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Functional evidence for the partial conservation of arsenic tolerance mechanisms in *Marchantia polymorpha* and *Arabidopsis thaliana*

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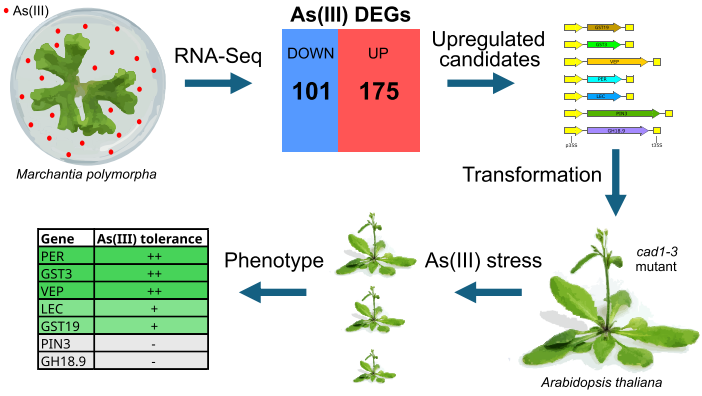
HIGHLIGHTS

- Arsenic is highly toxic for vascular and non-vascular plants (e.g liverworts).
- Seven arsenic-responsive liverwort genes were overexpressed in *Arabidopsis*.
- Most liverwort genes improved arsenic tolerance in *Arabidopsis cad1–3* mutant.
- A 1-cys peroxiredoxin gene conferred the highest tolerance to arsenic in *cad1–3*.
- Liverworts are valid models to elucidate arsenic detoxification in plants.

GRAPHICAL ABSTRACT



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ABSTRACT

Arsenic is a widespread metalloid that even at low concentrations is highly toxic to most plant species. While the transcriptional responses associated to arsenic tolerance have been widely investigated in vascular plants, comparatively little is known in their sister lineage, the bryophytes. Most importantly, functional evidence of whether the same genes play major roles in arsenic tolerance responses in these two anciently diverged land plant lineages is currently largely missing. In this study, we identified by RNA-Seq a highly reliable set of differentially expressed genes (DEGs) responding to arsenite toxicity in the model bryophyte *Marchantia polymorpha*. We then explored the evolutionary level of functional conservation of seven upregulated DEGs by *Agrobacterium*-mediated transformation in the highly arsenic-sensitive *cad1–3* mutant of *Arabidopsis thaliana* as a representative of tracheophytes, and characterized fresh weight, malondialdehyde production and total arsenic content in dry biomass of transgenic lines. While two of the tested *M. polymorpha* DEGs did not significantly enhance arsenic tolerance, the remaining five DEGs, when overexpressed in *cad1–3*, conferred maximal levels of

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tolerance, measured as biomass accumulation, between 56 % and 100 % of WT Col-0 plants. Among them, a putative 1-cys peroxiredoxin restored growth, protection from lipid peroxidation and capacity to accumulate arsenic to levels indistinguishable from those of WT. These results provide functional evidence for the considerable conservation of arsenic tolerance responses between *M. polymorpha* and *A. thaliana*, suggesting that *M. polymorpha* can be a valid model for the identification of evolutionarily deeply conserved genes for the genetic improvement of crops for arsenic tolerance.

1. Introduction

Arsenic (As) is a relatively common element in the earth's crust, where it is present in more than 240 different minerals, with concentrations averages of 5–10 mg/kg and about 1.5 mg/kg in sedimentary and igneous rocks, respectively [1]. This metalloid, which is not essential for plant growth, is mainly present in soils and groundwater in its inorganic form, either as As(III) (arsenite) or as As(V) (arsenate) oxidation states [2]. Both As(III) and As(V) are toxic for plants, albeit with different mechanisms of action [3]. As(III), the most toxic As species for plants, has a high affinity towards sulfhydryl (-SH) groups present in proteins, co-factors and other important compounds controlling cellular redox homeostasis like glutathione (GSH) [4]. It can, therefore, negatively impact proper protein folding and functioning as well as interfere with redox control leading to reactive oxygen species (ROS) production and oxidative stress [5]. On the other hand, As(V) is known to be a phosphate analog, thus interfering in a wide range of fundamental biochemical phosphorylative reactions like respiration and the dark phase of photosynthesis, glycolysis, phospholipid metabolism, and cellular signaling [5].

In order to protect themselves from As toxicity, plants have evolved different tolerance strategies that can be loosely classified based on whether they are specifically concerning the metalloid itself or non-specifically targeting the cellular damage it causes. The first type of strategies encompass As immobilization in the rhizosphere or regulation of its uptake, translocation, speciation, sequestration and extrusion [4, 6]. The second type of strategies are instead general tolerance mechanisms that are in common among different types of primary abiotic and biotic stresses that secondarily trigger oxidative stress and activate the plant antioxidant defense system [7,8].

The first line of defense against As starts outside of plant cells, through avoidance mechanisms aiming at decreasing the access of this toxic metalloid in the cytoplasm. Phyto-immobilization by root exudates, precipitation in iron plaques, biosorption in cell walls are among the major mechanisms of As avoidance used by plants [6]. If, despite these avoidance mechanisms, the bioavailable As still present in the rhizosphere enters the root cells, a series of tolerance mechanisms are responsible to mitigate the toxic effects caused by the metalloid. Among them, As uptake from soil into root cells and its translocation from roots to shoots involve no less than 12 different families of membrane transporters, although none of them is selective specifically for this metalloid [9,10]. Therefore, their strict transcriptional, translational, and post-translational regulations are fundamental lines of defense against As toxicity in plants [11]. In contrast, despite being very important as a tolerance strategy, As speciation does not seem to be a highly regulated process because the majority of plant intracellular As(V) is converted to As(III) either enzymatically or non-enzymatically by antioxidant metabolites [12]. Given the high toxicity of As in the cytoplasm, plants evolved different mechanisms to store this metalloid away in other cellular compartments less sensitive to damage [13]. The vacuole is by far the major intracellular compartment for As sequestration, which in plants is mainly mediated by chelation with proteins like metallothioneins and thiol-containing peptides like GSH and especially phytochelatins (PCn) in the case of As(III) [14]. Knockout of *phytochelatin synthase* (*PCS*), coding for the enzyme responsible for the As- and heavy-metal-activated biosynthesis of PCn, confers hypersensitivity to arsenic and various essential and non-essential heavy metals in both *Arabidopsis* and rice [15–17]. In the As hyperaccumulator fern *Pteris vittata*, As(III) can be also directly transported into vacuoles by the ACR3 transporter [18]. Two additional routes of As(V) sequestration in the vacuole are those relying on either its direct transport across the tonoplast [19] or, at least in ferns, via the import of its derivative 1-arseno-3--phosphoglycerate in cytoplasmic vesicles possibly followed by fusion to the tonoplast [20]. As can also be actively or passively extruded from cells through several membrane protein channels like ACR3, PIN2 and various NIP transporters [11].

The general tolerance mechanisms to As toxicity, on the other hand, heavily rely on activation of the complex antioxidant defense system present in plants [10]. Primarily in chloroplasts and mitochondria, but to a lower extent also in peroxisomes, endoplasmic reticulum and cell walls, As causes the generation of various reactive oxygen species, including superoxide anion radicals (O2•−), hydroxyl radicals (•OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) [3]. Superoxide anion radicals are quickly dismutated to H_2O_2 either spontaneously or in a reaction catalyzed by superoxide dismutases, a class of enzymes that are often considered the first line of defense against ROS among antioxidant system (AOS) enzymes. Then, H_2O_2 is detoxified to water by a wide range of peroxidative enzymes, like ascorbate peroxidases (APX), glutathione peroxidases (GPX), catalases (CAT), guaiacol peroxidases (GOPX) or peroxiredoxins (PER) [10,21,22]. By contrast, glutathione-S-transferases (GST) are involved in detoxification to arsenicals, possibly by conjugating to glutathione As(III) or the toxic by-products derived from As-driven oxidative stress [10,23]. Most of these AOS enzymes use as electron donors either GSH or ascorbate, which are two of the main plant antioxidants taking part also in non-enzymatic ROS detoxification [8].

