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Manuscript Draft

Manuscript Number: STOTEN-D-19-04903R1

Title: Integrative response of Arsenic Uptake, Speciation and Detoxification by Salix atrocinerea

Article Type: Research Paper

Keywords: Salix, arsenic, non-protein thiols, speciation, phytochelatins, gene expression

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Abstract: Despite arsenic (As) being very toxic with deleterious effects on metabolism, it can be tolerated and accumulated by some plants. General genetic mechanisms responsible for As tolerance in plants, including Salix species, have been described in transcriptomic analysis, but further experimental verification of the significance of particular transcripts is needed. In this study, a Salix atrocinerea clone, able to thrive in an As-contaminated brownfield, was grown hydroponically in controlled conditions under an As concentration similar to the bioavailable fraction of the contaminated area (18 mg kg-1) for 30 days. At different time points, i.e. short-term and long-term exposure, biometric data, As accumulation, phytochelatin synthesis, non-protein thiol production and expression of target genes related to these processes were studied. Results showed that S. atrocinerea presents a great tolerance to As and accumulates up to 2,400 mg As kg-1 dry weight in roots and 25 mg As kg-1 dry weight in leaves. Roots reduce As V to As III rapidly, with As III being the predominant form of As accumulated in root tissues, whereas in the leaves it is As V. After 1 d of As exposure, roots and leaves show de novo synthesis and an increase in non-protein thiols as compared to the control. Integrating these data on As accumulation in the plant and its speciation, non-protein thiol production and the kinetic gene expression of related target genes, a fundamental role is highlighted for these processes in As accumulation and tolerance in S. atrocinerea. As such, this study offers new insights in the plant tolerance mechanisms to As, which provides important knowledge for future application of high-biomass willow plants in phytoremediation of As-polluted soils.

Response to Reviewers: Major comment:
1) The authors present novel findings related to arsenic species shifts and transport which is certainly of interest to the field. The gene expression element of the work is, in my opinion, quite poor in relying very heavily on assumptions and the work of Yanitch et al 2018 as well as, importantly, relying on qPCR which isn’t really up to the contemporary standard in the field. As such I think the gene expression

represents a major flaw preventing publication as it would be a step backwards in the field and could be unsound (I've outlined the methodological flaws below). If the authors can shift the focus of this manuscript away from very limited gene expression towards the more novel results and discussion about arsenic fate and non-protein thiolic compounds, I would consider this suitable for publication.

2) Abstract:
2.1) The major discussion point here is that "analysis of transcript levels of target genes… highlighted a fundamental role for certain gene in As accumulation and tolerance in S. atrocinerea". The target genes were already identified as being of potential importance using RNAseq and differential expression analysis. Do the author's believe the qPCR is an important addition to that work? I'm concerned as the targeted nature of the analysis actually precluded any novel genetics findings.

2.2) The subsequent major conclusion point ends with "new possibilities for genotype selection and genetic engineering… in phytoremediation". Neither of these statements are appropriate given the very limited qPCR work on genes from the Yanitch paper. The genetic engineering point really seems very far away from something relevant for the abstract of this work – can the author's rephrase this to discuss why understanding arsenic accumulation, fate and plant tolerance is important?

3) Methods:
3.1) Line 152: the assessment of As III, As V, monomethylarsenic acid and dimethlyarsenic acid is a real strength of this work in my eyes. Can the authors focus the results and, in particular, the discussion around considering these novel findings and their potential meaning (in place of the expression levels of selected genes).

3.2) Lines 183-206: The authors use qPCR to estimate expression levels of genes selected from the Yanitch paper. While qPCR was the gold standard for gene expression study, such approaches seem outdated when next generation sequencing is available for exploring an organism's gene expression of a new treatment, especially when extensive subsequent interpretation is made. I don't believe the approach taken by the authors prevents publication of the work per se and I do recognise that modern sequencing approaches can be expensive. However, the extensive focus on a very limited number of genes selected from a different system here, alongside very extensive discussion, is not an appropriate use of this qPCR data. Ultimately the work provides generates a more limited version of the Yanitch findings (as it is limited to their findings). I'd strongly recommend removing the majority of gene expression data from the MS and focusing on the novel analysis of non-protein thiolic compounds, which are interesting results that aren't discussed at length.
3.3) On a more technical note, the referenced Li paper doesn't establish these reference genes are appropriate for this species of willow or (more importantly) for arsenic stress, and cites a number of studies where most of these reference gene are not stable.

3.4) I do know the graynorm algorithm approach (Remans et al., 2014), no data about stability of these genes in the system is presented. Are the authors sure that arsenic won't impact the stability of these genes? For example, one of the three reference genes used for the roots was identified as DE in roots of the Yanitch paper (EF1-alpha), and reference genes used for leaves were identified as DE in leaves (ACT7 and alpha-TUB2) – each being the same genes from L. Smart's assembly. I'm not sure

the DNAJ mentioned in the paper's table was used as this isn't consistent with the text.

4) Results + discussion

4.1) The section starting on line 255: this represents interesting findings which I think are the strength of the presented research – the subsequent section on gene expression opens with a general pattern of some of the Yanitch et al selected genes showing the same increases observed in their paper (but assessed with less sound methodology). I'm particularly uncomfortable with the presentation of differential gene expression based on reference gene normalisation and then Tukey HSD – one has to consider the background of complexity from which these transcripts are being amplified. As an example, the statement in line 353: "there were no changes in the expression levels of GS, nor PCS transcripts," - isn't justifiable using this methodological approach.

4.2) The discussion lines: 382-464 were well written and interesting given the manuscripts novel findings (as was the conclusion section). The subsequent, quite long, discussion presents the gene expression as independent from Yanitch et al paper but with persistent comparisons. The selection of these genes prevents this analysis from being independent. It might be potentially of interest to focus on the significant differences from their study, although the limited approach (not assessing all the transcripts) would make this a bit unconvincing.

5) Minor comments

We thank the reviewer for the valuable comments and we took them into account when revising the manuscript. Especially the emphasis on As speciation and non-protein thiol production was more focussed upon and the gene expression analyses were discussed in function of these processes. This really improves the focus and novelty of the current manuscript.

Hereafter, we provide a point-by-point reply to the reviewer's comments:

Response 1): We fully agree with the reviewer that presenting gene expression data on its own is insufficient to draw conclusions on the As tolerance mechanisms of willow species. That is why in our manuscript, we complemented these data with metabolic data on As speciation and non-protein thiol compounds to integrate the data set and not solely rely on the gene expression outcome.

Whereas the reviewer mentions that qPCR is not the standard to the field anymore, it is still the standard validation technology for NGS data sets. In addition to the work of Yanich et al. (2018), who presented transcriptome data of willow (other species) exposed during 30 days (1 time point) to As, the current manuscript followed a selection of genes over time, which provides kinetic insights (instead of a snapshot) in the plant responses to As exposure. However, we do agree with the reviewer that we should better present our selection of genes and might limit them to the genes related to the As uptake (phosphate transporters and aquaporins), speciation (arsenate reductases), and to the non-protein thiol production (glutathione and phytochelatin synthases) and sequestration into the vacuoles (ABC transporters) in the manuscript. Other genes, more in general related to the stress response are put into supplemental data. This will improve the focus of the manuscript towards the As speciation and thiol metabolism as suggested by the reviewer.

Response 2.1: In order to better highlight the focus of our manuscript, we rewrote part of the abstract to emphasise on the integration of the metabolic and gene expression data as was proposed by the reviewer. In addition, we emphasize on the kinetic experimental set-up for the experimental verification of the significance of particular transcripts, which is highly relevant in studying tolerance mechanisms and which will provide novel findings on the plant's coping mechanisms with As stress. Nevertheless, as we are not working with full transcriptome datasets, but focus on a selection, therefore, we rephrased all the parts where 'the genetic importance' is too much put forward.
Response 2.2: We do agree with the reviewer that making the relation between genetic engineering and phytoremediation based on our results is too ambitious. Therefore, as mentioned in the previous comment, we omitted the parts on 'genetic engineering'. Furthermore, we briefly addressed why it is important to understand As tolerance in relation to phytoremediation.

Response 3.1: We do agree with the reviewer that As speciation is very important when considering plant tolerance mechanisms to As exposure. At present, it is often overlooked in studies investigating As stress responses. Therefore, together with the other metabolic findings, we highlighted this in the integrated discussion in which gene expression is part of it to support the observations.

Response 3.2: As mentioned previously, qPCR is the golden standard to validate gene expression results that are obtained during NGS. Nevertheless, we do agree with the reviewer that it is only used for a selection of genes and it does not provide a full overview of the transcriptome response. Therefore, integrating the gene expression data from a selection of genes that are related to the metabolic data (see earlier comment) is a very powerful tool to understand the mode of action of plants to As stress. Furthermore, the present study was performed in a kinetical experimental setup (from short-term, i.e. 1 day, to long-term, i.e. 30 days exposure and time points in between), in which the dynamics of plant responses are addressed. Therefore, the discussion was rewritten in the light of this major suggestion focussing on the genes involved in the metabolic processes reported of As uptake, accumulation, speciation and synthesis of non-protein thiols.
Response 3.3: We understand that there might be a confusion to the selection of reference genes that were chosen to normalize the expression of the genes of interest. We wanted to make the selection for reference genes as extensive as possible based on studies in which Salix is exposed to abiotic stress. Therefore, we also included published data from Li et al. (mainly drought stress), because we saw in several studies that some reference genes might be unstable under As exposure, as well as in our data as provided in Supplementary Fig. 1. Using the Graynorm algorithm, the best selection of at least 3 genes is proposed, with best normalisation outcome proposed.
Response 3.4: Concerning the genes we used to find the optimal selection for normalisation, we provided a list in supplemental data (Supplementary Fig. 1). As reviewed, we also noticed that EF1-alpha in roots and ACT7 and alpha-TUB2 in leaves were differentially expressed genes in the study of Yanitch et al. (2017). However, we do not observe this in our data. In contrast to Yanitch et al. (2017) another Salix species was used and a different arsenic concentration, which might make a big difference in stability. In addition, we also performed the Graynorm algorithm to clarify our selection of reference genes.

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Response 5: All minor comments were addressed and changed according to the reviewer’s suggestions.

*Title Page

Integrative response of Arsenic Uptake, Speciation and Detoxification by *Salix atrocinerea*

Reply to reviewer's comments

Manuscript Number: Stoten-D-19-04903

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in Salix atrocinerea

The manuscript describes a pot trial treating hydroponically grown willow with Arsenate and then measuring the arsenic concentration and species in roots and leaves as well as measuring some specific non-protein thiolic compounds and some gene expression.

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~~Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*~~

Integrative response of Arsenic Uptake, Speciation and Detoxification by *Salix atrocinerea*

Alejandro Navazas[a,b], Sophie Hendrix[a], **Ann Cuypers**[a], **Aida González**[b,*,c*]

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Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

Highlights

- *Salix atrocinerea* accumulates and tolerates high As concentrations in its tissues.
- Inside the roots As V rapidly reduces to As III and accumulates.
- ~~*De novo* synthesis and accumulation of thiols occurs in As-exposed plantlets.~~
- As exposure decreased P and increased Ca and Fe concentrations in roots.
- Transcriptional regulation of As transporters and reductases are key for tolerance.
- ~~An early regulation of the flavonoid pathway alleviates As toxicity in leaves.~~
- *De novo* synthesis and accumulation of thiols occurs in As-exposed plants.

