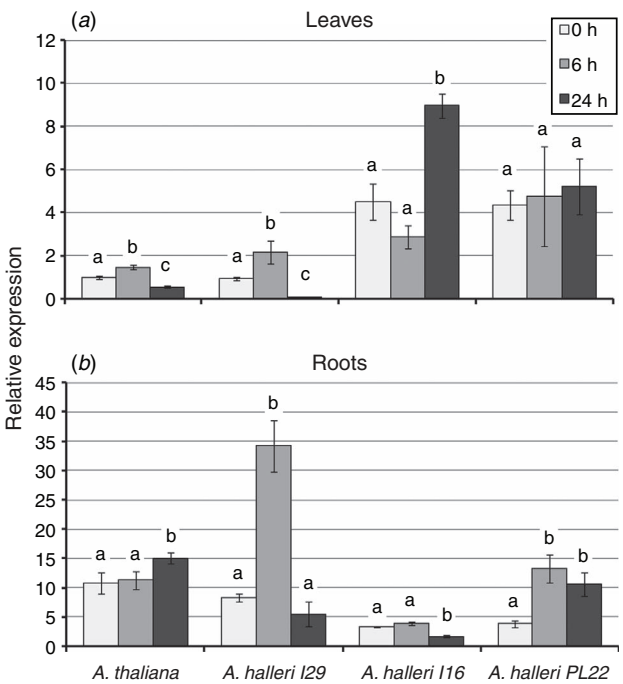

Fig. 4 *MYB59* expression in (*a*) leaves and (*b*) roots of plants under Cd treatment. Two-week-old plants of *Arabidopsis thaliana* and *A. halleri* populations I29, I16 and PL22 were cultivated in hydroponics for 1 week in standard Hoagland solution, then transferred in Hoagland solution supplemented with 10 μM $CdSO_4$. Relative expression levels were determined by real-time PCR, setting *MYB59* expression in *A. thaliana* leaves under control conditions as reference.

of *MYB59*. In roots, expression profiles were significantly different among the genotypes; in particular, I29 revealed a consistent upregulation at 6 h, whereas PL22 had a more moderate increase after both 6 h and 24 h and I16 showed negative modulation at 24 h (Fig. 4*b*). The variability in

MYB59 response to Cd treatment in roots can be related to the different adaptation of the populations taken into consideration. Indeed, *A. halleri* population I29 is non-metallicolous and has only a moderate ability to accumulate Cd (Meyer *et al*. 2015), and interestingly *MYB59* expression in leaves under Cd treatment has a profile remarkably similar to that of *A. thaliana* in the same conditions, likely associated with stress response as observed in the latter (Fasani *et al*. 2019). On the other hand, the two metallicolous populations I16 and PL22 have a contrasting regulation of *MYB59*, coherently with their different accumulation capacity. Given their tolerance to extremely high levels of Cd in soil (Meyer *et al*. 2015), it is improbable that *MYB59* upregulation is due to the perception of the imposed Cd treatment as stress. However, it must be considered that MYB59 participates in the control of mineral homeostasis by negatively regulating Ca compartmentalisation, as well as by promoting K root-to-shoot translocation (Du *et al*. 2019; Fasani *et al*. 2019). Interestingly, both Ca and K transporters have been found as involved in the response to Cd in PL22 (Corso *et al*. 2018); moreover, this hyperaccumulating population absorbs and transfers to shoots higher levels of essential elements (including K and Ca) than non-hyperaccumulator I16 (Corso *et al*. 2018). Also the different modulation of *MYB59* isoforms (see Fig. S4) finds its place in this context of altered mineral homeostasis and signalling. In particular, *MYB59.3* is the major contributor of total *MYB59* transcripts in the non-metallicolous population I29 upon Cd treatment. MYB59.3 is the transcription factor positively regulating K^+ translocation in *A. thaliana* (Du *et al*. 2019). Moreover, evidence suggests that the three isoforms, here defined MYB59.1, MYB59.2 and MYB59.3, may play different roles in expression regulation and that *MYB59* alternative splicing occurs differently upon nutrient deficiency (Nishida *et al*. 2017; Du *et al*. 2019). On the whole, the different modulation of *MYB59* in the three *A. halleri* populations fits into the scenario of substantially different strategies for Cd tolerance and accumulation (Corso *et al*. 2018). This is also supported by the different expression profiles of *CAX1*, *CNGC12* and *GSTF10* in the three *A. halleri* populations upon Cd treatment (Fig. 5; see Fig. S5). The latter two genes are involved in the control of Ca fluxes upon stress (Urquhart *et al*. 2011) and in protection against oxidative stress (Sappl *et al*. 2009), respectively. Notably, *CNGC12* and *GSTF10* were found as differently expressed in I16 and PL22 (Corso *et al*. 2018), coherently with the results observed in this work. This evidence further reinforces the model of a complex and independent adaptation to metalliferous environments, including specific strategies associated with Ca signalling and control of redox status.