While functional genetic and genomic studies on the molecular mechanisms of As tolerance have been carried out extensively in vascular plants, comparatively little is known about the same mechanisms in the other major lineage of land plants, the bryophytes [24]. Physiological studies indicated that both mosses and liverworts, the two major clades of bryophytes, can accumulate and tolerate significant amounts of As [25,26], although interspecific differences have been reported among taxa [27]. Recently, functional studies in the model liverwort *Marchantia polymorpha* demonstrated that PCn play a primary role in the detoxification towards cadmium and other metals [28–31], but contribute marginally to As detoxification in this species [30]. Indeed, mutant plants unable to synthesize PCn because of total loss of function of the single *phytochelatin synthase* (Mp*PCS*) gene present in *M. polymorpha* displayed levels of sensitivity towards As(III) comparable to those of wild type (WT) plants [32]. Further work demonstrated by CRISPR-CAS9 mediated knockouts and overexpression that in *M. polymorpha* the single-copy *Arsenic Compounds Resistance 3* (Mp*ACR3*), coding for a plasma membrane-localized As (III)/H+ antiporter, plays *in vivo* the major role in As(III) tolerance rather than *PCS* [33]. A recent study, besides confirming the function of MpACR3 as an As(III) transporter in the heterologous host *Saccharomyces cerevisiae* using the *Δacr3* mutant background, further assessed the transcriptomic responses of a local *M. polymorpha* ecotype exposed to 40 μM As(III) in non-axenic conditions [27]. Consistently to the high antioxidant capacity and the putative functions of the differentially expressed genes (DEGs) associated with As tolerance in *M. polymorpha* [27], several of the AOS enzymes previously reported in bryophytes to mediate the defense against oxidative stress caused by heavy-metals

[34] could take part also in As tolerance. However, whether the same gene networks play major roles in arsenic tolerance responses in bryophytes and vascular plants until now has not been functionally validated.

The goal of the present study was, thus, to provide the first assessment of the functional conservation of the antioxidant defense systems of the two extant lineages of land plants, the bryophytes and the tracheophytes, towards As toxicity. To this aim, a highly reliable set of *M. polymorpha* subsp. *ruderalis* DEGs responding to arsenite toxicity in axenic cultures was identified. The evolutionary level of functional conservation was then assessed in tracheophytes for seven upregulated DEGs through genetic complementation tests of the highly arsenic-sensitive *cad1–3* genotype of *A. thaliana*, harboring a loss of function mutation in the *AtPCS1* gene. The results presented indicate for the first time a considerable degree of functional conservation of the responses to arsenic toxicity between both lineages of land plants, highlighting the utility of *M. polymorpha* as a model for the identification of evolutionarily deeply conserved genes for arsenic tolerance.

2. Materials and methods

2.1. Plant materials and growth conditions

Marchantia polymorpha L. subsp. *ruderalis* Bischl. & Boissel.-Dub. (Marchantiales, Marchantiophyta) Cam-2 (UK Cambridge-2 WT) female gametophytes, *Arabidopsis thaliana* (L.) Heynh. *cad1–3* mutant and transgenic plants were used in this study. *M. polymorpha* was cultivated as in a previous study [35] in half-strength Murashige and Skoog (MS) medium supplemented with 1 % (w/v) sucrose and 1 % (w/v) agar under long-day photo period (16 h light and 8 h dark) at 21 °C with a light intensity of 60 μmol m^{-2} s^{-1} in the growth chamber, its propagation was generally originated from gemmae. For *Arabidopsis* plants, seeds were sterilized as former description [36], stratified at 4°C for three days and germinated in half-strength MS agar medium containing 1 % (w/v) sucrose; one week after germination the plants were transferred into pots containing commercial soil under standard long-day conditions at 23 °C with a light intensity of 100 – 120 μmol m^{-2} s^{-1} and relative humidity of 60 % in the growth chamber.

2.2. Sample collection and lipid peroxidation analysis

Ten-day-old Cam-2 gemmae grown in the medium mentioned above were transferred either to fresh medium or to fresh medium containing different concentrations of As(III) (65, 100, 200, 300, 400 and 500 μM of $NaAsO_2$) for two weeks, the fresh weight was recorded, and plant materials were collected in liquid nitrogen and stored at − 80 °C. For *Arabidopsis* transgenic lines and the *cad1–3* mutant, two-week-old plants growing in standard long-day condition in half strength hydroponic solution developed by [37] were transferred to the fresh medium supplemented without or with 10 μM As(III) for three or seven days. Before harvesting, the roots of young seedlings were rinsed in MilliQ water and blotted quickly in paper towels, subsequently entire seedlings were collected in 2 ml safe-lock Eppendorf tubes, snap frozen in liquid nitrogen and stored at −80 °C for later use.

Lipid peroxidation of the sample was estimated based on malondialdehyde (MDA) production according to the [38]. At least three biological replicates for each condition were carried out for the entire treatment.

2.3. Elemental assay

For total As analyses of *M. polymorpha* wild-type plants, 12-day-old Cam-2 plants were transferred to the fresh medium as indicated above supplemented without or with 500 μM As(III) and kept for 24 h. In the case of *Arabidopsis* transgenic plants, two-week-old plants growing in hydroponic solution as mentioned above were transferred to the fresh medium supplemented without or with 10 μM As(III) and maintained for three days.

Afterwards, both types of plant materials (Cam-2 and *Arabidopsis* transgenic plants) were collected, washed with ice-cold buffer (1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$ and 5 mM MES, pH 5.6) for 10 min to get rid of apoplastic As, blotted dry in paper towels and dried at 65°C till no changes in dry weight. The dried plant materials were acid digested with ultrapure nitric acid (67–69 %, Carlo Erba, Milan, Italy) before Inductively Coupled Plasma – Mass Spectrometry (Agilent 7800, Agilent Technologies, Santa Clara, CA, USA) quantification of As like in previous studies [33,39].

2.4. Total RNA extraction, RNA-Seq library construction and sequencing

For transcriptomic analyses, 10-day-old gemmae were transferred either to fresh medium or to fresh medium containing 65 μM As(III) in the form of $NaAsO_2$ for 24 h, and collected for RNAseq library preparation.

Total RNA was extracted from about 100 mg of plant material harvested using a Spectrum Plant Total RNA Kit (Sigma-Aldrich®) according to the manufacturer's instructions, and purified total RNA was treated with Amplification-Grade DNase I (Sigma-Aldrich®) to eliminate the traces of genomic DNA. The integrity and quality of extracted total RNA were analysed in Bioanalyzer 2100 (Agilent Technologies). The 150 bp paired-end library construction for Illumina sequencing with Novaseq 6000 in Novogene company limited, United Kindom (UK) was performed as previously reported [40] with a small modification: the unique dual index (UDI) adaptors from Illumina were used for library preparation. A total of 8 libraries were sequenced (four biological replicates of the control plants without As treatment and four replicates of the plants treated with 65 μm As(III)). An average of 55.6 ± 7.6 million PE reads were obtained for each sample, with more than 92 % of them mapping back to *M. polymorpha* JGI3.1 transcripts (see Supplementary Table S1). The quality of the raw data was checked using FastQC (version 0.11.9) (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data (no date). Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Accessed: 21 November 2021)) and the unstranded paired-end reads were aligned to the reference genome of *M. polymorpha* (using the genome assembly and annotation GCA_003032435.1) using the Subread aligner (version 1.6.3) [41]. Raw read counts were extracted using the featureCounts read summarization program (version 1.6.3) [42]. All raw RNA-Seq read data were deposited in the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/) under the BioProject accession code PRJNA1252024.

2.5. Quantification of differential gene expression and GO enrichment

The differential expression analysis was performed with edgeR v.3.24.1 [43] using the trimmed mean of M values (TMM) method for library size normalization and implementing the edgeR quasi-likelihood test with robust settings (robust=TRUE with estimateDisp and glmQLFit functions) [44]. For each pairwise comparison, genes with a FDR-adjusted p-value below 0.05 and a fold change above 1.5 were considered differentially expressed [45]. We used a threshold of fold change more stringent than the standard one adopted in many publications (| LogFC |>1.0, corresponding to a fold change of 2) following the rationale that the most interesting candidate genes for conferring As tolerance when overexpressed in *A. thaliana* would likely be among those with a stronger upregulation in *M. polymorpha*.

Venn diagrams between DEGs identified in this study and by Dutta et al. [27] have been drawn with the online version of DeepVenn v1 [46] (available at https://www.deepvenn.com/). ShinyGO v0.82 [47] was used for analysis of GO enrichment using a False Discovery Rate (FDR) cutoff of 0.05, a maximal number of 10 pathways to report, each with a minimum size of 10 and a maximum size of 5000. For result display, the options "remove redundancy", "abbreviate pathways" and "show

pathway IDs" were selected. GO enrichment was analyzed separately for up- and down-regulated DEGs in each of the Biological Processes, Cellular Component and Molecular Function GO categories selecting by FDR and sorting the results according to fold enrichment.