Abstract

Despite arsenic (As) being very toxic with deleterious effects on metabolism, it can be tolerated and accumulated by some plants. General genetic mechanisms responsible for As tolerance in plants, including *Salix* species, have been described in transcriptomic analysis, ~~although~~but further experimental verification of the significance of particular transcripts is needed. In this study, a *Salix atrocinerea* clone, able to thrive in an As-contaminated brownfield, was grown hydroponically in controlled conditions under an As concentration similar to the bioavailable fraction of the contaminated area (18 mg ~~K~~gkg^{-1}) for 30 days. At different time points, i.e. short-term and long-term exposure, biometric data, As accumulation, phytochelatin synthesis, non-protein thiol production and expression of target genes related to ~~arsenic tolerance~~these processes were ~~measured~~studied. Results showed that *S. atrocinerea* presents a great tolerance to As and accumulates up to 2,400 mg As ~~K~~gkg^{-1} dry weight in roots and 25 mg As ~~K~~gkg^{-1} dry weight in leaves. Roots reduce As V to As III rapidly, with As III being the predominant form of As accumulated in root tissues, whereas in the leaves it is As V. After 1 d of As exposure, roots and leaves show *de novo* synthesis and an increase in non-protein thiols as compared to the control. ~~Analysis~~Integrating these data on ~~transcript levels~~As accumulation in the

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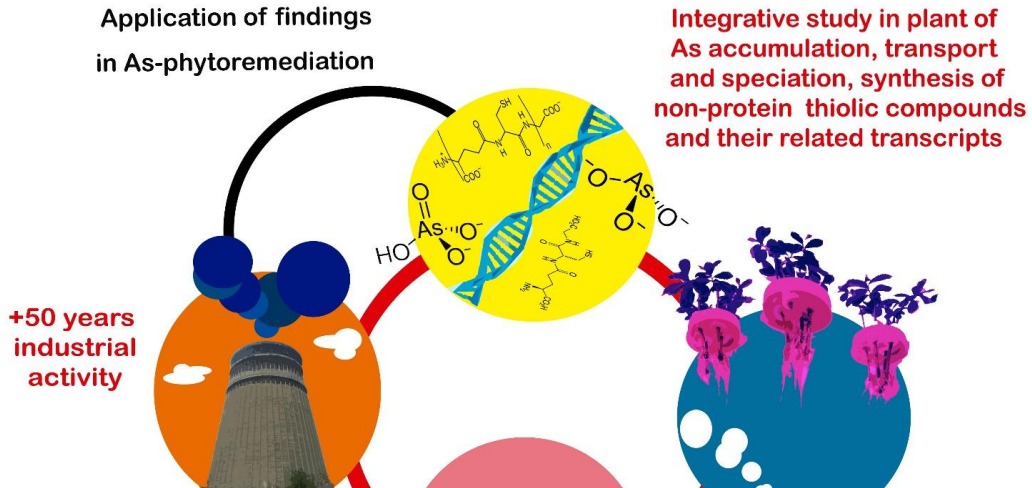
plant and its speciation, non-protein thiol production and the kinetic gene expression of related target genes ~~involved in As entry into the roots, thiol metabolism, storage in vacuoles and As stress related responses, highlighted~~ , a fundamental role is highlighted for ~~certain genes~~these processes in As accumulation and tolerance in *S. atrocinerea*. ~~This~~As such, this study~~, integrating the arsenic accumulation and speciation, non-protein thiol and gene response of *S. atrocinerea*~~ offers new ~~possibilities for genotype selection and genetic engineering~~insights in the ~~use~~plant tolerance mechanisms to As, which provides important knowledge for future application of high-biomass willow plants in phytoremediation~~.~~ of As-polluted soils.

Keywords: ***Salix*, arsenic, non-protein thiols, speciation, phytochelatins, gene expression, ~~abiotic stress~~**

Graphical Abstract



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1 Introduction

Arsenic (As) is a metalloid widely spread in the upper Earth's crust although at very low concentrations. The overall mean value of the total ~~arsenic~~As for different soils is estimated as 6.83 mg ~~K~~gkg^{-1} soil. ~~Nonetheless, arsenic~~However, As soil concentrations may range from 0.1 to more than 1,000 mg ~~K~~gkg^{-1} in some locations due to both anthropological and geological factors (Kabata-Pendias, 2010). ~~Arsenic~~Concerning its toxicity, As is the only known human carcinogen for which there is adequate evidence of carcinogenic risk for both exposure routes, inhalation and ingestion (Smith et al., 2009). Therefore, As has been defined as a group 1 carcinogen and is placed in the highest health hazard category by the international agency for research on cancer (Naidu et al., 2006). By the use of natural resources, humans release ~~arsenic~~As into the air, water and soil (Mandal and Suzuki, 2002). Sixty percent of the anthropogenic As emissions can be accounted to only two

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sources: Cu-smelting and coal combustion. Nevertheless, the application of herbicides, Pb and Zn smelting, glass production, wood preservation, waste incineration and steel production are also responsible for As emissions (Matschullat, 2000). According to the European Commission (2000), air contributes less than 1% of the total ~~arsenic~~As exposure since most of this emitted As ends up retained in the water and soils, making these the major sources of As exposure to humans.

Once inside the cell, ~~arsenic~~As toxicity depends on its speciation state. Arsenite (As III) has a high affinity for sulfhydryl groups found in the amino acid cysteine. As such, it inactivates a wide range of enzymes by disrupting protein structure and impairs the metabolism by preventing protein–protein interactions (Ehlrich, 1990). This affects many key metabolic processes in the cell such as fatty acid metabolism, glucose uptake and glutathione production (Paul et al., 2007; Ahsan et al., 2008; Wang et al., 2015). Arsenate (As V) is a phosphate analogue and can substitute inorganic phosphate affecting ATP synthesis and therefore interrupting the production of energy, carbon metabolism and nucleic acid synthesis (Singh et al., 2011; Spratlen et al., 2017). This can also negatively affect DNA repair and methylation and thus impact on gene expression (Reichard and Puga, 2010). Therefore, removal or ~~reduction~~lowering of ~~arsenic~~As concentrations from highly As-polluted soil and water is an environmental priority. Among the most eco-friendly cleanup technologies and opposite to traditional excavation and disposal in landfills, ~~emerges the~~ phytoremediation ~~that can cope with the above mentioned contamination challenge (Kidd et al., 2015).~~emerges. This green technology, already described more than two decades ago by Raskin et al. (1994~~) exploit~~), exploits the ability of certain plants species to accumulate metal(loid)s in their tissues, thus reducing their concentrations or attenuating their mobility in the environment

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~~(PilonSmits, 2005).~~, and therefore offering a solution to the above-mentioned pollution challenge (Pilon-Smits, 2005; Kidd et al., 2015).

It is well known that toxic metal(loid)s induce loss of plant biomass, among other deleterious effects, mainly associated with growth inhibition (Gill et al., 2015). Plants differ in As tolerance, from sensitive plant species like all major crops, to tolerant plants such as certain ecotypes of the grass

Holcus lanatus (Quaghebeur and Rengel, 2003), as well as hyperaccumulators like *Pteris vittata* (Chinese break fern), which can accumulate 2% of its dry weight as ~~arsenic~~As (Wang et al., 2002). However, hyperaccumulator species are usually limited by a low biomass production, which may pose serious restrictions to this cleaning procedure (Shelmerdine et al., 2009, Fernández et al., 2010). Some plant species and soil biota populations, usually autochthonous to polluted soils, are able to colonize and thrive in highly polluted environments, even when high concentrations of metals are found in their cells and tissues. This is the case of *Salix atrocinerea* (grey willow). ~~About~~So far, about 450 species of *Salix* worldwide have been described (Argus, 1995), with some of them reported as ~~efficient~~suitable in phytoremediation processes because of their high growth rate and deep-rooting traits (Kuzovkina and Quigley, 2005; Janssen et al., 2015). ~~Whereas~~Nevertheless, the focus on the use of *Salix* for ~~arsenic~~As uptake is still low because it is not a metal(loid) hyperaccumulating species. However, some investigations have highlighted its phytoremediation potential for As (Purdy and Smart, 2008; Puckett et al., 2012; Yanitch et al., 2017). In addition, complementary studies exploring the feasibility of high biomass plants to extract metals from polluted soils such as willow, concluded that the high biomass compensates for the moderate metal concentrations found in the aboveground tissues (Hammer et al., 2003; Ruttens et al., 2011).

Understanding ~~arsenic~~As tolerance in plants is ~~potentially~~ useful ~~knowledge~~ to know whether plants avoid As uptake and, thus, reduce ~~human arsenic~~the As intake by humans and the As-associated health problems (Song et al., 2010), or enhance As uptake and its removal by phytoremediation (Yang et al., 2012). To achieve this, it is necessary to study the As behavior from

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the soil to its accumulation in the aboveground plant tissues. Although ~~arsenic~~As is toxic and not essential for plants it is effectively absorbed through various transporters into the roots, mainly as As V, the most thermodynamically stable and hence dominant species in aerobic environments (Quaghebeur and Rengel, 2003). ~~Specific~~ As ~~V~~ such, specific As transporters ~~have been identified that~~ include the high affinity phosphate uptake systems for As V (Shin et al., 2004; Catarecha et al., 2007;

LeBlanc et al., 2013), while As III uses the silicon transporters ~~are responsible for As III influx~~ (Xu et al., 2015; Lindsay and Maathuis, 2016). Once inside the plant cells, a small amount may be transported to the xylem but the majority is reduced to As III by arsenate reductases (Ellis et al., 2006; Duan et al., 2007; Zhao et al., 2009). In this form, As can be exported back into soil, transported via the xylem to stem and leaves, or complexed with thiol-rich molecules like metallothioneins (MTs), glutathione (GSH) or, more stably, by phytochelatins (PCs) (Schmöger et al., 2000; Hartley-Whitaker et al., 2001; Dave et al., 2013; Batista et al., 2014). Then ~~this~~these As-PCs complexes can subsequently be transferred from the cytosol into the vacuole by ABC transporters for storage in order to prevent cell damage (Song et al., 2010). ~~This~~Therefore, this suggests that ~~PCs~~non-protein thiols (NPTs) compounds play an important role in decreasing As toxicity in plants~~.~~ and preventing its transport from roots to shoots.

Apart from the works on arsenic with *Salix* of Purdy and Smart (2008), Puckett et al. (2012), and more recently the extensive transcriptomic study by Yanitch et al. ~~(2017), only limited, but not integrated~~(2017) that have provided unequivocal useful information to understand the tolerance of *Salix* to As, still an integrative approach concerning the tolerance mechanisms of *Salix* to As is ~~available and this information has hardly any emphasis on~~needed. Besides, special attention needs to be paid to the speciation state of As~~.~~, since this determines its uptake and also its tolerance by the plant. In the current study, a *S. atrocinerea* clone, previously selected for its As accumulation~~,~~

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(unpublished data), was grown hydroponically in the presence of As V. Samples were harvested at different time points to kinetically study ~~arsenic~~As accumulation~~,~~ and its chemical speciation in ~~the different plant tissues,~~roots and shoots. In addition, the production of NPTs as well as the ~~synthesis of phytochelatins as a mechanism implied in As detoxification. In addition, gene~~ expression of the main transcripts ~~related to the entry of arsenic into the roots and its storage into vacuoles, thiol metabolism and As stress related responses was analyzed.~~involved in the genetic response behind As tolerance were also measured. Therefore, this study aims to describe the As uptake and ~~transport pathways as well as possible~~accumulation in *S. atrocinerea*, together with the changes in the mechanisms involved in ~~arsenic~~As tolerance ~~in grey willow~~ at different biological organization levels.

2 Material and Methods

2.1 Plant material and hydroponic culture conditions

~~2.1~~

Salix atrocinerea plants were selected from an *in vitro* willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). ~~Non lignified stem~~Stem cuttings of 15 cm length were placed on cellulose plugs in a hydroponic system containing 50 mL of 1/10 Woody Plant Medium

(pH 5.7) (Lloyd, 1981) with an aeration system to prevent lack of oxygen (Moreno-Jimenez et al., 2010). After 3 weeks of growth, 48 cuttings were exposed to 0 and 18 mg L^{-1} As. This As concentration was similar to that found at the exchangeable fraction of the Nitrastur brownfield soil. The As was added as sodium heptahydrate arsenate, $Na_2HAsO_4.7H_2O$~~.~~, since in this form the As is freely soluble.