In this view, we propose that MYB59 is likely not a direct determinant for Cd tolerance and accumulation, consistently with what was observed in *A. thaliana* (Fasani *et al*. 2019). In fact, it also lacks the high and constitutive expression generally observed for genes playing key roles in hyperaccumulation (Hanikenne and Nouet 2011). On the contrary, MYB59 may be rather associated with the different regulation of mineral nutrition and Ca signalling in hypertolerant and hyperaccumulator populations. In this capacity, this transcription factor is likely indirect part of the complex and till now poorly understood network underlying Cd hypertolerance.

In conclusion, MYB59 transcription is differentially modulated in *A. halleri* populations adapted to different edaphic conditions and characterised by specific behaviours towards Cd. The results achieved in this work support MYB59 role in Ca signalling, also suggesting a convergent evolution of its regulation in the adaptation to metalliferous environments. Moreover, *MYB59* response to Cd reflects the specific strategies adopted by different *A. halleri* populations to cope with Cd, in line with the variability in mineral nutrition already observed.

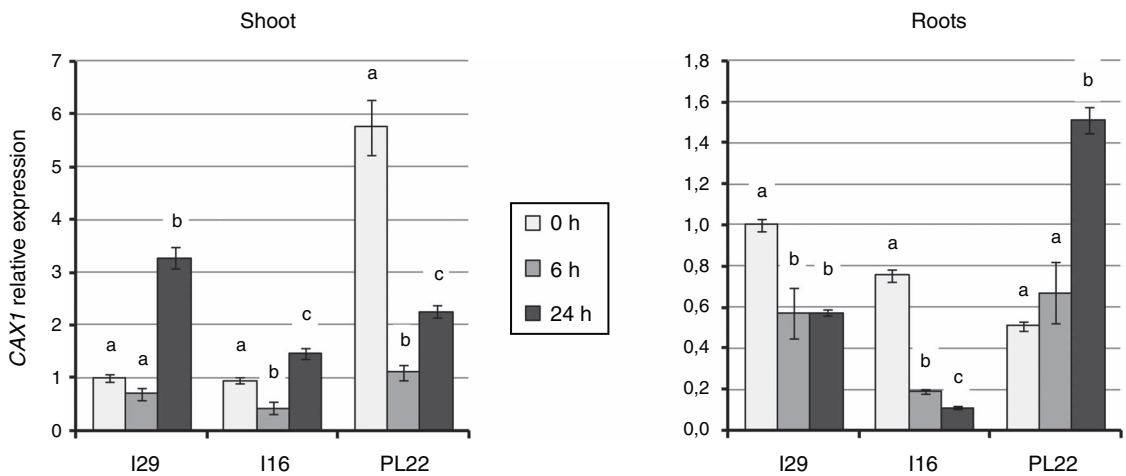

Fig. 5 *CAX1* expression in leaves and roots of plants upon Cd treatment. Two-week-old plants of *Arabidopsis halleri* populations I29, I16 and PL22 were cultivated in hydroponics for 1 week in standard Hoagland solution, then transferred in Hoagland solution supplemented with 10 µM $CdSO_4$. Relative expression levels were determined by real-time PCR, setting *CAX1* expression in *A. halleri* I29 under control conditions as reference.

Conflicts of interest

The authors declare no conflicts of interest.

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