2.6. Quantitative real-time PCR (qRT-PCR) analyses

1 μg of extracted total RNA was used for cDNA syntheses using SuperScript™III Reverse Transcriptase (Invitrogen) as former description [48]. For real-time qRT-PCR analysis, both *MpAPT* and *MpACT* were used as reference genes for *M. polymorpha* [49] using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) in a Bio-Rad C1000 Thermal Cycler detection system. The RefFinder software [50] was used to examine the stability of reference genes and the fold changes for qRT-PCR was calculated with $2^{-\Delta\Delta CT}$ approach [51]. Primers for qRT-PCR analyses were designed with Primer3Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) using the same parameters indicated in [48], and a standard curve of qPCR reaction for each primer pair was assessed from six series of a 4-fold dilution to determine the amplification efficiency. All reactions were carried out in triplicate for qRT-PCR analyses. Primer sequences are listed in Supplementary Table S2.

2.7. Plasmid construction and cad1-3 mutants transformation

Candidate genes to be overexpressed in the *A. thaliana cad1–3* mutant background were selected as follows: (1) DEGs were ranked by LogFC and FRD; (2) the top 150 DEGs were functionally annotated based on the information available in The MarpolBase database ([52]; https://marchantia.info/); (3) genes with unknown function were discarded, (4) the selection was further refined by excluding genes belonging to multigenic families with more than three closely related paralogs (Blastn E-value $\leq 10^{-10}$); (5) the remaining candidates were re-sorted based on whether they belonged to gene families with known association to either arsenic or oxidative stress in general. The full-length cDNAs were individually amplified using primer pairs (see Supplementary Table S2) with Phusion High Fidelity DNA Polymerase (Thermo Scientific), cloned into pENTR/D TOPO vector (Invitrogen) and recombined into the destination vector pK7WG2 under the constitutive Cauliflower mosaic virus (CaMV) 35S promoter [53] to generate the final construct (the numbering of the constructs and the corresponding gene names are provided in Table 1). The seven constructs were independently transformed into *A. tumefaciens* strain GV3101-pMP90RK by electroporation and further transformed into the A. thaliana *cad1–3* mutants by the floral dip method [54]. T1 transgenic lines were screened on solid MS medium supplemented with 50 mg l^{-1} kanamycin. Single-copy transgenic plants were used for As(III) sensitivity analyses. In addition, two T3 homozygous transgenic lines overexpressing gene Mp*7g05690* (construct No. 4; Table 1) were selected for As(III) tolerance and elemental assays. All sequences used for preparing these constructs were verified by bidirectional Sanger sequencing with a 96-capillary 3730xl DNA Analyzer (Thermo Scientific).

2.8. Statistical analyses

Statistical significance among means for all experiments was assessed based on Student's t-tests carried out for each measurement versus the respective control (untreated control, or *cad1–3* genotype, depending on the experiment). For numbers of t-tests higher than 4, the false discovery rate (fdr) correction for multiple testing was applied and the corrected p-value reported. The number of stars, unless otherwise specified, represent the level of statistical significance of each Student's *t*-test according to the following coding conventions: *: $p <= 0.05$; **: $p <= 0.01$; ***: $p <= 0.001$; ****: $p <= 0.0001$. At least $n = 3$ biological replicates were analyzed for each experiment.

3. Results

3.1. Physiological responses of Cam-2 exposure to As(III)

It has been shown that *M. polymorpha* can withstand rather high amounts of As(III) [33] compared to flowering plants, but the kinetics of uptake and levels of toxicity can vary with experimental conditions. We, therefore, first evaluated the rapidity of As accumulation capacity of WT Cam-2 plants under our experimental conditions by subjecting 12-day-old gametophytes to a 24-hour As(III) treatment. As shown in Fig. 1A, Cam-2 plants could accumulate significantly higher amounts of As(III) relative to untreated plants. The physiological responses of Cam-2 exposed to As(III) were, thus, investigated further by treating 10-day-old Cam-2 plants with different concentrations of As(III) for 14 days. The plants grew progressively smaller (Fig. 1B) and accumulated less biomass (Fig. 1C) with increasing As(III) concentrations, resulting in plant death at 500 μM As(III) under the experimental conditions used. In addition, the intensity of oxidative stress (lipid peroxidation) resulting from As(III) treatment was also quantified by measuring the MDA concentrations in the same experimental conditions. No differences in MDA concentrations between control samples and samples treated at different concentrations of As(III) up to 200 μM were observed (Fig. 1D). The first statistically significant increase of MDA relative to control plants was detected in the samples treated with 300 μM. and the treatments with higher concentrations (400 and 500 μM As(III)) further increased lipid peroxidation.

3.2. RNA-Seq identification of candidate genes responsive to As(III) in M. polymorpha

To investigate further the early transcriptionally responsive genes involved in As(III) tolerance, 10-day-old Cam-2 plants after 24-hour treatment without and with 65 μM As(III) were prepared for whole transcriptome sequencing. A total of 288 differentially expressed transcripts were identified using a cutoff of $|LogFC| > 1.5$ and $FDR < 0.05$. Retaining only the transcripts for genes present in the latest *M. polymorpha* genome annotation (MpTak_v7.1), a total of 276 differentially expressed genes (DEGs) were identified. Among them, 175 DEGs were upregulated, while 101 were downregulated (Supplementary Table S3). Among the top 10 up- and down-regulated DEGs, one and six,

Table 1
The seven *Marchantia polymorpha* genes functionally characterized in this study by complementation of the *Arabidopsis cad1-3* mutant. The CDS length corresponds to the major isoform if more than one exist. The gene IDs correspond to MarPol annotation MpTak_v7.1 in Marpol Base.

Gene ID	Gene symbol	Construct No.	Annotation	Fold-Change	CDS (bp)	Reference
Mp*8g10560*	MpGST19	1	Glutathione S-transferase [EC:2.5.1.18]	5.97	654	[55]
Mp*5g04090*	MpGST3	2	Glutathione S-transferase [EC:2.5.1.18]	5.07	642	[55]
Mp*5g20060*	NA	3	Delta4–3-oxosteroid 5beta-reductase [EC:1.3.1.3]	75.88	1149	
Mp*7g05690*	NA	4	Peroxiredoxin 6 [EC:1.11.1.7 1.11.1.27 3.1.1.-]	6.59	660	
Mp*1g00460*	NA	5	Lectin domain of ricin B chain profile.	84.92	651	
Mp*6g07950*	MpPIN3	6	Auxin efflux carrier family protein.	4.80	1374	[56]
Mp*5g17165**	MpGH18.9	7	Glycosyl hydrolases family 18; Chitin recognition protein.	399.90	1161	[57]

* In previous *Marchantia polymorpha* genome annotations the ID of this gene was Mpzg00570.