Plants were cultured under a 12~~-~~ h light photoperiod and 22~~-~~ °C/18~~-~~ °C with 65% relative humidity. Light was provided by a combination of blue, red and far-red Philips Green-Power LED

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modules, simulating the photosynthetically active radiation (PAR) of sunlight. The PAR level reached 170 µmol m^{-2} s^{-1} at the plant apex level.

~~170 µmol m^{-2} s^{-1} at the plant apex level.~~

After 1, 3, 10 and 30 days (d), plants were carefully removed from beakers and roots exhaustively rinsed with tap water first, and 3 times with double de-ionized water at 4 °C. Leaves were rinsed only once in distilled water. To determine the influence of the treatments on plant growth, fresh and dry weights and lengths of roots and leaves were measured. Leaves and root samples of at least 3 different plants were analyzed individually for each treatment. Plant material was homogenized with liquid nitrogen and stored at −80 °C until further use.

2.2 Analysis of essential elements, arsenic and arsenic speciation

Nutrients, such as boron (B), calcium (Ca), iron (Fe), phosphorus (P), and zinc (Zn), together with As, were determined in leaves and roots of *S. atrocinerea*. For this, 100 mg of dry powdered samples were dissolved in 8 mL of 50% nitric acid solution (Sigma, Aldrich, USA) using a microwave at 800 W during 15 min (Multiwave3000, Anton Paar). The solutions were diluted up to 50 mL with ultrapure water and filtered through a 0.45 µm polytetrafluorethylene (PTFE) filter prior to their analysis. Plant samples were analyzed by ICP-MS (Agilent Technologies 7700 ICP-MS) using isotopic dilution analysis (IDA) as previously described (Gallego et al., 2015).

To determine the As speciation in leaves and roots, 100 mg of ~~finely-ground sample~~dry powdered samples were extracted in 2.5 mL of 0.3 M nitric acid solution at 95 °C for 90 min (Huang et al., 2012). The extracts were centrifuged at 3000 *g* during 15 min and the supernatants were

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filtered through a 0.45 µm PTFE membrane filter. The solutions were neutralized by the addition of NaOH. The ~~arsenic~~As species were separated through a mobile phase of 0.2 M EDTA dissolved in 2 M PBS (Phosphate Buffered Saline; pH 6.0) in a separation column with a 1260 Infinity HPLC coupled to a 7700 ICPMS (both from Agilent Technologies). Identification of ~~arsenic~~As species was confirmed by spiking real extracts with a mixture of standard solutions: As III, As V, monomethylarsenic acid (MMA), and dimethylarsenic acid (DMA).

2.3 Analysis of non-protein thiolic compounds

The extraction and analysis of non-protein thiols (NPTs) were carried out ~~in~~from 150 mg of fresh weight leaves and roots of *S. atrocinerea* following the protocol described by Fernández et al. (2012). The high-performance liquid chromatography (HPLC) separation was performed using a chromatograph Waters 600 (Waters Corporation) with a post-column derivatization with Ellman's reagent (Ellman, 1959). The sample (100 µL) was injected into a Kromasil 100 C18 5 µm (250 ~~×~~ × 4.6 mm) column (Scharlau) and eluted with solvent A (acetonitrile: H_2O, 2: 98 (v/v) to which 0.05% trifluoroacetic acid (TFA) was added) and solvent B (acetonitrile: H_2O, 98: 2 (v/v) also with 0.05% TFA). Samples were separated using a linear gradient (0–25% in 25 min and 25–50% in 5 min) of solvent B at 1.5 mL min^{-1} flow for 30 min. The derivatized thiols were detected at 412 nm using a Waters 996 photodiode array detector and the obtained peaks were identified by comparison with the standards of GSH and a mix of PCs. The quantitative changes in the thiol compounds observed were calculated by the integration of their peak areas at 412 nm ~~of absorbance~~ converted into nmol and quantified as GSH equivalents.

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2.4 Gene expression analysis

Isolation of RNA was carried out using the protocol described by Chang et al. (1993) with slight modifications. Frozen leaves or roots (100 mg) were homogenized with 550 µL of buffer containing 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP), 100 mM ~~TrisHCl~~Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermidine and 2% β-mercaptoethanol. Then, it was extracted twice by adding 550 µL of chloroform:isoamyl alcohol (24:1) and centrifuged at 14,000 *g* for 20 min at 4 °C. After addition of 10 µL LiCl (10 M), RNA was precipitated overnight at 4 °C and harvested by centrifugation at 14,000 *g* for 20 min at 4 °C. The pellet obtained was washed with 75% ethanol and resuspended in RNase free water. The concentration of RNA was determined spectrophotometrically at 260 nm using Nanodrop equipment (Isogen Life Science) and the RNA quality was tested using the Experion™ automated electrophoresis system (Bio-Rad). DNA was removed using a TURBO DNA-free Kit (Ambion) and the cDNA synthesis was performed using PrimerScript RT reagent Kit (Takara) with equal amounts of RNA input (1 µg). Finally, the cDNA was ten-fold diluted using a 1/10 dilution of TE (Tris-EDTA) buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at −20 °C.

~~PrimerScript RT reagent Kit (Takara) with equal amounts of RNA input (1 µg). Finally, the cDNA was ten-fold diluted using a 1/10 dilution of TE (Tris-EDTA) buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at −20 °C.~~

Reverse Transcription quantitative PCR (RT-qPCR) was performed with an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. Gene forward and reverse primers (***Supplementary Table 1***) were designed using Primer 3 (Untergasser et al., 2012), according to sequences of genes obtained in the *Phytozome* nucleotide database of the closely related species *Salix purpurea* v1.0, for which the whole genome has been sequenced (Goodstein et al., 2012~~). Genes measured were selected based on a transcriptomic study of Yanitch et al. (2017)~~

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addressing different interesting aspects of arsenic metabolism. Only), only for three genes, highaffinityhigh-affinity phosphate transporter (*HAPO4*), arsenite-inducible RNA-associated protein (*AIP-1*) and metallothioneins (*MT1A*), their sequences were obtained from willow reference sequences annotated at the NCBI (National Center for Biotechnology Information) (O'Leary et al., 2016). Their specificity was verified *in silico* using BLAST (http://www.arabidopsis.org/Blast/index.jsp). The genes measured (***Supplementary Table 1***) were selected based on different genetic aspects behind As tolerance (Konlechner et al., 2013; Puckett et al., 2012; Yanitch et al., 2017). The qPCR efficiency of the primers was determined using a standard curve consisting of a two-fold dilution series of a pooled sample. Only primers with an efficiency between 90 and 110% were used for qPCR analysis and their amplification specificity of the primers was validated by melting curves. PCR amplifications were done in a total volume of 10 µL containing 2 µL cDNA sample, 5 µL SYBR Green, 0.6 µL of primers (10 µM300 nM) and 2.4 µL RNase free water. The reaction cycle was as follows: 20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C. Gene expression was calculated relatively as $2^{-\Delta Cq}$, in which ΔCq represents each corresponding quantification cycle (Cq) value minus the minimum Cq value observed (Schmittgen, 2008). Gene expression was normalized with a factor suggested by the Graynorm algorithm (Remans et al., 2014) based on the expression of six reference genes from *Salix* selected from literature (Li et al., 2016; Zhang et al., 2017).

In roots *AFR2, OUT*normalization factor based on the expression of six reference genes from *Salix* selected from literature under As and other abiotic stresses (Li et al., 2016; Zhang et al., 2017). The 6 selected candidate reference genes, *α-TUB2, Alpha-tubulin 2; ACT7, Actin 7; ARF2, ADP-ribosylation factor 2; DNAJ, Chaperone protein DnaJ 49; EF1α, Elongation factor 1-alpha* and *OTU; OTU-like cysteine protease* (***Supplementary Table 1***) are also orthologs of genes in *S. purpurea*. The primer sequences, amplicon length, PCR amplification efficiency and correlation coefficient are shown in ***Supplementary Table 1***. To evaluate the stability of the 6 candidate reference genes (RG) at the transcript level under As exposure, the gene expression levels were determined by the average Cq values (***Supplementary Fig. 1***). In order to detect the stabilities of 6 candidate RGs, the best combination of RG for normalization of our transcripts of interest was suggested by the Graynorm algorithm (Remans et al., 2014). In roots *AFR2, OTU* and *EF1α* were the three most stable reference genes in all the sample sets according to the *GrayNorm* algorithm and the combination of the three was used for normalization. (***Supplementary Fig. 1A***). In leaves a different combination of genes than that obtained in roots showed the most stable pattern in all the sample sets,

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and therefore a combination of *α-TUB2*, ~~*OUT and ACT7 was used for*~~*OTU* and *ACT7* was used for normalization (***Supplementary Fig. 1B***). In both roots a leaves the suggestion selected by *GrayNorm* corresponded to the genes less affected by As exposure.

~~normalization.~~

~~Heat~~A principal component analysis and heat maps were constructed to compare expression levels between different genes and samples at different time points.

2.5 Statistical analysis

To evaluate the effects of ~~arsenic~~As toxicity in *S. atrocinerea* over the different time points on the measured variables, depending on the number of variables to compare a one-way or a two-way Analysis of variance (ANOVA) was performed. Log transformation was applied to approximate ~~normality~~ when it was necessary (e.g. to determine statistical significance of gene expression data, datasets were first log-transformed). Data normality was tested using the Shapiro-Wilk test, while homoscedasticity was verified via Bartlett's and Levene's tests. If data did not meet the normality assumption, a non-parametric Kruskall-Wallis test was used, followed by the Wilcoxon rank sum test. When the F ratio was significant ($p \leq 0.05$), Tukey's least significant difference test (HSD, $p \leq 0.05$) was employed to compare between individual means~~. A principal component~~ of different data groups (e.g. different treatments). In the gene analysis ~~(PCA)~~the previous was performed ~~using the log-transformed data of gene expression~~on both the normalized and the non-normalized data, although only the first are presented both were taking into account to establish the significance of the ~~different treatments and exposure periods.~~results. Results are expressed as the mean ± standard deviation of at least three independent replicates. ~~Data~~All data were analyzed using R (version 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae (version 1.2e4, ~~http://tarwi.lamolina.edu.pe/~fmendiburu).~~ http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level $p \leq 0.05$.

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3 Results

3.1 Plant growth and nutrient analysis

After 30 d of exposure, no external symptoms of phytotoxicity (data not shown) nor growth reduction, measured as dry weight, were observed between plants grown on control or As-containing medium (**Fig. 1**).

With regard to nutrient concentrations, total P concentration significantly decreased in roots from 10 d onwards in As-exposed plants as compared to controls, whereas in leaves the P concentration was lower in As-exposed plants as compared to controls at 3 and 10 d. However, P concentration in leaves was similar in both treatments after 30 d of exposure (**Table** ~~2~~1). Accumulation of Ca increased in ~~Asexposed~~As-exposed roots along the exposure time when compared to control conditions. However, in leaves a Ca decrease was observed in As-exposed plants, except at 3 d (**Table** ~~2~~1). Although the B concentration was slightly higher in roots of As-exposed plants, this increase was only significant at 3 d. In leaves ~~there was,~~ an increase in B concentration at 3 d was observed and this increase was maintained in As-exposed plants till the end of the experiment. For Zn concentrations, ~~an opposite accumulation trend was observed between roots and leaves of As-exposed plants at 1 d with~~there was an increase in roots and a decrease in leaves as compared to control conditions~~.~~ at 1 d of As exposure. However, no differences were observed in ~~Zn concentrations~~for this elements at other time periods. Fe concentrations were higher in roots of As-exposed plants throughout the experiment, whereas the opposite trend was observed in leaves (**Table** ~~2).~~ 1).

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With regard to the pH of the culture medium, we generally observed a decrease during the first 3 days of ~~exposure and~~the experiment. However, an increase from 10 d onwards was observed under As exposure as compared to control medium (**Fig.** ~~2).~~ **2A**).

3.2 Arsenic accumulation and speciation

We observed that roots of *S. atrocinerea* accumulated As concentrations ranging from 180 mg As ~~K~~gkg^{-1} dry weight at 1 d to more than 2,400 mg As ~~K~~gkg^{-1} dry weight after 30 d of exposure (**Table** ~~3~~**2**). In leaves, ~~arsenic~~As accumulation was much lower, although after 30 d of exposure, it reached an As concentration higher than that present in the culture medium (**Table** ~~3~~**2**). Although only As V was added to the culture medium, 4% of As III was observed in the medium after 1 d of exposure and it decreased to 0% by the end of the experiment (**Fig.** ~~2~~**2B**). Total ~~arsenic~~As concentration in the medium decreased 14 % due to plant uptake and no spontaneous ~~arsenic~~As speciation was detected in the medium when *S. atrocinerea* was not present (data not shown).

In plant tissues, the ~~arsenic~~As was detected as As III or As V, but no ~~arsenic~~As methylated species were observed (**Table** ~~3~~**2**). In roots, As V was more abundant (91%) during the first 3 d of exposure but after 10 d, As III was the predominant ~~arsenic~~As form (**Fig.** ~~2~~**2C**). In leaves, As V was the predominant speciation form observed throughout the experiment and As III was only detected at 3 d of exposure in low quantity (18%) (**Fig.** ~~2).~~ **2C**).

3.3 Analysis of non-protein thiolic compounds

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In roots of control plants, only GSH was observed and present at a 2-fold higher concentration than that observed in the As-exposed roots (**Table** ~~4). Nevertheless, already after 1 d of exposure~~3). However, in roots and leaves of *S. atrocinerea* plants exposed to As, changes in the concentrations of non-protein thiols (NPTs) ~~in roots and leaves~~ were already observed ~~in Asexposed plants~~ after 1 d and this trend was maintained over time (**Table** ~~4). Interestingly~~**3**). Besides, in the roots of ~~Asexposed~~As-exposed plants the total concentration of NPTs increased over time (up to 4.5-fold higher after 30 d compared to 1 d) and it was always higher than that observed in leaves. This NPTs increase in roots under As exposure was mainly due to an increment of *de novo* synthesized compounds such as PC_2, Cys-PC_2, PC_3, desGly-PC_3, Cys-PC_3 and also two unidentified thiolic compounds that were named TC_1 and TC_2 (**Table** ~~4). 3~~).

In leaves of control plants, the thiolic compounds GSH, desGly-PC_4, and TC_3 were detected (Table ~~4~~3), whereas under ~~arsenic~~As exposure we observed *de novo* synthesis of desGly-PC_2 at increasing concentrations over time. In both control and As-exposed plants, GSH concentrations in leaves were always higher than those observed in roots and were initially higher in As-exposed plants than in control plants (**Table** ~~4~~3). This increase in GSH, together with *de novo* synthesis of desGly-PC_2, accounted for a higher NPTs concentration at 1 d and 3 d in leaves of As-exposed plants. However, after 10 and 30 d of exposure, the total NPTs concentration in leaves of plants exposed to As did not significantly differ from that observed in leaves of plants grown under control conditions (**Table** ~~4). 3~~).

3.4 Gene expression

In general, the gene expression ~~patterns differed~~ pattern in roots between control and As-treated samples ~~in roots~~differed due to the prominent regulation of transcripts related to As transport, As V

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reduction to As III, thiol metabolism and vacuolar transports. In this way changes were observed in transcripts coding for the phosphate transporter (PHO1~~, the~~), aquaporins (NIP1, SIP1 and SILICON~~, the~~), boron transporter (BORON~~, the~~). As V reductase CDC25-like tyrosine phosphatase (~~arsenate reductase;~~ CDC25-1), ~~the proteins related to thiol metabolism such as~~ glutathione synthase (GS) and ~~metallothioneins (MT1A), the vacuolar transporters like CAX2 2, white brown complex~~ ABC transporter (WBABCT ~~and ABCG and the~~) (**Fig. 3**). This differential regulation was also accompanied by changes in transcripts for As stress-related proteins like cellulose synthase (CSA), arsenite inducible protein (AIP-1) and aminocyclopropane-1-carboxylate synthase (ACCS~~).~~

~~) ()~~. In leaves, regulation of the transcripts measured was not so noticeable as in roots and differences in gene expression between ~~leaves from~~ control ~~plants~~ and ~~leaves from Asexposed~~ As-exposed plant were due ~~to the expression of *NIP1, CDC25 1* and *CDC25 2* transcripts and genes of the flavonoid pathway coding for chalcone synthases (CHS1, CSH2 and CSH3), flavanone 3hydroxylase (F3H), flavonoid 3' hydroxylase (FLH), dihydroflavonol 4 reductase (2HFLR) and anthocyanidin reductase (ANR); and also the alterations in~~ mainly to the overexpression of ACCS (***Supplementary Fig. 2***). A heat map representation of the other transcripts measured in this study with a fold regulation lower than two can be found for both roots (***Supplementary Fig. 3A***) and leaves (***Supplementary Fig. 3B***).

To establish the kinetic gene expression ~~observed for *MT1A, BORON* and *PHO1*.~~

~~3.4.1 Principal Component Analysis~~

~~A~~ of related target genes, a principal ~~components~~component analysis (PCA) was performed using the ~~normalized~~ gene expression ~~levels data, allowing us to identify the correlations among the measured genes simultaneously.~~data obtained in leaves and roots of *S. atrocinerea* plants collected at 1, 3, 10 and 30 d. According to PCA component 1, roots of plants exposed to ~~arsenic~~As for 1 d showed the highest gene expression for *GS*,

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NIP1, *SIP1*, *CSA*, *AIP-1*, vacuolar transporter (*CAX2-2*), glutathione reductase (*GR*) and *ACCS*, whereas the expression of *BORON*, *PHO1*, *MT1A*, *CDC25-2*, *WBABCT*, vacuolar transporter (*ABCG*) was higher at later time points. On the other hand, PCA component 2 in roots (24% of the total variation) indicated that the increased expression of *ABCG*, clustered samples at 3 d, whereas the decrease in expression for phytochelatin synthase (*PCS*) and transcripts for a high-affinity phosphate transporter (*HAPO4*) at 3 d separated this group from the rest (**Fig.** ~~3).~~ **4A**).

In leaves, according to PCA component 1, the differential gene expression collected from plants growing under ~~arsenic~~As exposure at 1 and 3 d, had a more similar pattern than that observed at 10 and

30 d. Main differences were attributed to the up-regulation of genes involved in the flavonoid pathway *CHS3*, *CHS2*, *ANR*, *F3H*, *FLH*, *2HFLR*, *BORON* and *CHS1* expression at 1 and 3 d and of *CDC25-2* at 10 and 30 d. Component 2 (20% of the total variation), however, indicated a separation between the initial (1 d) and the last time point (30 d) from the intermediate points (3 and 10 d) as a consequence of lower *ACCS* and higher *ABCG2* expression in these intermediate points (**Fig.** ~~3).~~ **4B**).

~~**3.4.2 Differential gene expression in roots**~~

~~Concerning As transporters, transcripts encoding for PHO1 were 2-fold up-regulated at 1 d and kept steady throughout the experiment in roots of As-exposed willows (Fig. 4). Likewise, transcripts encoding for HAPO4 were 1.5-fold up-regulated after 10 and 30 d of arsenic exposure (Fig. 4) in comparison to control plants. Changes involving transcripts coding for arsenite transport related proteins were also detected. As such, *NIP1* transcripts were 12, 5.5, 5 and 4-fold up-regulated in the presence of As at 1, 3, 10 and 30 d, respectively (Table 5). For transcripts encoding for SIP1 a 5, 2 and 1.8-fold up-regulation was observed at 1, 3 and 10 d, respectively (Table 5). On the contrary, there was a 2-fold down-regulation at 3 d in *SILICON* transcripts (Fig. 4). Interestingly, *BORON* transcripts were~~

~~7-fold down regulated at 1 d and 2.7-fold up-regulated at 3, 10 and 30 d in response to As exposure (Table 5).~~

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Regarding genes involved in arsenic reduction, two transcripts of CDC25-like tyrosine phosphatase, an arsenate reductase, *i.e. CD25-1* and *CD25-2*, were measured in roots of As-exposed plants. The transcript levels of *CD25-1* showed an up-regulation of 2-fold at 1 and 3 d, 2.5-fold at 10 d and 3-fold at 30 d after As exposure (Table 5), whereas no differential expression was observed in the transcript levels for *CD25-2* (Fig. 4).

Concerning genes related to the thiol metabolism, expression levels of *GS* transcripts were 17, 5, 5 and 3-fold up-regulated after 1, 3, 10 and 30 d As exposure, respectively (Fig. 4). Transcripts for *GR*, an enzyme involved in the turnover of GSH, were only found to be 2-fold overexpressed at 1 d after arsenic exposure, whereas no changes were detected in glutathione transferase (*GST*) transcript levels. In As-exposed roots, there was a 1.5-fold up-regulation in *PCS* transcripts at 1 d after exposure and *MT1A* was 2-fold down-regulated under arsenic exposure at 1, 3 and 10 d. For transcripts related to the vacuolar accumulation of metals and PC-metal complexes, a 1.5 and 2-fold up-regulation of *CAX2-1* and *CAX2-2* was respectively observed under arsenic exposure at 1 d (Fig. 4). At 3 d there was a 1.8fold increase in *ABCG* transcripts (Fig. 4). In addition, a 2, 4 and 2-fold increase in *WBABCT* transcripts was detected at 3, 10 and 30 d, respectively (Table 5).

Studying the arsenic-stress related genes, an up-regulation of 40, 8, 16 and 11-fold was observed after As exposure for transcript levels of *AIP-1* at 1, 3, 10 and 30 d, respectively (Table 5). One transcript encoding an ACCS, producing the ethylene precursor ACC, was up-regulated throughout the experiment in the As-exposed plants, but transcript quantities decreased from 30-fold at 1 and 3 d to 9 and 8-fold at 10 and 30 d, respectively (Table 5). There were no changes in transcripts associated to the ethylene receptor protein ER under As exposure (Fig. 4). For cellulose synthesis, there was a 2fold down-regulation in transcript levels of *CSA* at 10 and 30 d after As exposure (Fig. 4).

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3.4.3 Differential gene expression in leaves

Changes observed in transcripts encoding for As-related transporters in leaves were minor and showed a different trend than that seen in roots. Transcripts associated to *PHO1* were 1.3, 1.4, 1.5-fold down-regulated in leaves of As-exposed willows at 1, 3 and 10 d, respectively (Fig. 4). However, transcript levels of genes encoding for HAPO4 remained unchanged (Fig. 4). For arsenite transportrelated proteins, transcript levels of genes encoding NIP1.1 were not differentially regulated in the presence of As and *SIP.1* expression was 1.5-fold up-regulated but only at 3 d (Fig. 4). In contrast to the roots, *SILICON* transcript levels were 1.4-fold up-regulated in As-exposed plants, but also only at 3 d. In leaves, *BORON* transcript levels were 1.8-fold up-regulated at 1 and 3 d and 1.5-fold upregulated at 30 d of As exposure (Fig. 4).

Regarding arsenic reduction in leaves, transcript levels for *CDC25-1* were 1.7-fold up-regulated from 3 d on throughout the experiment. In addition, *CDC25-2* transcript levels were 1.4-fold downregulated at 1 d but 1.7-fold up-regulated at 10 and 30 d (Fig. 4).