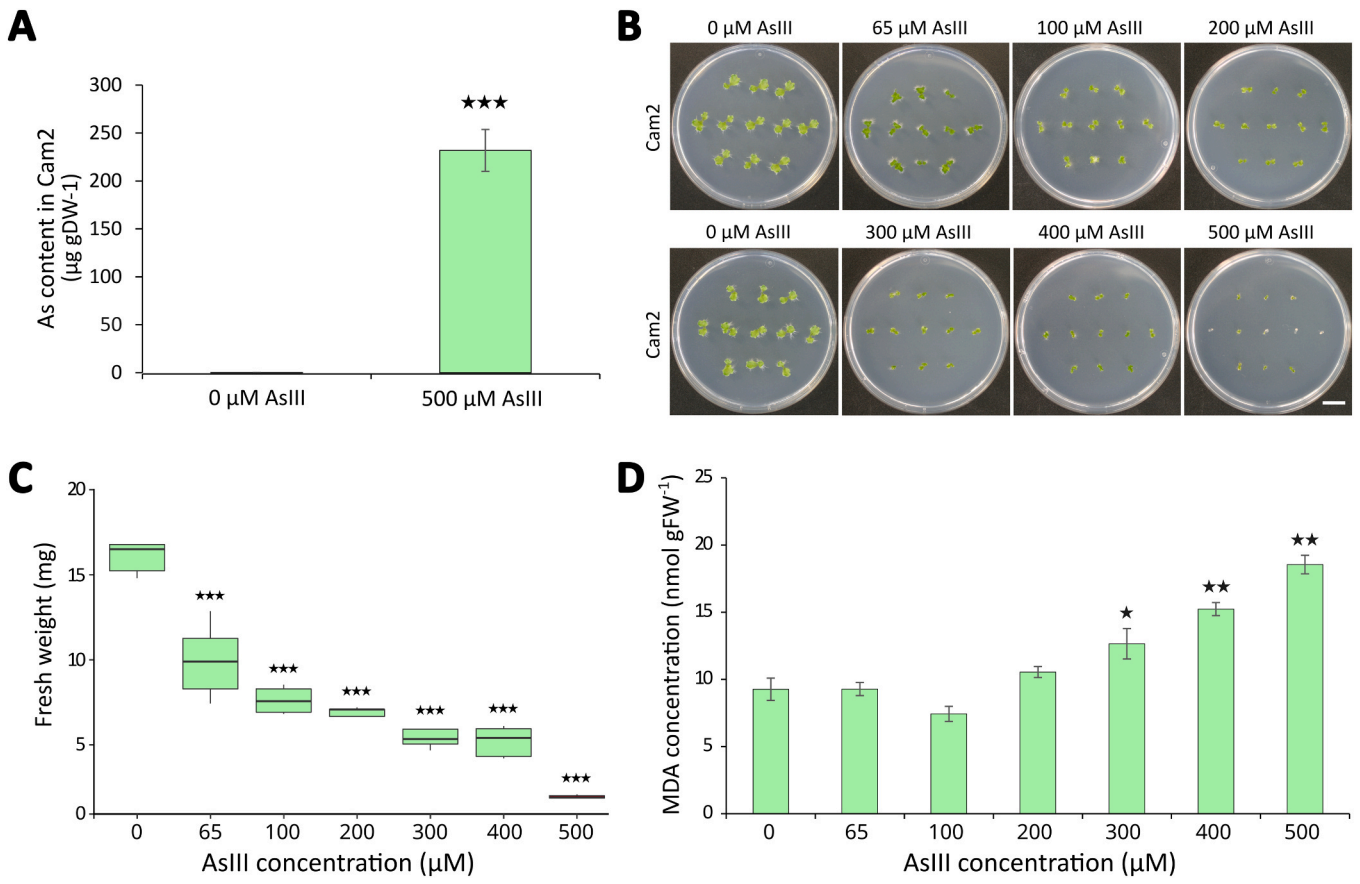


Fig. 1. Responses of Cam-2 exposed to different concentrations of As(III). A) Total As accumulation after 24 h exposure to 500 µM of As(III). B) Phenotypic variation in plant growth; C) Fresh weight quantification; D) lipid peroxidation assessed through MDA amounts at increasing levels of As(III) exposure. Scale bar in (B) corresponds to 12 mm.

respectively, have no predicted function (Table 2). Among the up-regulated DEGs, two are heat shock proteins and two are peroxidases, suggesting that oxidative stress responses have been triggered by As(III) treatment. The gene with the highest up-regulation (about 400 times higher than in the untreated condition) is a predicted chitinase. On the other hand, the gene with the highest down-regulation (about 50 times lower than in the controls) is a predicted lipoxygenase. The DEGs identified in this study were compared with those from [27] after 24 h exposure to As(III), which for simplicity will be called from now on Dutta's experiment. By using as cutoffs $|LogFC| > 1.0$ and $FDR < 0.05$, the total number of DEGs we identified was about one third of that reported in the other study (552 vs.1487, respectively; Supplementary Table S4). Among up-regulated DEGs, 141 were in common between studies, while 148 and 623 DEGs were specific to our experiment and Dutta's experiment, respectively. In the case of the down-regulated DEGs, only 48 shared DEGs were identified, while 676 and 215 were

Table 2
Top up- and down-regulated *M. polymorpha* genes that are differentially expressed in As(III) treated versus untreated gametophytes. NA: not available.

MpTak_v7.1 ID	Gene ID	Transcript ID	Regulation type	logFC	Predicted function
Mp*5g17165*	Mapoly0196s0008	PTQ27496	Up-regulated	8.64	Glycosyl hydrolases family 18; Chitin recognition protein.
Mp*5g07120*	Mapoly0136s0009	PTQ29680	Up-regulated	6.95	Plant heme peroxidase [EC:1.11.1.7]
Mp*7g07910*	Mapoly0076s0003	PTQ34756	Up-regulated	6.79	Hsp20/alpha crystallin family
Mp*8g05000*	Mapoly0081s0001	PTQ34270	Up-regulated	6.49	NA
Mp*1g00460*	Mapoly0103s0041	PTQ32070	Up-regulated	6.41	Lectin domain of ricin B chain profile.
Mp*5g20060*	Mapoly0190s0002	PTQ27605	Up-regulated	6.25	Delta4–3-oxosteroid 5beta-reductase [EC:1.3.1.3]
Mp*5g16170*	Mapoly0185s0004	PTQ27726	Up-regulated	5.35	Small hydrophilic plant seed protein
Mp*5g15340*	Mapoly0071s0075	PTQ35462	Up-regulated	5.23	Zinc-binding dehydrogenase
Mp*5g01690*	Mapoly0161s0035	PTQ28540	Up-regulated	5.21	Plant heme peroxidase [EC:1.11.1.7]
Mp*5g23120*	Mapoly0010s0143	PTQ46761	Up-regulated	5.09	Hsp20/alpha crystallin family
Mp*8g13290*	Mapoly0110s0010	PTQ31517	Down-regulated	−3.40	NA
Mp*8g10080*	Mapoly0008s0214	PTQ47464	Down-regulated	−3.52	NA
Mp*7g13040*	Mapoly0003s0312	PTQ49483	Down-regulated	−3.63	GDSL-like Lipase/Acylhydrolase
Mp*2g23910*	Mapoly0069s0041	PTQ35696	Down-regulated	−3.88	NA
Mp*3g01490*	Mapoly0007s0141	PTQ47740	Down-regulated	−4.04	NA
Mp*5g18390*	Mapoly0084s0087	PTQ34025	Down-regulated	−4.09	NA
Mp*2g16790*	Mapoly0109s0020	PTQ31588	Down-regulated	−4.22	Zinc finger, C3HC4 type (RING finger)
Mp*1g06950*	Mapoly0043s0086	PTQ39845	Down-regulated	−4.34	NA
Mp*3g09880*	Mapoly0085s0038	PTQ33819	Down-regulated	−4.98	POT (proton-dependent oligopeptide transport) protein
Mp*5g21680*	Mapoly0106s0031	PTQ31835	Down-regulated	−5.41	Plant lipoxygenase signature

those specific to our analysis and Dutta's analysis, respectively. A cross check of the consistency in the identification of up- and down-regulated DEGs uncovered in total 13 DEGs with opposite directions of differential expression in the two experiments (Supplementary Table S4). Nine genes (Mp*1g01450*, Mp*6g15610*, Mp*1g22360*, Mp*1g26380*, Mp*3g17610*, Mp*3g22960*, Mp*7g05870*, Mp*8g02940* and Mp*zg00500*) were up-regulated in Dutta's dataset but down-regulated in ours, while four genes (Mp*5g12260*, Mp*8g12880*, Mp*3g22470* and Mp*7g05690*) were down-regulated in Dutta's dataset and up-regulated in ours.

To obtain a general overview of what are the main functional changes upon As(III) exposure in the experimental conditions tested, GO enrichment analysis was carried out. Both up- and down-regulated DEGs were enriched in the same two Cellular Component GOs, namely GO:0048046 apoplast and GO:0005576 extracellular region (Supplementary Table S5 and S6). By contrast, while down-regulated DEGs were not significantly enriched in any GO from Biological Process and Molecular Function, several GOs from these categories were enriched for up-regulated DEGs. In the case of GOs from the Biological Process category, the three most significant enrichments (lowest FDR) were those in GO:0042221 response to chemical, GO:0050896 response to stimulus and GO:0070887 cellular response to chemical stimulus (Fig. 2A; Supplementary Table S7). In general, among the top ten most enriched GOs the majority refer to upregulated DEGs functioning in cellular detoxification, as well as oxidative chemical stresses (Fig. 2A; Supplementary Table S7). Accordingly, network analysis identified a major cluster of GOs related to the detoxification of oxidative stress related to chemicals (6 of the top 10 GOs; Fig. 2B), along with a second cluster related to negative regulation of catalytical and molecular functions (Fig. 2B). Regarding the GOs from the Molecular Function category, the most significant enrichments associated to up-regulated DEGs were in GO:0033293 monocarboxylic acid binding, GO:0016491 oxidoreductase activity and GO:0031406 carboxylic acid binding (Fig. 2C; Supplementary Table S8). Network analysis highlighted two major clusters, the first related to very specific antioxidant activities in response to oxygen peroxide while the second related to more general activities of binding to monocarboxylic acids, lipids and abscisic acid (Fig. 2D; Supplementary Table S8).

Seven of the up-regulated DEGs (Mp*1g00460*, Mp*5g04090*, Mp*5g17165*, Mp*5g20060*, Mp*6g07950*, Mp*7g05690* and Mp*8g10560*) were selected for validation by qRT-PCR. Four of the genes (Mp*5g04090*, Mp*5g20060*, Mp*7g05690*, Mp*8g10560*) were consistently found to be up-regulated also in Dutta's dataset. One of the seven genes, Mp*7g05690*, contrary to what observed in our RNA-Seq results, had been identified as down-regulated under As(III) stress in Dutta's experiment (see above). The remaining two genes (Mp*5g17165* and Mp*6g07950*) were not found to be differentially expressed in Dutta's dataset. We, thus, carried out qRT-PCR validation of the genes' expression levels in the same conditions used for RNA-Seq. For six genes (Mp*1g00460*, Mp*5g04090*, Mp*5g17165*, Mp*5g20060*, Mp*6g07950*, Mp*7g05690* and Mp*8g10560*) the primer designed provided good amplification efficiencies and confirmed with excellent agreement the results obtained in the RNA-Seq experiment (Fig. 3). On the other hand, the primers for gene Mp*5g17165* did not amplify well, preventing us from confirming its upregulation upon As(III) stress. As overexpression of this gene in the *Arabidopsis cad1–3* mutant did not result in significant phenotypic complementation, no further primers were designed to verify its expression.

3.3. Functional characterization of seven DEGs by complementation of the cad1-3 mutant of Arabidopsis thaliana

Based on the RNAseq data analysis, a set of seven differentially upregulated genes (Table 1) were selected for *Agrobacterium*-mediated plant transformation in the *AtPCS1* knockout mutant background (*cad1–3*), which is highly sensitive to As(III). The selected candidate genes spanned a wide range of up-regulation fold-changes values, displaying from around 5–400 times increases in transcription following As (III) treatment. The predicted functions of these genes range from glutathione S-transferase (Mp*8g10560* and Mp*5g04090*), to delta4–3-oxosteroid 5beta-reductase (Mp*5g04090*), peroxiredoxin (Mp*7g05690*), lectin (Mp*1g00460*), auxin transporter (Mp*6g07950*), and chitinase (Mp*5g17165*).

A total of 12 independent single-copy transgenic lines for each gene were used to assess As(III) sensitivity. Fresh weight analyses of 10-day-old plants indicated that most of the transgenic lines transformed with Mp*5g04090*, Mp*5g20060* and Mp*7g05690*, were significantly more tolerant to As(III) compared with *cad1–3* plants (Figs. 4C, 4D and 4E). By contrast, only around 30 % of the transgenic lines generated from Mp8g10560 and Mp1g00460 transformations were more tolerant than *cad1–3* plants (Figs. 4B and 4F). Finally, almost all transgenic lines

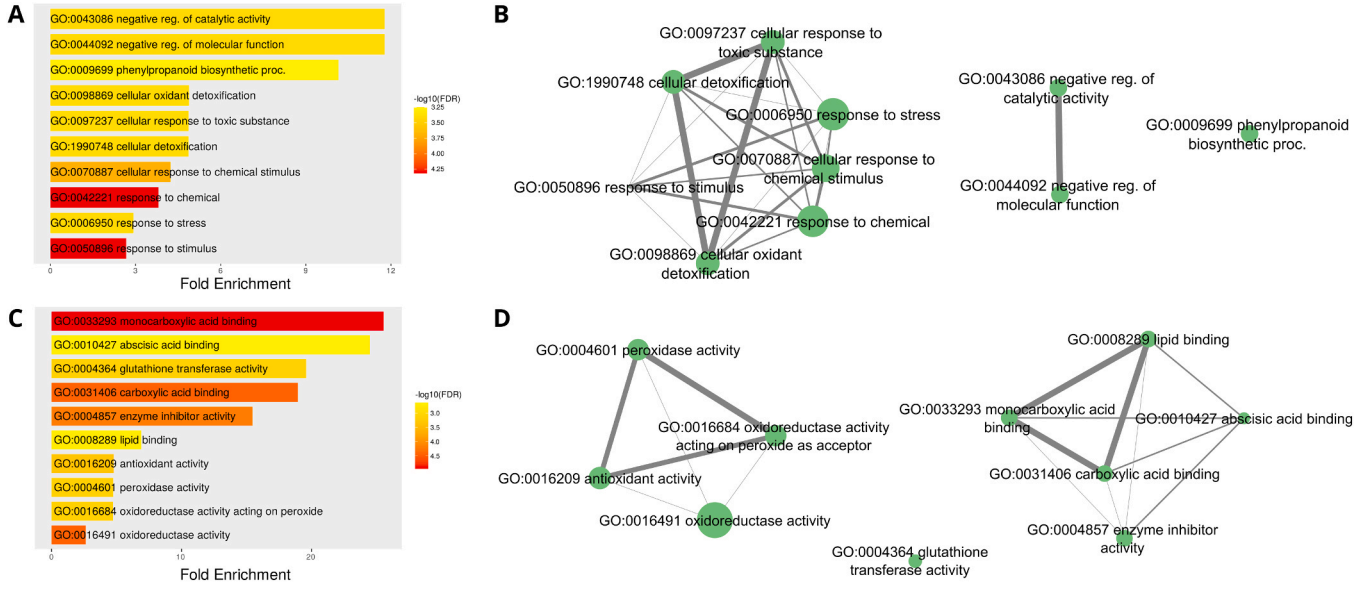


Fig. 2. GO enrichment of up-regulated DEGs upon As(III) treatment. The top 10 most enriched GO terms in the Biological Processes (A, B) and Molecular Function (C, D) categories are reported. In the barplots (A, C), the GO terms are sorted from top to bottom in decreasing order of fold-enrichment and the bars are color-coded according to FDR-corrected p-values. The networks (B, D) provide the relationships among different GO terms.

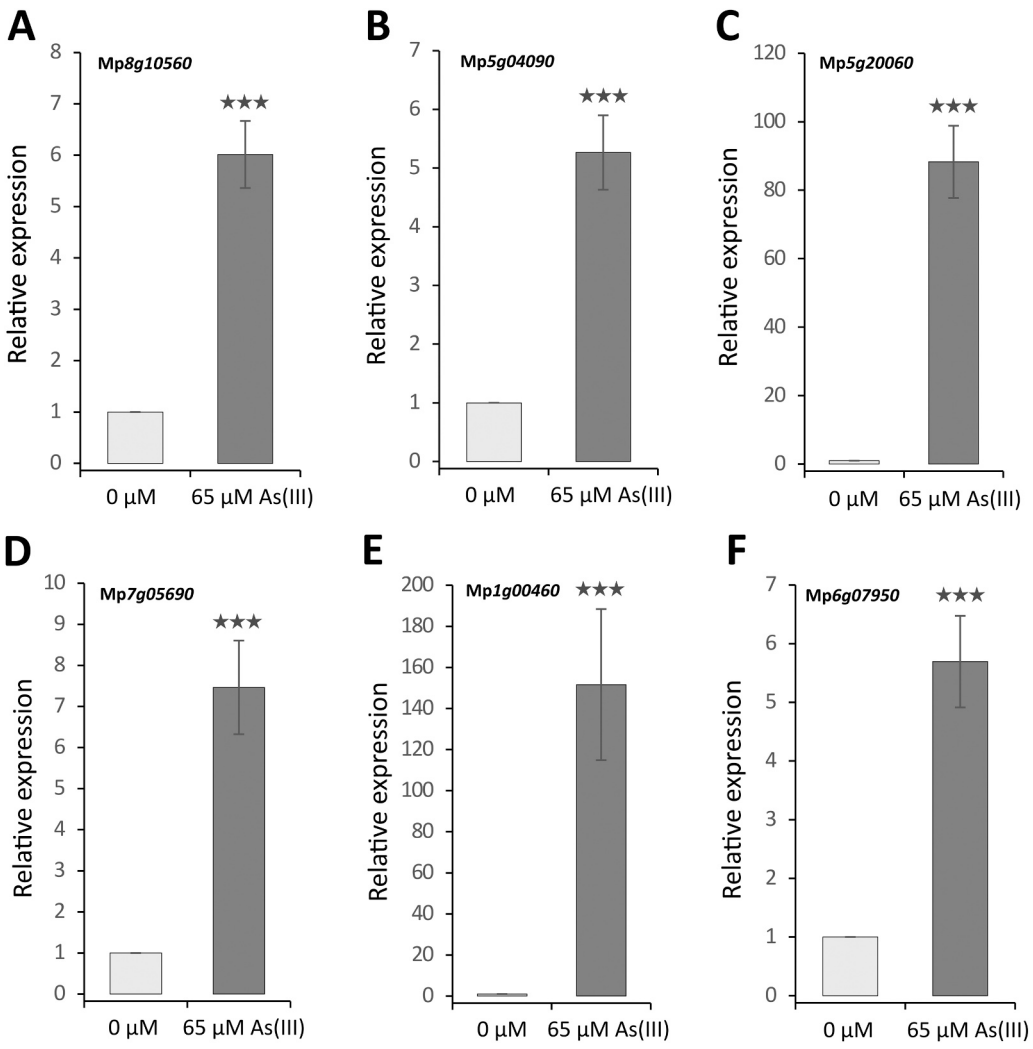


Fig. 3. qRT-PCR validation of expression induction for selected DEGs after exposure to As(III).