~~With regard to gene expression of the thiol metabolism related genes, there were no changes in the expression levels of *GS*, nor for *PCS* transcripts, which is in contrast to what is observed in roots. However, similar as in roots, no changes were detected in *GST* transcript levels (Fig. 4). Opposed to the pattern in the roots, *MT1A* transcripts were up-regulated under arsenic exposure throughout the experiment, with a 2, 1.7, 1.5, 1.9 fold increase at 1, 3, 10 and 30 d, respectively (Fig. 4). Vacuolar transport dynamics in leaves of As-exposed plants were represented by a 1.4, 1.7 and 1.5-fold upregulation of *ABCG* at 1, 3 and 10 d, respectively; and by a 1.6-fold up-regulation in transcript levels of *CAX2-1* at 1 d. No differences were observed in transcript levels of *CAX2-2* or *WBABCT* (Fig. 4).~~

~~For the As stress-related genes, a 2.3-fold up-regulation in transcript levels of *AIP-1* after 30 d As exposure was observed (Fig. 4). *ACCS* transcripts were 3, 2 and 4 fold higher up-regulated after As exposure at 1, 10 and 30 d, respectively (Table 5). In leaves, transcript levels of *ER* were 1.5-fold upregulated in *S. atrocinerea* plants after 30 d under arsenic exposure (Fig. 4). Concerning the cellulose biosynthesis, there was a 2-fold down-regulation in transcript levels of *CSA* after 30 d of As exposure (Fig. 4).~~

~~Transcript levels for genes encoding enzymes regulating the phenylpropanoid and flavonoid pathways were differentially expressed in leaves in response to As treatment (Fig. 4). For the initial starting point of the biosynthetic pathway, the levels of three transcripts encoding a chalcone synthase (CHS) were measured (*CHS1*, *CHS2* and *CHS3*): *CHS1* was 1.3 and 1.5-fold down-regulated at 10 and 30 d, respectively; whereas *CHS2* transcripts were 1.6 and 1.4 fold up-regulated at 1 and 3 d, respectively. Also *CHS3* transcripts were 1.6 and 1.3 fold up-regulated at 1 and 3 d, respectively; but 2.5 fold down-regulated at 10 and 30 d. For the remainder of the pathway, transcript levels of *F3H* were 1.5 fold up-regulated at 1 d and 1.5 fold down-regulated at 10 and 30 d. A similar trend was observed for *FLH* and *2HFLR* transcripts. No changes in the flavonol synthase transcripts (*FLS*) were detected under As exposure. With regard to transcript levels of key enzymes regulating the production of anthocyanins, it was observed that anthocyanidin synthase transcripts (*ANS*) were 2-fold downregulated at 10 d and transcript levels of the gene encoding for ANR were 1.5-fold downregulated at 10 and 30 d in As-exposed leaves of *S. atrocinerea*.~~

4 Discussion

The total pollutant concentration of a certain element in the soil is not a representation of the amount ~~of metal~~ that is available (exchangeable) for the ~~plants~~plant uptake, neither a good indicator to establish plant toxicity limits. Therefore, ~~for a successful~~when phytoremediation ~~process~~processes will rely on the use of certain plant species that tolerate and accumulate high concentrations of metal(loid)s, it is very important that the studies conducted in the laboratory under controlled conditions, on which the basic physiological knowledge is set, are based on well reflected pollutant concentrations. Many hydroponic studies have used higher As concentrations than those found in soil solution, and their environmental relevance has been questioned (Fitz and Wenzel, 2002). According to this, some authors propose that hydroponic cultures should include As doses in the range of 0 – 10 µM to allow the extrapolation of the results to As-polluted soils (Moreno-Jimenez et al., 2010).

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Nonetheless, the fact that *S. atrocinerea* plants used in this study already grow on ~~the Nitrastura~~ brownfield with an As exchangeable fraction of 18 mg ~~K~~g~~k~~g^{-1} (data not shown), suggests that the As dose could be increased for this hydroponic assay. Furthermore, this As concentration matches that recommended in previous hydroponic studies with willow (Purdy and Smart, 2008) and it has already been used in analyzing differential As gene expression under hydroponic conditions (Puckett et al., 2012). Although some authors have reported that willows have the capability to translocate As from roots to aboveground tissues (Tlustoš et al., 2007; Puckett et al., 2012; Sylvain et al., 2016~~), in our case,~~) the As accumulation in leaves ~~did~~does not reach those quantities ~~accumulated by~~ present in hyperaccumulating species like *Pteris vittata* (Caille et al., 2004~~) -~~), as it was our case, but the phytoremediation potential is compensated with a higher biomass ~~as it has also been proposed by Jiang~~(Meers et al~~. (2015) for other species.~~., 2007, Witters, 2009). Furthermore, after 30 d of As exposure, *S. atrocinerea* did not show any phytotoxic symptoms and was capable of accumulating a higher As concentration than that present in the culture medium, showing therefore a bioaccumulation factor higher than 1~~. In its natural habitat, *S. atrocinerea*~~, which is ~~also able to thrive in a brownfield environment under~~an added value for the ~~presence of other metal(loid)s at toxic concentrations (e.g. Zn, Pb). This multi metal(loids) tolerance, together with a great biomass, highlights the potential of *S. atrocinerea* in~~ phytoremediation of ~~polluted environments.~~ As,

It ~~is well known~~has been reported that exposure to toxic metalloids, such as As, can disturb the nutrient profile of the plant and hence lead to toxicity (Lou et al., 2010~~) and also that arsenate uptake and tolerance to the induced toxicity is intimately linked to phosphate nutrition. Some authors propose that an increased phosphate accumulation will lead to a reduced arsenate uptake (Meharg and Macnair, 1994). Therefore, a higher cytoplasmic phosphate accumulation may enable phosphate to compete more effectively with arsenate for ATP synthesis, decreasing arsenate toxicity within the plant cells. Nonetheless, different studies have seen a reduction of phosphate uptake in plants exposed to arsenate, which indicates that arsenate uptake occurs via the phosphate transporters and can also replace phosphate groups in biomolecules (Wang et al., 2002; Patra et al., 2004). Our results showed that *S. atrocinerea* does not rely on P accumulation to prevent As toxicity and that As uptake decreased P accumulation in roots and its subsequent transport to the leaves. We can suggest that Ca accumulation observed in the roots and its reduced translocation to the leaves is a response to As accumulation in the roots of *S. atrocinerea*. Ca is an essential plant macronutrient,~~), and also that As V uptake and tolerance to its induced toxicity is intimately linked to phosphate nutrition. In the soil, As is mainly present in its As V form (Cordos et al., 2006) and once it is in contact with the roots, As

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V can enter via phosphate transporters (Maciaszczyk-Dziubinska et al., 2012). Therefore, changes in transcripts encoding for As V-related transporter proteins could be expected and, in our case, the As V added to the hydroponic solution caused a differential regulation of transcripts for phosphate transporters. The up-regulation of *PHO1* in roots of *S. atrocinerea* from the onset of the As exposure (**Fig. 3**) and that observed at 10 and 30 d of transcripts encoding for a high-affinity phosphate transporter protein (*HAPO4*) (***Supplementary Fig. 3A***), relate to the first lower and then similar P concentrations in roots of As-exposed *S. atrocinerea* as compared to non-exposed plants (**Table 1**). It has been suggested that reduced uptake of As V is a well-known mechanism of As V resistance employed by many plant species, which is achieved through a reduction of the phosphate/arsenate uptake system in resistant plants (Meharg and Hartley-Whitaker, 2002). Moreover, it is thought that this reduction decreases As V influx to a level at which the plant can detoxify As, presumably by constitutive mechanisms (Catarecha et al., 2007). However, according to our results of As accumulation and a lower concentration of P under As exposure as compared to the control condition, it can be suggested that the transcript upregulation of phosphate-related transcripts in roots is based on preventing As V competition and avoiding P deprivation. Therefore, since As does accumulate at high concentrations in roots of willow, a more effective detoxification mechanism than inhibition of phosphate transporters as seen in other plants would be necessary in *S. atrocinerea*. After 30 d, As concentration in leaves of *S. atrocinerea* reached levels higher than toxicity levels established for non-tolerant plants (1-20 mg As kg^{-1} dry weight; White and Brown, 2010). Under these conditions, a differential regulation of As V-related transporters in leaves of *S. atrocinerea* was observed. The decrease in *PHO1* transcripts at 1, 3 and 10 d (***Supplementary Fig. 3B***), is a similar response to that of As resistant species, where avoiding As uptake in leaves by reducing phosphate uptake constitutes a tolerance mechanism (Meharg and Hartley-Whitaker, 2002). However, at 30 d the down-regulation ceased and there were no differences in transcript levels of *PHO1* compared to those observed in leaves of control plants and it matched with a similar P concentration in leaves of both treatments (**Table 1**). According to the Ca concentrations observed in plants of *S. atrocinerea* (**Table 1**), it can be suggested that Ca accumulation in the roots and its reduced translocation to the leaves is a response to As accumulation. Ca is an essential plant macronutrient and it plays an important role in cell wall and membrane stabilization and regulates nutrient uptake as well as different stress responses (Ahmad et al., ~~2015). Rahman et al. (2015) suggested that Ca supplementation improves the tolerance of rice seedlings to As by reducing As uptake and enhancing their antioxidant defense. More recently, Ji et al. (2017) have identified a Ca-dependent protein kinase (CPK31) responsible for As III tolerance in *Arabidopsis*. CPK31 is an interacting protein of NIP1.1, an aquaporin involved in As III uptake. Similarly to the *nip1.1* mutants, the loss-of-function mutants of *CPK31* improved the tolerance against As III but not As V, and accumulated less As III in roots than that of the wild-type plants. This might indicate that this Ca-dependent CPK31 protein might be a target to regulate NIP1.1 for As III tolerance.~~

~~In multiple studies~~2015), including an increase of the antioxidant defense under As exposure and reducing As uptake (Rahman et al., 2015). In multiple studies, it has been shown that micronutrient accumulation is affected by As exposure, but it can also have an impact on As uptake and hence As toxicity (Srivastava et al., ~~2017).~~2017). It has been proposed that B channels might

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have a role in As transport into the cell (Yanitch et al., 2017), which is also reflected in our results with increased *BORON* transcript levels in roots (**Fig. 3**) and leaves (***Supplementary Fig. 3*B**). Furthermore, boric acid transporter NIP5.1 from *Arabidopsis* is also permeable to As III (Mitani-Ueno et al., 2011), and our data showed that B accumulation in plant tissues changes along the time of exposure to As (**Table 1**), with *BORON* transcripts 5-fold down-regulated at 1 d (**Fig. 3**), when As III concentration in the medium was the highest (**Fig. 2B**). In leaves, *BORON* transcripts are induced at 1 and 3 d in response to As (***Supplementary Fig. 3*B**), coinciding at 3 d with the highest B concentration (**Table 1**). Whereas Zn is described as an indispensible micronutrient, which mitigates As toxicity by modulating ROS and the antioxidant function in plants (Das et al. 2016) or ~~improve~~by improving the thiol metabolism (Srivastava and Srivastava, 2017), no major changes were detected in Zn concentration~~, appart~~ apart from the ~~decrease~~increase at 1 d~~, highlights the tolerance of *S. atrocinerea* to~~ in As~~.~~ exposed plants. With regard to Fe, our data showed that Fe translocation to leaves was more affected by As than any of the other elements, with an increased Fe concentration in roots exposed to As, whereas in leaves ~~a Fe decrease was observed~~it decreased (**Table ~~2~~1**). Shaibur et al. (2008) described that one of the symptoms of As toxicity is the formation of Fe plaques in roots and, as also seen in our case, Fe:P ratios in the roots of the As-exposed plants were higher than those observed in the control roots. This suggests that, in the liquid culture medium, As may have been adsorbed with Fe on the surface of the roots, forming Fe-As plaques. Thus, the iron plaque formed on the root surface will act as a natural As barrier and reduce As uptake by the plant and its translocation~~.~~ to shoots.