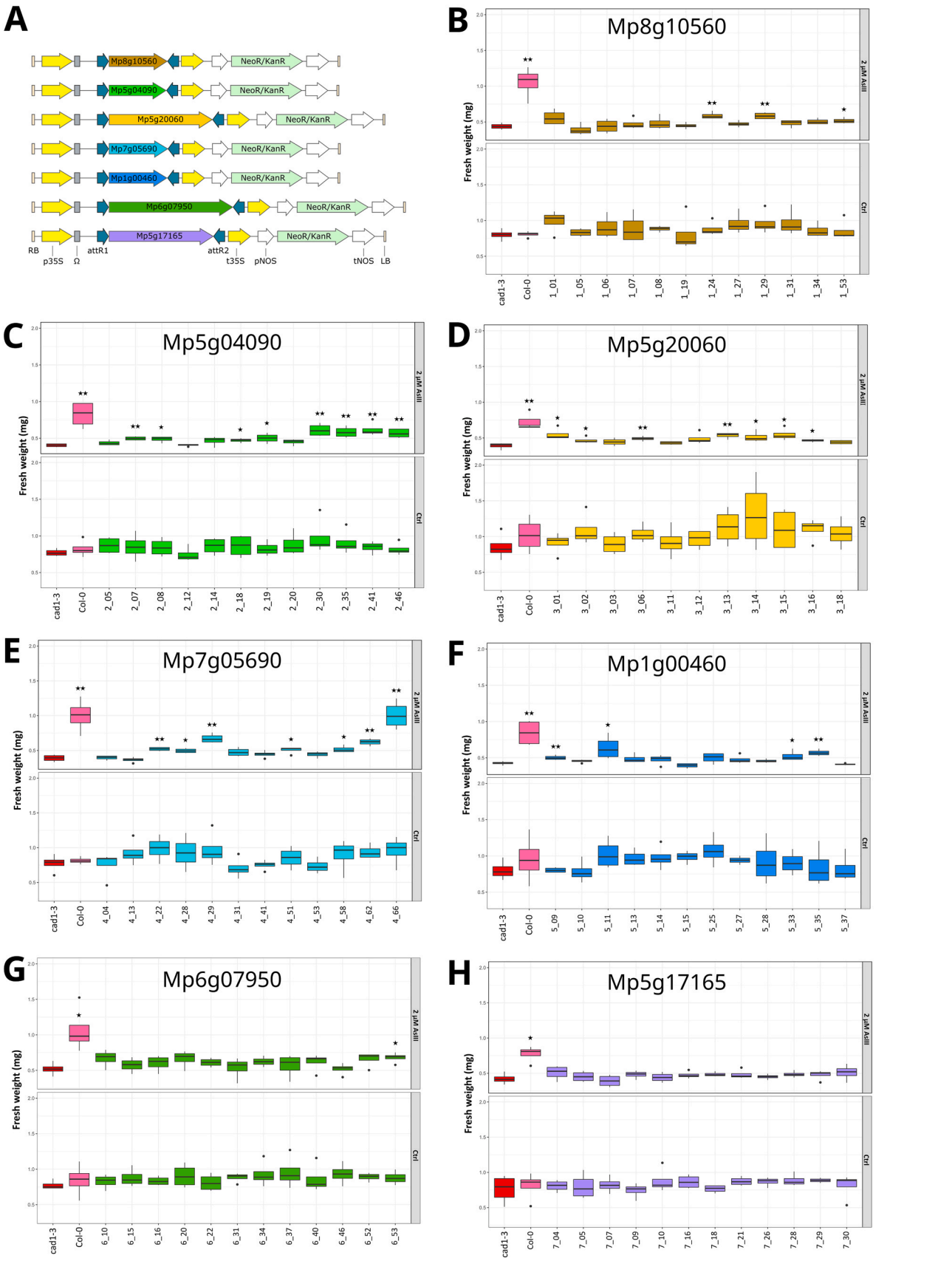
derived from Mp*6g07950* and Mp*5g17165* transformations showed no differences in As(III) tolerance relative to *cad1–3* plants (Figs. 4G and 4H).

3.4. *Mp7g05690 overexpression complements As(III) hypersensitivity of the cad1-3 mutant*

To characterize more in detail the As(III) tolerance of transgenic plants, two transgenic lines (04–29 and 04–66 in Fig. 4, from now on referred to as MpPL-1 and MpPL-2 for simplicity) of the Mp*7g05690* transformation were selected. The biomass accumulation of the transgenic lines, Col-0 and *cad1–3* was measured from 10-day-old plants growing in different concentrations of As(III) (2 µM and 3 µM). The root lengths in both transgenic lines were dramatically longer than in *cad1–3* plants and very similar to Col-0 wild-type plants under both concentrations of As(III) tested (Fig. 5A), although in control conditions all three genotypes had comparable root lengths. Accordingly, also the whole-plant fresh weights of both transgenic lines treated with different concentrations of As(III) were significantly higher than that of *cad1–3*. Moreover, no differences in fresh weight of all different genotypes tested were detected in the control condition (Fig. 5B).

To explore more in depth the physiological responses of transgenic plants, oxidative stress induced by 10 µM As(III) treatment was evaluated by measuring the MDA content at different time points. The MDA content was significantly lower in both transgenic lines and col-0 wild-type plants than in *cad1–3* mutants after three days of As(III) treatment ($p = 0.0031$ for Col-0, $p = 0.0449$ for MpPL-1, $p = 0.0416$ for MpPL-2 according to pairwise Student's *t*-tests against the *cad1–3* mutant; Fig. 5C). Also after seven days of As(III) exposure, both transgenic lines and col-0 wild-type plants displayed very similar MDA contents, which were in all cases significantly lower compared with that of *cad1–3* mutants ($p = 0.0001$ for Col-0, $p = 0.0003$ for MpPL-1, $p = 2.59E-05$ for MpPL-2 according to pairwise Student's *t*-tests against the *cad1–3* mutant; Fig. 5D). No differences in MDA content were found among all genotypes examined both under three-day and seven-day control conditions (Figs. 5C and 5D).

Furthermore, to estimate the capacity to accumulate arsenic in planta among the different genotypes, elemental analysis was carried out using 12-day-old plants which were exposed to 10 µM As(III) for three days. This analysis indicated no significant difference for total arsenic contents among *cad1–3* mutants, col-0 and transgenic lines under control condition, although very limited amounts were detected in all these genotypes as expected (Fig. 5E). By contrast, after As(III) exposure, all genotypes accumulated sizable amounts of the metalloid. While no differences were observed between transgenic lines and Col-0 wild-type plants, significantly higher amounts of arsenic were scored both for transgenic lines and col-0 wild-type plants relative to *cad1–3* mutants ($p = 0.0008$ for col-0, $p = 0.0183$ for MpPL-1, $p = 0.0015$ for MpPL-2 according to pairwise Student's *t*-tests against the *cad1–3* mutant; Fig. 5E).



(caption on next page)

Fig. 4. Constructs of seven *M. polymorpha* As tolerance candidate genes and phenotypic complementation of *A. thaliana cad1–3* plants overexpressing them. A) summary of the T-DNA region for the seven plant binary constructs used for *A. thaliana* transformation. RB: *A. tumefaciens* T-DNA right border; p35S: Cauliflower mosaic virus 35S promoter; Ω (Omega): 5′-leader sequence of tobacco mosaic virus (TMV) translational enhancer; attR1, attR2: bacteriophage λ recombination sites; t35S: Cauliflower mosaic virus 35S terminator; pNOS: *A. tumefaciens* nopaline synthase promoter; tNOS: *A. tumefaciens* nopaline synthase terminator; NeoR/KanR: *Escherichia coli* neomycin phosphotransferase gene conferring resistance to kanamycin; RB: *A. tumefaciens* T-DNA right border. The features are drawn to scale and the colors of *M. polymorpha* candidate genes correspond to those of the corresponding *A. thaliana* transgenic lines overexpressing them in panels B-H. The panels show the results of the As(III) tolerance complementation tests of the *cad1–3* mutant using the seven candidate genes from *M. polymorpha*: B) Mp*8g10560*; C) Mp*5g04090*; D) Mp*5g20060*; E) Mp*7g05690*; F) Mp*1g00460*; G) Mp*6g07950*; H) Mp*5g17165*.

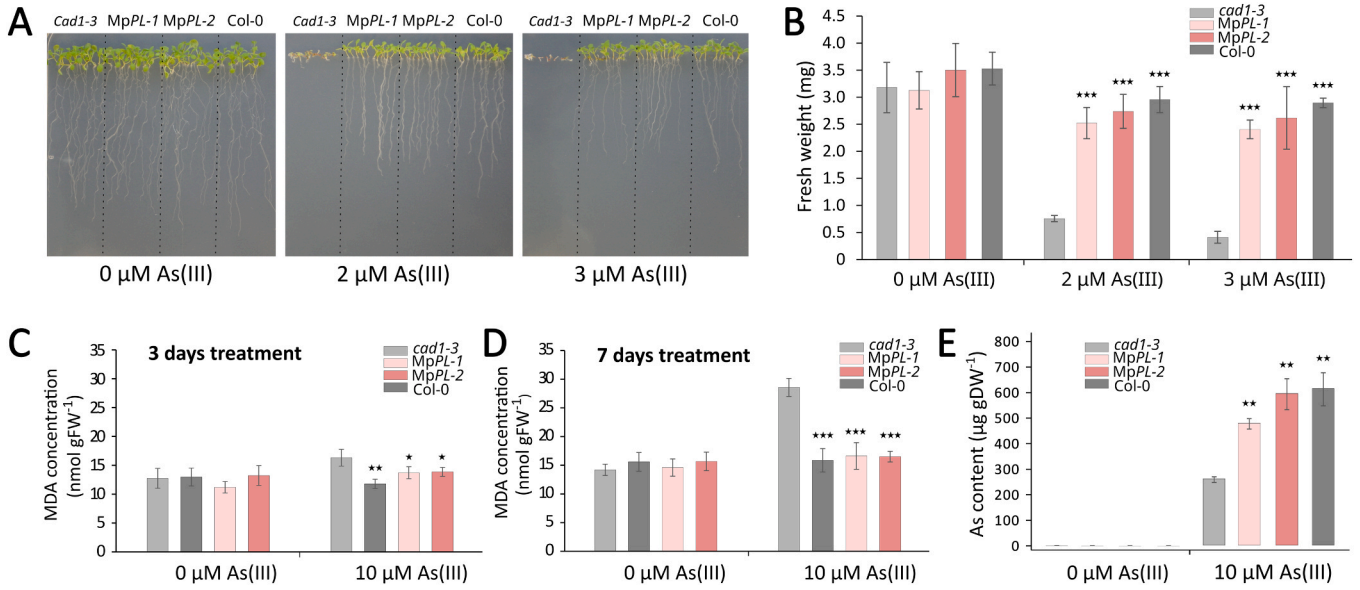


Fig. 5. Characterization of two selected *A. thaliana* transgenic lines overexpressing the peroxiredoxin-like protein encoded by Mp*7g05690* in the *cad1–3* mutant background. Phenotype of complementation lines MpPL-1 and MpPL-2 compared to that of *cad1–3* and Col-0 grown at different As(III) concentrations (A). Corresponding fresh weight of the four genotypes (B). MDA concentrations after three days (C) and seven days (D) of treatment with As(III). Total As content in plants grown for three days after As(III) treatment(E).

The characterization of the native expression of Mp*7g05690* in *Marchantia polymorpha* upon As(III) exposure revealed that the gene is transiently induced by the metalloid, with the peak of expression (25x upregulation compared to time 0) at 12 h after treatment, to further decline at 24 h (Fig. 6).

4. Discussion

4.1. Comparison with previous RNA-Seq studies on As(III) responses in Marchantia

Compared to the only other transcriptomic study published on the As (III) responses in *M. polymorpha*, using the same cutoffs we identified about two thirds less DEGs after 24 h of treatment with the metalloid (Supplementary Table S3). Also the functions of the up-regulated DEGs,

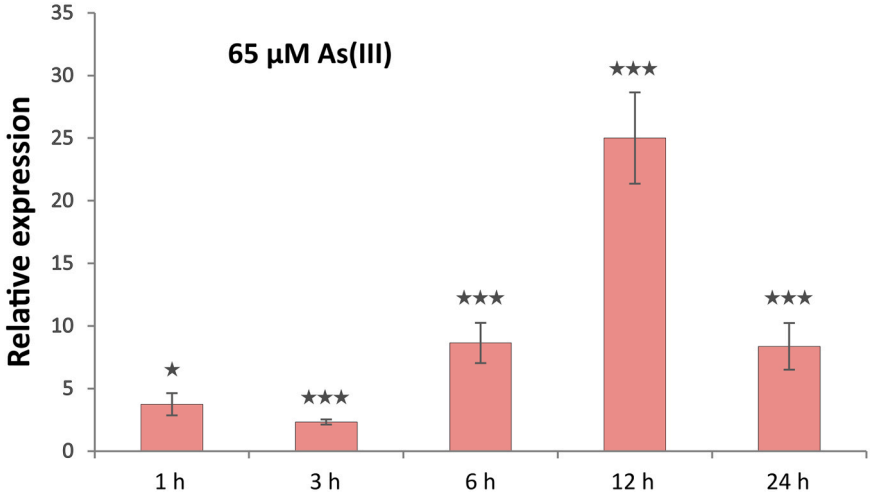


Fig. 6. Time-course analysis of the expression pattern of Mp*7g05690* in *M. polymorpha* exposed to As(III) stress.

although largely congruent for those involved in a chemically induced oxidative stress, showed more GO terms associated with biotic stress in Dutta's dataset than in ours (Fig. 2; Supplementary Table S4). Strikingly, while many GO terms were enriched for down-regulated DEGs in Dutta's dataset, no Molecular Function and Biological Process GOs were enriched for down-regulated DEGs in our study. Several factors related to the different *M. polymorpha* genotypes and experimental conditions used likely contribute to these differences. Recent population genomics studies and the sequencing of *M. polymorpha* pangenome indicate that substantial gene flow and recombination events occurred across the geographical range of subsp. *ruderalis* [58,59], although local adaptations and considerable phenotypic variability have been reported [59,60]. Part of the observed differences in As(III) transcriptional responses may, therefore, stem from ecotypic differentiation and an overall higher responsivity of the Indian accession compared to Cam-2. The fact that the transcriptional responses are stronger in Dutta's experiment notwithstanding a lower nominal concentration of As(III) used (40 μM of exogenously applied As(III) versus 65 μM As(III) in our study), however, suggests that also differences in the experimental conditions used likely account for the differences among the two transcriptomics studies. In particular, the MS-agar medium used here allows us to precisely define the amount and chemical form of As supplied to the plant, providing a tight control on the level of stress applied. On the other hand, peat moss, constituting 1/3 in weight of soilrite, is known to accumulate significant, although variable, amounts of both organic and inorganic arsenicals [61–63], suggesting that the actual amount of bioavailable As in Dutta's experiment could be higher than the nominal 40 μM As(III) exogenously applied. Additionally, some of the organoarsenicals present in peat moss have been reported to be more toxic to plants than their inorganic counterparts [64,65]. Thus, the stronger transcriptional response observed in Dutta's experiment despite the use of a nominal As(III) concentration lower than that in our study could be possibly also ascribed to the type of substrate used to carry out the experiments. Moreover, the peat-derived component of soilrite can potentially contain bacterial contaminants, which could explain the higher proportion of biotic stress-related GOs in Dutta's dataset than in ours. The higher number of enriched GOs for the down-regulated DEGs in Dutta's dataset, on the other hand, could result from the lower number of biological replicates used (two vs. four in our study), which could affect especially lowly expressed genes.