Besides the impact of other elements in the medium on As uptake, the speciation of As also plays an important role in the accumulation of As and tolerance by the plant (Moreno-Jimenez et al.,

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2010). The ~~arsenic~~As was added to the culture medium as As V and after 24 h a 4% reduction to As III was observed (**Fig.** ~~2~~**2B**). This chemical reduction can be attributed to metabolic activities of *S. atrocinerea* since no speciation was detected when the plant cuttings were not present in the culture medium. ~~It is known that~~For this observation, two possible explanations can be given. On one side, plants might induce changes in the pH and in the redox potential of the culture medium~~, affecting the As~~ as it was observed in this study (**Fig. 2A**), and those changes might affect the speciation~~. Furthermore, it is also known that arsenite efflux from the plant to the medium can be linked to the proton gradient across the plasma membranes or dependent~~ of ~~the plant metabolism (e.g. arsenite efflux by yet unidentified transporters) (Xu et al.,~~ As. For an example, it~~2007; Park et al., 2016). It~~ has been proposed that protons released from organic acids (R-COOH) and excreted by plant roots may contribute to the reduction of As V to As III, while increasing the pH as the process consumes H^+ (Park et al., 2016). Interestingly, only ~~arsenate~~As V was detected after 30 d ~~matching~~ and it matched with the highest pH value~~,~~ in the medium; whereas the highest ~~arsenite~~As III concentration ~~in the medium~~ was detected at ~~24 h~~1 d, when the pH was the lowest (**Fig.** ~~2). Possibly,~~**2A**). Therefore, another possible explanation for the presence of As III in the medium is a direct efflux of As III from the plant to the medium that can be linked to the proton gradient across the plasma membranes or dependent of the plant metabolism (e.g. direct As III from plant cells to the medium) (Xu et al., 2007; Park et al., 2016). Taking transcriptional regulations into account, since willow plants were able to induce the occurrence of As III in the medium, differences in transcript levels of genes encoding for As III transport were expected in roots of As-exposed plants.

In our case, we observed a noticeable up-regulation of the transcripts encoding the aquaporin NIP1.1, reported for As III uptake into the roots (Ma et al., 2008), and in transcripts for SIP1 at 1 d of As exposure (**Fig. 3**). This up-regulation diminishes over time, probably as a consequence of a very

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active As V reduction to As III during the first days of exposure, with a lot of free As III initially in the cytoplasm. This transcript up-regulation for As III transporters in roots, suggests that As V reduction has occurred even before its entry into the roots, which is supported by the presence of 4% of As III in the medium at 1 d of exposure (**Fig. 2B**), and which coincides with the highest up-regulation of *NIP1* and *SIP1*. In leaves, where As was mainly present as As V, no changes in transcript levels for the aquaporin transcripts were observed (***Supplementary Fig. 3B***).

Once inside the cell, since As V has no affinity for the "-SH" groups in the PCs, the first step in As detoxification is As reduction (Finnegan and Chen, 2012). The main mechanism for As V reduction is the presence of As V reductases where GSH acts as electron donor (Dhankher et al., 2002). Arsenate reductases are believed to have evolved from the CDC-25 (cell division cycle) dual-specificity tyrosine phosphatases (Duan et al., 2007). Based on homology with the yeast As V reductase, ACR2P, Bleeker et al. (2006) identified a CDC25-like plant candidate and showed that it had arsenate reductase activity like it was also observed in other assays (Dhankher et al., 2006). ~~arsenite~~In our study, As V reduction to As III was observed in roots right after As uptake with an increasing As III concentration in root tissues from 9% to 70% by the end of the study (**Fig. 2C**). This coincides with the *CDC25-1* up-regulation in roots of plants exposed to As (**Fig. 3**), whereas no changes were observed for *CDC25-2* (***Supplementary Fig. 3A***).

Another mechanism to reduce As V in the plant is through a non-enzymatic reduction, where GSH is implied, but this process is relatively slow, so according to the NPT data we can attribute the large up-regulation observed in *GS* transcripts along the As-exposure time to PC production as a detoxification mechanism. This was reflected by the increased NPT concentrations in *S. atrocinerea* roots after As uptake (**Table 3**). Our results showed that although there was a clear up-regulation of *GS* transcripts in roots (**Fig. 3**), GSH concentrations of As-exposed plants remained constant over time and lower than those in the roots of control plants (**Table 3**). However, since PCs use GSH as a building substrate, the decreasing concentrations of GSH are consistent with its use in PCs or other NPTs. This fast increase in NPT concentrations and the As III presence in the roots, support our

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observations of As speciation in the medium; where it seems that As III efflux by roots occurs right after ~~arsenate~~As V uptake and that this efflux diminishes once the ~~arsenite~~As III is complexed with thiols and stored in the vacuoles ~~(Zhao et al., 2009).~~. Therefore, an increase in NPTs under increased As III presence points towards an As-PC complex formation possibly leading to less As III efflux. In support to this explanation, Raab et al. (2005) found that in sunflower roots the amount of As not complexed by thiols fell from 90% of total As after 1 h exposure, to 43% after 4 d of exposure. ~~Similarly, in our case we observed an increase in As III in roots but also an increase in thiols, pointing towards As-PC complex formation possibly leading to less As III efflux.~~

~~With each new transcriptomics experiment, massive quantities of information on gene expression are generated with the purpose to produce a list of candidate genes for functional analyses. Yet an effective strategy remains elusive to prioritize the genes on these candidate lists. Based on the study of Yanitch et al. (2017), we have selected a set of genes related to different aspects of As metabolism~~

~~(uptake and transport across plant cells, thiol metabolism, storage in vacuoles and stress related responses) based on the expression pattern previously observed and the design of efficient primers to measure gene expression.~~

~~In the soil arsenic is mainly present in its As V form (Cordos et al., 2006) and once it is in contact with the roots, As V can enter via phosphate transporters and As III via aquaporins (MaciaszczykDziubinska et al., 2012). Therefore, changes in transcripts encoding for As-related transporter proteins could be expected. In our case, As V was added to the hydroponic solution and a differential regulation of transcripts for *PHO1* was observed. An up-regulation of *PHO1* in roots of *S. atrocinerea* (Fig. 4) might indicate that As V enters via these transporters from the onset of the As exposure. It has been suggested that reduced uptake of arsenate is a well-known mechanism of arsenate resistance employed by many plant species, which is achieved through a reduction of the phosphate/arsenate uptake system in resistant plants (Meharg and Hartley-Whitaker, 2002). Moreover, it is thought that this reduction decreases arsenate influx to a level at which the plant can detoxify As, presumably by constitutive mechanisms (Meharg and Macnair, 1994). This is also supported by the study of Catarecha et al. (2007) who identified an arsenate-tolerant mutant of *A. thaliana*, *pht1.1-3*, which harbours a semidominant allele encoding for the high-affinity phosphate transporter (PHT1.1). This allowed the *pht1.1-3* mutant to decrease arsenate uptake in the short-term and increase As accumulation over a longer period since mutant plants showed a better growth and produced more biomass. Taking this information into account, together with our accumulation data and the fact that transcripts encoding for a high-affinity phosphate transporter protein (HAPO4) were up-regulated at 10 and 30 d of arsenic treatment, the idea of *S. atrocinerea* as an As-tolerant plant is reinforced. Moreover, As accumulation did not cause phytotoxic symptoms in the plants. The fact that *S. atrocinerea* does not show an As-excluding behavior, is an added value for phytoremediation. Thus, since As is accumulated in plant tissues of willow, a more effective detoxification mechanism than inhibition of phosphate transporters would be necessary in this plant as suggested by Yanitch et al. (2017).~~

In addition, although PCs were synthesized in *S. atrocinerea* in response to As exposure and their concentration increased over time in roots (**Table 3**), ~~Arsenic~~ accumulation in leaves of ~~*S. atrocinerea* revealed that after 30 d the level for toxicity (1-20 mg As Kg^{-1} dry weight) established by White and Brown (2010) for non-tolerant plants was exceeded. In these conditions, a differential regulation of~~

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arsenate-related transporters in leaves could be expected. In this way, a down-regulation in the expression of *PHO1* transporter was observed, indicating a similar response to that observed in arsenic resistant species (Meharg and HartleyWhitaker, 2002), where avoiding As uptake is a tolerance mechanism. Since at 30 d the downregulation ceased and there were no differences in transcript levels of *PHO1* to those observed in leaves of control plants, this indicates the need to absorb P to maintain the P homeostasis in the plants. This is reflected in our data, since after 1 d the P concentration in leaves of As exposed plants is lower than that under control conditions, whereas at 30 d no differences were observed. According to Yanitch et al. (2017) who also observed a transcriptional up regulation for *PHO1* in roots and a *PHO1* downregulation in leaves, this suggest that higher *PHO1* transcript levels in roots can counteract arsenate competition and avoid P deprivation.

Since willow plants were able to induce the occurrence of arsenite in the medium, differences in transcript levels of genes encoding for arsenite transport were also expected in roots of As exposed plants. Especially an up regulation in transcripts encoding the aquaporin NIP1.1 that has been reported previously as responsible for arsenite uptake into the roots (Ma et al., 2008) was observed (Table 5). This suggests that arsenate reduction has occurred even before its entry into the roots, which is supported by the presence of 4% of As III in the medium at 1 d of exposure (Fig. 2), and which coincides with the highest up regulation of *NIP1.1*. Another aquaporin, *SIP.1* was also up-regulated up to 10 d. In leaves, where As was mainly present as arsenate, no changes in transcript levels for the aquaporin *NIP1.1* was observed (Fig. 4). These results differ from those of Yanitch et al. (2017) as was also seen for *SILICON* and the *SIP.1* regulation pattern. Despite arsenite uptake, As metabolism is different in different species and growth conditions and a different gene expression pattern can be expected (Ma et al., 2008; Li et al., 2009).

Interestingly, in a study of Yanitch et al. (2017) it has also been proposed that B channels might have a role in As transport into the cell, which is also reflected in our results with increased *BORON* transcript levels in roots and leaves (Table 5 and Fig. 4). This is interesting since it has been shown that boric acid transporter NIP5.1 from *Arabidopsis* is also permeable to arsenite (Mitani-Ueno et al., 2011) and our data showed that B accumulation in roots is increased under arsenic treatment. It is interesting that *BORON* transcripts in leaves are induced at 1 and 3 d in response to arsenic treatment (Fig. 4) coinciding at 3 d with the highest B concentration (Table 2).

Once inside the cell, since As V has no affinity for the "-SH" groups in the PCs, the first step in As detoxification is As reduction (Finnegan and Chen, 2012). The main mechanism for arsenate reduction is the presence of arsenate reductases where GSH acts as electron donor (Dhankher et al., 2002). Arsenate reductases are believed to have evolved from protein tyrosine phosphatases

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~~(PTPases), which include the CDC 25 (cell division cycle) dual specificity tyrosine phosphatases (Duan et al.,~~

~~2007). Based on homology with the yeast As(V) reductase, ACR2P, Bleeker et al. (2006) identified a CDC25-like plant candidate and showed that it had arsenate reductase activity like it was also observed in other assays (Dhankher et al., 2006). In both studies, they focused on maximizing As uptake and increasing translocation from roots to shoots with a phytoremediation purpose. In our study, arsenate reduction to arsenite was observed in roots right after As uptake with an increasing arsenite concentration in root tissues from 9% to 70% by the end of the study (Fig. 2). This coincides with the *CDC25 1* up regulation in roots of plants exposed to As (Table 5), whereas no changes were observed for *CDC25 2* (Fig. 4). In the study by Dhankher et al. (2006), As accumulation in shoots of *Arabidopsis* was enhanced, through enhanced As V translocation in knockdown *ACR2* lines. Therefore, *CDC25 1* offers a target to increase As translocation in *S. atrocinerea*, where by decreasing As reduction and its further complexation to PCs and sequestration into the vacuole, it can be transported and accumulated in aboveground tissues.~~