4.2. Candidate genes weakly complementing As(III) hypersensitivity in Arabidopsis

The high number of biological replicates and the relatively stringent fold-change cutoff we used, allowed us to identify a highly reliable set of DEGs, as demonstrated by the qRT-PCR validation (Fig. 3). The two genes which did not complement the As(III) hypersensitivity of the *cad1–3* mutant are putatively involved in auxin transport and degradation of chitin. Homologs of both genes have been associated to As(III) tolerance responses, but whether their involvement is direct or not remains to be demonstrated. The protein coded by Mp*6g07950*, MpPIN3, has the highest homology to the PIN1 auxin efflux transporter, a plasma membrane localized isoform playing a major role in determining auxin basipetal movement and organ initiation and development in the shoots of *A. thaliana* [66,67]. The PIN1 protein has the same specificity towards auxin as its root-specific isoform, PIN2 [68,69], which has been in turn suggested to function as an arsenite efflux transporter in roots, although functional proof of its direct As transport has not been provided [70]. Thus, currently, the reasons for the lack of As(III) tolerance by MpPIN3 overexpressing lines are not clear. Overexpression of PIN1 in *A. thaliana* causes root shortening and agravitropic growth associated to higher auxin concentrations in root tips, possibly because of subcellular mislocalization associated to ectopic expression in epidermal cells [71,72]. It is, thus, possible that the lack of complementation in the *cad1–3* lines overexpressing Mp*6g07950* could result from a dominant phenotype related to auxin transport deregulation rather than As(III) toxicity. Alternatively, the MpPIN3 protein could be mistargeted in *A. thaliana* cells compared to PIN2, preventing it from enhancing As(III) tolerance. Regarding Mp*5g17165*, chitinases have been previously demonstrated to be responsive, in addition to biotic stresses like fungal and bacterial infections, also to heavy metals and As in several angiosperm families [73], with some isoforms showing metal(loid)-specific inducibility [74–77]. Overexpression of fungal chitinases in tobacco conferred elevated tolerance to cadmium and copper and other abiotic as well as biotic stresses and enhanced the overall peroxidase activity of the plants, leading to the suggestion that they could be involved in the detoxification of the resulting ROS [78]. This explanation is, however, at odds with the lack of protection towards As(III) in *A. thaliana* plants overexpressing Mp*5g17165*. One plausible explanation for this apparent incongruence is that the fungal chitinases may have been recognized by the immune system of host plants and have activated defense responses [79], which were instead not activated by the plant chitinase in our study because of the low homology to fungal enzymes [80]. Under this scenario, thus, upregulation of plant chitinases would be just a side-product of ROS resulting from any type of stress and, therefore, would not play any role in As tolerance. In-depth characterization of the Mp*5g17165* overexpressing lines will be needed to test this hypothesis.

4.3. Candidate genes with intermediate levels of complementation of As (III) hypersensitivity in Arabidopsis

The modest increase in As(III) tolerance in Mp*1g00460* and Mp*8g10560* overexpression lines, on the other hand, suggests that the protection towards As toxicity provided by both genes is rather low and thus sensitive to transgene insertion site in the genome of *A. thaliana* (Figs. 4B and 4F). The protein coded by Mp*1g00460* is homolog to plant lectins, a class of plant proteins able to bind carbohydrates usually involved in defense, but also in response to abiotic stress like drought, cold and salinity especially when secreted to the apoplast [81]. The lack of significant homology to angiosperm lectins and of previous association to As tolerance, however, does not currently allow to infer possible mechanisms of action for this gene. By contrast, the GST coded by Mp*8g10560* (also called MpGST19) [55] based on its high homology to the single copy PpUre2p1 from the moss *Physcomitrium patens* belongs to the Ure2p class, a group of bacterial-like GSTs lacking from seed plants [82,83]. PpUre2p1 can use 7-chloro-4-nitrobenzo-2-oxa-1,3diazole (NBD-Cl), and to a lesser extent 1,2-dichloro-4-nitrobenzene (DCNB) and cumene hydroperoxide (Cum-OOH) as substrates [82], but its *in vivo* targets allowing it to enhance As tolerance in *M. polymorpha* are currently unknown.

4.4. Candidate genes with high levels of complementation of As(III) hypersensitivity in Arabidopsis

The other GST gene used for *Arabidopsis* transformation, Mp*5g04090* (also called MpGST3) [55], belong to class Phi, a plant-specific abundant group of GSTs present in all land plants with variable substrate specificities and involved in abiotic stress responses [83]. While an unusual Phi-type GST from the As hyperaccumulator fern *Pteris vittata* has been demonstrated to act as an arsenate reductase [20], the MpGST3 protein has higher homology to other *P. vittata* GSTs (data not shown). As also a Lambda-type GST from rice could confer As as well as heavy metals tolerance to *Arabidopsis* possibly by enhancing its antioxidant capacity [84], the function of *M. polymorpha* GSTs against As toxicity probably is indirect and can be ascribed to their general involvement in ROS detoxification. Another gene enhancing As tolerance when overexpressed in *Arabidopsis* is Mp*5g20060*, which codes for a progesterone 5β-reductase/iridoid synthase-like enzyme (PRISE) ortholog of the *Arabidopsis* VEP1 gene [85]. PRISEs detoxify methyl vinyl ketone and other reactive electrophile species [85,86] resulting from trienoic fatty acid peroxidation [87]. The increased tolerance towards As(III) in

transgenic *Arabidopsis* lines is, thus, most likely due to the protective role of this enzyme towards the oxidative stress triggered by the metalloid. The *M. polymorpha* DEG conferring the highest level of tolerance to As (III) in the *cad1–3* mutant is Mp*7g05690*, coding for a peroxiredoxin of the 1-cys subclass named PER1 [21]. In Dutta's experiment, Mp*7g05690* was down-regulated under As(III) stress, but it was not among the genes whose expression was validated by qRT-PCR. On the other hand, our validation of the differential expression of Mp*7g05690* by qRT-PCR excludes a possible error in our results and confirms that the gene is upregulated in Cam-2 gametophytes under the experimental conditions we used. Given that two additional genes (Mp*5g17165* and Mp*6g07950*) among those that we confirmed to be up-regulated by qRT-PCR were not found to be differentially expressed in Dutta's dataset, we consider that the discrepancy in transcriptional responsiveness of Mp*7g05690* to As (III) stress between our and Dutta's study, therefore, could be most likely due to genotype differences among the *Marchantia polymorpha* ecotypes used in the two studies. PER1 in vascular plants is mainly expressed in seeds [88], where it is thought to enhance seed dormancy by detoxifying H_2O_2 and other ROS which are normally produced during seed germination [89]. Overexpression of various PER1 genes in model systems like tobacco and *Arabidopsis* consistently enhanced oxidative stress tolerance [88–91], but the ability to detoxify ROS from As(III) stress has never been functionally tested. Our results clearly demonstrate that, when overexpressed in *Arabidopsis*, Mp*7g05690* can restore growth, total As content and lipid peroxidation comparable to WT levels, thus suggesting that As tolerance in plants can be achieved by increasing the antioxidant capacity of cells in alternative to the dominant mechanism based on PCn-mediated vacuolar sequestration. Interestingly, the gene is transiently induced in M. polymorpha upon As(III) stress (Fig. 6), suggesting that its role may be confined to early responses to oxidative stress.

5. Conclusions

Taken together, the results obtained point to a considerable degree of conservation in the mechanisms of As detoxification in both lineages of land plants, the bryophytes and the tracheophytes, with the majority of *M. polymorpha* DEGs tested contributing to tolerance to this metalloid in *A. thaliana*. Given the generally low redundancy present in its genome, therefore, *M. polymorpha* can be a valid model for the identification of evolutionarily deeply conserved genes for the genetic improvement of crops for As tolerance through either biotechnological or conventional breeding approaches. The general picture emerging from the current study is that As tolerance is achieved through the cooperative action of many genes involved at different levels in ROS detoxification, suggesting that stacking of multiple genes could be necessary to this aim. Worth of note, our results point to the possible equivalence between sequestration and ROS detoxification mechanisms in conferring As(III) tolerance to plants, implying that the pivotal role played by PCS and phytochelatins in this regard could have been the result of evolutionary contingency, although possible fitness tradeoffs warrant further experiments to confirm this hypothesis.

Environmental implication

Inorganic arsenic (As) is a recognized carcinogen, considered the most hazardous element at the global level due to its ubiquitous presence as environmental pollutant of either natural or anthropic origin. The current work demonstrates that mechanisms of As tolerance are significantly conserved in the bryophyte *Marchantia polymorpha* and *Arabidopsis thaliana*. Thus, *M. polymorpha* can be a valid model for the identification of evolutionarily deeply conserved genes for the genetic improvement of crops for As tolerance through either biotechnological or conventional breeding approaches.

CRediT authorship contribution statement

Luigi Sanità di Toppi: Writing – review & editing, Supervision, Resources, Funding acquisition. **Roberto Larcher:** Writing – review & editing, Supervision, Resources. **Alessandro Cestaro:** Writing – review & editing, Funding acquisition, Formal analysis. **Daniela Bertoldi:** Writing – review & editing, Investigation, Formal analysis. **Mingai Li:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Claudio Varotto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Enrico Barbaro:** Writing – review & editing, Investigation, Formal analysis. **Paolo Sonego:** Writing – review & editing, Formal analysis. **Cong Wang:** Writing – review & editing, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.140001.

Data availability

Data will be made available on request.

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