~~Another form to reduce accumulated arsenate in the plant is through a non enzymatic reduction where GSH is implied. Since this process is relatively slow, we can attribute the large up regulation observed in *GS* transcripts to PCs production as a detoxification mechanism. This was shown by the increased total thiol concentrations in *S. atrocinerea* roots after As uptake (Table 4). Our results showed that although there was a clear up regulation of *GS* (Table 5), GSH concentrations in roots of As exposed plants remained constant over time and lower than those of the control plants, as also reported by Hasanuzzaman et al. (2017). In addition, although phytochelatins were synthesized as a detoxification mechanism under As exposure and their concentration increased over time in roots,~~ there was only a slight increase, in transcripts coding for PCS at 1 d, similar to the behavior observed in transcripts of *GR* (***Supplementary Fig.*** ~~4~~3A). This suggests that the induction of *PCS* expression is unlikely to play a significant role in regulating PC biosynthesis (Cobbett 2000). ~~It is known~~This agrees with Rea el al. (2004), who reported that PCS enzymes are expressed constitutively at relatively high levels and are generally unaffected by exposure of cell cultures or plants to heavy metal(loid)s ~~(Rea et al., 2004).~~ As ~~reported~~described in other plant species, ~~phytochelatin~~PC-based sequestration is considered to be essential for ~~arsenic~~As tolerance, where hypertolerant ecotypes

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present higher ~~PCs concentration~~ PC concentrations under ~~arsenic~~As exposure compared to ~~nontolerant~~non-tolerant ecotypes ~~and in both cases the plants exhibited arsenic hypersensitivity when PC synthesis was blocked by buthionine sulfoximine (BSO)~~ (Meharg and Hartley-Whitaker, 2002; Schat et al., 2002; Fernández et al., 2013). The ~~big~~7-fold increase in ~~NPTs~~ NPT concentration observed in our case in roots of willow after 1 d of As exposure, ~~could be~~it is then related to a fast ~~arsenate~~As V reduction to ~~arsenite in the plant~~As III and to the need to synthetize ~~longerchain~~longer-chain PCs to chelate ~~arsenite.~~the increasing concentrations of As III, and therefore maintaining cellular stability. As it has been reported~~,~~ by Sharma et al. (2016), longer chain PCs contribute to a more effective cellular detoxification due to a higher metal-binding capacity and formation of more stable ~~Ascomplexes~~As-complexes that will prevent the interaction with ~~sulphydryl~~sulfhydryl groups of other proteins and hence affect the metabolism ~~(Sharma et al., 2016). .~~

We also observed that in the roots, the organ where more As was accumulated, a greater PCs synthesis was present than in leaves where As accumulation is lower. Another interesting observation of our study is the presence of many unknown thiol products. This is in accordance with the results of Li et al. (2004) in *Arabidopsis*, where ~~arsenic~~As exposure resulted in the expression of many unknown thiol products, whereas cadmium induced ~~greater~~higher increases in traditional PCs (PC_2, PC_3, PC_4).

Most of the ~~arsenic~~As speciation experiments described in literature propose As III as being the predominant As form in leaves (~~Zhang et al., 2009;~~ Kertulis et al., 2005; Zhang et al., 2009; Yan et al., 2012; Park et al., 2016~~), however~~). However, in our case As V was the main As species observed in leaves throughout the experiment (**Fig.** ~~2). A possible explanation for the lack of arsenite observed in leaves could be that, despite~~**2C**). Despite As exposure caused *de novo* synthesis of desGly-PC_2 and

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778 ~~an~~the increase of desGly-PC_4 in leaves of As-exposed plants, a possible explanation for the lack of As
779 III ~~is~~observed in leaves, with the exception at 3 d, could be attributed to the relatively low
780 concentration of As in leaves as compared to roots (**Table 2)** , which might require a less effective
781 NPT response. Another explanation could be related to the stability of the As-thiol complexes present
782 in leaves, where As III could be mainly bound to GSH~~.~~ which was present at higher concentrations
783 than in roots, and represent the main NPT in leaves (**Table 3**). Since As III – GSH complexes are less
784 stable that As III – PCs, a dissociation of these complexes could take place with the consequent re-
785 oxidation of As III to As V (Bluemlein et al., 2009; Zhao et al., 2009). ~~This~~ In relation to this, the As
786 V presence in leaves of *S. atrocinerea* might explain the need for ~~constant~~the up-regulation of the
787 CDC25-like tyrosine phosphatases pathway ~~(Fig. 4). Taking into account that speciation~~observed at
788 10 and ~~distribution of arsenic in the~~30 d, when As increased in shoots (***Supplementary Fig. 3B***), and
789 exceeded plant ~~can provide important information and help to understand the mechanisms for arsenic~~
790 ~~accumulation, translocation,~~toxic limits (White and ~~transformation as noted by Zhang et al. (2002),~~
791 ~~our results suggest that the arsenic tolerance mechanism of *S. atrocinerea* relies on arsenate reduction~~
792 ~~in roots but not in leaves. Limited As translocation, and its presence as As V in leaves could be~~
793 ~~related to *S. atrocinerea* tolerance to As, with an effective As V reduction and complexation of As III~~
794 ~~to non protein thiolic compounds and further sequestration into the root vacuoles.~~Brown, 2010). In
795 contrast to PCs that rely on enzymatic synthesis, MTs, which are also important metal chelators in
796 plant cells, ~~MTs are encoded by genes and thus are~~are direct products of mRNA translation (Anjum
797 et al., 2015). Examples of MTs ~~examples~~ induction under metal exposure in *Salix* have been
798 described by Konlechner et al. ~~(2013). However, the metal~~(2013), and it is known that metals like Zn
799 ~~binds~~or Fe bind to MTs with the highest affinity ~~and also Fe and other essential metals~~ (Blindauer et
800 al. 2010). Therefore, in this study according to the differential transcription pattern of *MT1A* between

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roots and leaves, due to the Fe accumulation in roots and its reduction in leaves, it ~~might~~could be that *MT1A* induction in leaves (***Supplementary Fig.* 3B**) corresponds to the need of supplying enough Fe ~~in leaves~~ and that this up-regulation is not ~~be~~ involved in direct As chelation. ~~Anyway~~However, its induced expression in leaves forms part of the response to the As-induced stress.

Once ~~arsenate~~As V is reduced to ~~arsenite~~As III and complexed to NPTs to limit its toxicity, these complexes ~~could be~~are taken up by ABC transporters and stored in the vacuole. ~~It is known that As III-PCs complexes have a low stability and their storage into the acidic environment of vacuoles can improve their stability and counteract a redox-based destabilization of the complex (Schmöger et al., 2000).~~ ABC transporters constitute one of the largest protein families, present in organisms ranging from bacteria to humans, and have been identified as transporters involved in detoxification processes by transporting metal(loid)-PC complexes (Kang et al., 2011). It is known that As III-PCs complexes have a low stability and their storage into the acidic environment of vacuoles can limit its dissociation and As release back into the cytosol (Schmöger et al., 2000). Song et al. (2010) already ~~made emphasis in~~emphasized that engineering of vacuolar PC transporters in plants may be of potential use in phytoremediation ~~as they observed that *Arabidopsis* overexpressing *ABCC1* and *ABCC2* mutants resulted in arsenic hypersensitivity. Later on, it was also proven by Park et al. (2012) that overexpression of these transporters is also involved in greater vacuolar sequestration of cadmium and mercury.~~ According to this, in our study, the up-regulation ~~in~~observed of *WBABCT* transcripts ~~encoding for the white brown complex ABC transporter (*WBABCT*) (Table 5) highlight~~(**Fig. 3**) highlights its role in metal(loid)-PC complexes transportation and constitutes an interesting target gene to increase accumulation for phytoremediation purposes. ~~Since~~Interestingly, in leaves, since only As V was present ~~throughout the exposure time~~ and at low concentration compared

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to ~~As accumulated in~~ roots, no differential up-regulation of ~~the vacuolar transporter~~ *WBACT* transcripts was observed~~.~~ as compared to control (***Supplementary Fig. 3*B**).

Taking into account that speciation and distribution of As in the plant can provide important information and help to understand the mechanisms for As accumulation, translocation, and transformation as noted by Zhang et al. (2002), our results suggest that the As tolerance mechanism of *S. atrocinerea* relies on As V reduction in roots but not in leaves. Therefore, limited As V translocation by an effective As V reduction to As III and its complexation to NPT compounds and further sequestration into the root vacuoles, as supported by the gene expression, seems to be the reason for the tolerance of *S. atrocinerea* to As.

Under ~~arsenate or arsenite~~As accumulation, stress is induced in plant cells. ~~The~~ Although in leaves As detoxification processes are not really activated as seen in roots, *S. atrocinerea* plants respond to As in both roots and leaves by altering gene expression related to general stress. This response includes alterations at the gene level of transcripts related to the cell wall synthesis, as the down-regulation of ~~genes involved in cellulose biosynthesis could indicate a limitation in cell expansion and as such plant growth (Le Gall et al., 2015). Several transcriptome analyses in plants exposed to heavy metal(loid)s reveal that cell wall related genes are altered. A large scale rice transcriptome analysis under arsenate stress showed changes in gene expression including 40 cell wall related genes among 637 transcripts (Huang et al., 2012). Down regulated genes included~~ the cellulose synthase like A (*CSA*) ~~that is also downregulated in our study in roots and leaves of *S. atrocinerea* exposed to As (Fig. 4). Although no growth inhibition is observed in the current experimental time frame on hydroponics, long term experiments should be foreseen in the future.~~

~~Within a short experimental time frame, it is well described that ethylene is involved in cross communication between plant organs during stress, such as Cd stress (Schellingen et al., 2014). In our experiment,~~(***Supplementary Fig. 3*A,B**); ethylene biosynthesis, with *ACCS* ~~was~~notably up-regulated in roots ~~(Table 5), highlighting the presence of ACC or ethylene as an As stress response as was also previously seen by Yanitch et al. (2017) and that, according to transcript data as also seen in our data, ACC might act as a signaling molecule independent from ethylene synthesis (Yanitch et al., 2017).~~

~~It has been described that one of the most prominent features of the eukaryotic cellular response to arsenic is the induction~~and to a lesser extent in leaves, probably explained a low As translocation (***Supplementary Fig. 2***); and transcripts related to the synthesis of heat- shock ~~genes to grant tolerance to its toxicity (Levinson et al., 1980). Acclimatization to As toxicity leads to the elevated expression of heat shock genes and therefore, molecular chaperones are rapidly synthesized and deployed to prevent protein misfolding and to assist in their refolding to the native state (Morimoto, 1998; Stanhill et al., 2006). Arsenite inducible, cysteine- and histidine-rich RNA-associated~~

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~~protein~~proteins, like AIP-1~~-related, AIP-1, is~~, a highly conserved gene selectively activated by ~~arsenite~~As III in many cell types~~. Inactivation of the *Caenorhabditis elegans* homolog *aip-1* compromises survival of worms exposed to arsenite, but not to other stressors~~ (Sok et al., 2001~~). In~~), that in our experiment~~, transcripts for *AIP-1*~~ showed in roots at 1 d the highest up-regulation observed for any of the measured genes~~, and these results were only observed in roots, where arsenite accumulation increased drastically throughout the experiment (Table 5; Fig. 2).~~ (***Supplementary Fig. 2***), and it decreased overtime. Since this protein is As III-induced, and As III concentrations increase over time in roots, this suggest an effective complexation of As III from 1 d on with NPTs to prevent its toxicity. Another As-related response of willow ~~to arsenic contamination~~ is the biosynthesis of phenylpropanoids that may culminate with the increased production of tannins (Yanitch et al., 2017). In our study, we observed an early up-regulation of ~~chalcone synthase (*CHS1*, *CHS2*) and in other~~ selected genes (*CHS1*, *CHS2*, 2HFLR and F3H) in the flavonoid pathway at 1 ~~and 3~~ d (**Fig. 4 B** and ***Supplementary Fig. 3B***), whereas ~~at 10 and 30 d~~ no major changes in the expression pattern or a down-regulation were noticed~~.~~ later on as compared to control conditions. Therefore, by the information provided by the transcript levels, we suggest that *S. atrocinerea* relies on the phenylpropanoid pathway to cope with As toxicity during the early times of exposure, but further investigation at metabolic level is essential.

5 Conclusions

The selected *S. atrocinerea* clone naturally growing in an As-contaminated brownfield showed great tolerance when grown in the presence of a high concentration of ~~arsenic~~As and accumulated more than 2,400 mg As ~~K~~gkg^{-1} dry weight in its roots without showing phytotoxicity symptoms. Our findings reveal that ~~tolerance to arsenic in *S. atrocinerea* is associated with~~under the ~~following mechanisms: (I) increased arsenate~~presence of As V in hydroponic conditions, willow plants show a transcriptional regulation of genes involved in nutrient transporters, As V reduction ~~in roots, resulting in high arsenite concentrations in this organ, (II) increased *GS* expression in roots (III), *de novo* thiolic compound~~, glutathione synthesis and ~~accumulation in both roots and leaves leading to~~

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~~vacuolar As~~ sequestration~~, and (IV) mediated arsenic defense~~ of As into the vacuoles, together with genes involved in stress responses ~~by ACC signaling~~, which coincides with a rapid As III presence and accumulation in root tissues, altered nutrient profile and *de novo* synthesis and ~~chaperone induction.~~ increase of NPT compounds, all of which contribute to the tolerance to the metalloid by *S. atrocinerea*.

The high As accumulation together with a high biomass yield makes this willow species a potential tool for its use in As phytoremediation. Overall, a better understanding of the physiological mechanisms of tolerance to arsenic toxicity in *S. atrocinerea* was achieved through this study by experimental verification of the significance of particular transcripts complemented by ~~a comprehensive~~an integrative analysis of nutrient profile, As accumulation and speciation, as well as ~~non-protein thiolic~~NPT compounds synthesis. ~~More research is needed~~However, according to our observations, further ~~clarify the exact molecular and biochemical responses that confer tolerance to arsenic's deleterious effects. It must not be overlooked that gene expression levels are not always reflective of actual protein levels, and that post-translational modifications can play an important role in protein stability and activity. Therefore, targeted metabolomics and proteomics studies will help to resolve a plethora of physiological effects that can be applied to improve the capabilities for As phytoremediation in the field. Further~~ research should also focus on what happens in real ~~field conditions~~ polluted soils where, apart from As, there are~~,~~ usually~~,~~ other metal(loid)s at high concentrations ~~in As polluted soils~~ that can affect ~~As remediation.~~ the plant detoxification responses.

6 Conflict of Interest

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

AN: Conceptualization, Investigation, ~~Orignal~~Original draft; SH: Review; AC: Methodology, Formal analysis, Validation, Resources, Review and Editing; AG: Resources, Review and Editing~~.~~.

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9 Acknowledgments

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10 References

Ahmad, P., M. Sarwat, N. A. Bhat, M. R. Wani, A. G. Kazi and L. S. Tran (2015). Alleviation of cadmium toxicity in Brassica juncea L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. PLoS One 10(1): e0114571.

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Ahsan, N., Lee, D.G., Alam, I., Kim, P.J., Lee, J.J., Ahn, Y.-O., Kwak, S.S., Lee, I.J., Bahk, J.D., Kang, K.Y., Renaut, J., Komatsu, S., Lee, B.H. (2008) Comparative proteomic study of ~~arsenicinduced~~arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. ~~PROTEOMICS~~Proteomics. 8, 3561-3576.

Anjum, N. A., M. Hasanuzzaman, M. A. Hossain, P. Thangavel, A. Roychoudhury, S. S. Gill, M. A. M. Rodrigo, V. Adam, M. Fujita, R. Kizek, A. C. Duarte, E. Pereira and I. Ahmad (2015). "Jacks of metal/metalloid chelation trade in plants—an overview." Frontiers in Plant Science 6(192)

~~"Jacks of metal/metalloid chelation trade in plants—an overview." Frontiers in Plant Science 6(192)~~

Argus, G.W. (1995) Salicaceae Willow Family: Part Two: Salix L. Willow. Journal of the Arizona-Nevada Academy of Science 29, 39-62.

Batista, B.L., Nigar, M., Mestrot, A., Rocha, B.A., Barbosa Junior, F., Price, A.H., Raab, A., Feldmann, J. (2014) Identification and quantification of phytochelatins in roots of rice to long-term exposure: evidence of individual role on arsenic accumulation and translocation. J Exp Bot 65, ~~14671479.~~ 1467-1479.

Bleeker, P.M., Hakvoort, H.W., Bliek, M., Souer, E., Schat, H. (2006) Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant Holcus lanatus. Plant J 45, 917-929.

Blindauer, C. A. and O. I. Leszczyszyn (2010). "Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more." Nat Prod Rep 27(5): 720-741.

~~Gong, J. M., D. A. Lee and J. I. Schroeder (2003). "Long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis." Proc Natl Acad Sci U S A 100(17): 10118-10123~~

Bluemlein, K., Raab, A., Feldmann, J. (2009) Stability of arsenic peptides in plant extracts: ~~offline~~off-line versus on-line parallel elemental and molecular mass spectrometric detection for liquid chromatographic separation. Anal Bioanal Chem 393, 357-366.

Caille, N., Swanwick, S., Zhao, F.J., McGrath, S.P. (2004) Arsenic hyperaccumulation by Pteris vittata from arsenic contaminated soils and the effect of liming and phosphate fertilisation. Environmental Pollution 132, 113-120.

Catarecha, P., Segura, M.D., Franco-Zorrilla, J.M., García-Ponce, B., Lanza, M., Solano, R., Paz-Ares, J., Leyva, A. (2007) A Mutant of the Arabidopsis Phosphate Transporter PHT1;1 Displays Enhanced Arsenic Accumulation. The Plant Cell 19, 1123-1133.

Chang, S., Puryear, J., Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113-116.

Cobbett, C. S. (2000). Phytochelatins and Their Roles in Heavy Metal Detoxification. Plant Physiology 123(3): 825-832.

Commission, European. 2000. Ambient Air Pollution by AS, CD and NI Compounds (Position Paper—Final), Luxembourg: Office for Official Publications of the European Communities.

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Cordos, E. A., T. Frentiua, M. Pontaa, l. Marginean, B. Abrahamb and C. Roman (2006). Distribution study of inorganic arsenic (III) and (V) species in soil and their mobility in the area of Baia-Mare, Romania. Chemical Speciation & Bioavailability 18(1): 11-25

~~Baia-Mare, Romania. Chemical Speciation & Bioavailability 18(1): 11-25~~

Das, I., Sanyal, S. K., Koushik G., and Das, D. K.. (2016~~. 'Arsenic~~). Arsenic mitigation in soil-plant system through zinc application in West Bengal ~~soils',~~soils. Bioremediation journal, 20: 24-37.

Dave, R., Singh, P.K., Tripathi, P., Shri, M., Dixit, G., Dwivedi, S., Chakrabarty, D., Trivedi, P.K., Sharma, Y.K., Dhankher, O.P., Corpas, F.J., Barroso, J.B., Tripathi, R.D. (2013) Arsenite tolerance is related to proportional thiolic metabolite synthesis in rice (Oryza sativa L.). Arch Environ Contam Toxicol 64, 235-242.

Dhankher, O. P., Y. Li, B. P. Rosen, J. Shi, D. Salt, J. F. Senecoff, N. A. Sashti and R. B. Meagher (2002). Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat Biotechnol 20(11): ~~11401145.~~1140-1145.

~~Dhankher, O.P., Li, Y., P Rosen, B., Shi, J., Salt, D., Senecoff, J., A Sashti, N., Meagher, R. (2002) Engineering Tolerance and Hyperaccumulation of Arsenic in Plants by Combining Arsenate Reductase and γ-Glutamylcysteine Synthetase Expressions.~~

Dhankher, O.P., Rosen, B.P., McKinney, E.C., Meagher, R.B. (2006) Hyperaccumulation of arsenic in the shoots of Arabidopsis silenced for arsenate reductase (ACR2). Proceedings of the National Academy of Sciences of the United States of America 103, 5413-5418.

Duan, G.L., Zhou, Y., Tong, Y.P., Mukhopadhyay, R., Rosen, B.P., Zhu, Y.G. (2007) A CDC25 homologue from rice functions as an arsenate reductase. New Phytol 174, 311-321.

Ehlrich, H.L. (1990) Geomicrobiology. Marcel Dekker New York.

Ellis, D.R., Gumaelius, L., Indriolo, E., Pickering, I.J., Banks, J.A., Salt, D.E. (2006) A novel arsenate reductase from the arsenic hyperaccumulating fern Pteris vittata. Plant Physiol 141, ~~15441554.~~1544-1554.

Ellman, G.L. (1959) Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82, 70-77.

~~Fernández-Fernández, R., I. Carballo, H. Nava, R. Sánchez-Tamés, A. Bertrand and A. González (2011). Looking for Native Hyperaccumulator Species Useful in Phytoremediation: 297330.~~

Fernandez, R., A. Bertrand, R. Reis, M. P. Mourato, L. L. Martins and A. Gonzalez (2013). Growth and physiological responses to cadmium stress of two populations of Dittrichia viscosa (L.) Greuter. J Hazard Mater 244-245: 555-562.

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Fernández, R., Bertrand, A., García, J.I., Tamés, R.S., González, A. (2012) Lead accumulation and synthesis of non-protein thiolic peptides in selected clones of Melilotus alba and Melilotus officinalis. Environmental and Experimental Botany 78, 18-24.

Fernández, R., Carballo, I., Nava, H., Sánchez-Tamés, R., Bertrand, A., González, A. (2010) Looking for native hyperacumulator species useful in phytoremediation. In: Handbook of Phytoremediation. Golubev, I.A (ed), Nova Science Publishers, Inc.New York, pp.297-330.

Finnegan, P. M. and W. Chen (2012). Arsenic Toxicity: The Effects on Plant Metabolism. Frontiers in Physiology 3: 182.

Fitz, W.J., Wenzel, W.W. (2002) Arsenic transformations in the soil–rhizosphere–plant system: fundamentals and potential application to phytoremediation. Journal of Biotechnology 99, 259-278.

Fu, S.F., Chen, P.Y., Nguyen, Q.T., Huang, L.Y., Zeng, G.R., Huang, T.L., Lin, C.Y., Huang, H.J. (2014) Transcriptome profiling of genes and pathways associated with arsenic toxicity and tolerance in Arabidopsis. BMC Plant Biol 14, 94.

Gallego, J.R., Esquinas, N., Rodríguez-Valdés, E., Menéndez-Aguado, J.M., Sierra, C. (2015) Comprehensive waste characterization and organic pollution co-occurrence in a Hg and As mining and metallurgy brownfield. Journal of Hazardous Materials 300, 561-571.

Gill, R.A., Zang, L., Ali, B., Farooq, M.A., Cui, P., Yang, S., Ali, S., Zhou, W. (2015) Chromium-induced physio-chemical and ultrastructural changes in four cultivars of Brassica napus L. Chemosphere 120, 154-164.

Gong, J. M., D. A. Lee and J. I. Schroeder (2003). "Long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis." Proc Natl Acad Sci U S A 100(17): 10118-10123

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., Rokhsar, D.S. (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res 40, D1178-D1186.

Hammer, D., Kayser, A., Keller, C. (2003) Phytoextraction of Cd and Zn with Salix viminalis in field trials. Soil Use and Management 19, 187-192.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W.T., Schat, H., Meharg, A.A. (2001) Phytochelatins Are Involved in Differential Arsenate Tolerance in Holcus lanatus. Plant Physiol 126, 299-306.

Hasanuzzaman, M., K. Nahar, T. I. Anee and M. Fujita (2017). Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. Physiology and molecular biology of plants : an international journal of functional plant biology 23(2): 249-268.

Huang, J.H., Fecher, P., Ilgen, G., Hu, K.N., Yang, J. (2012) Speciation of arsenite and arsenate in rice grain – Verification of nitric acid based extraction method and mass sample survey. Food Chemistry 130, 453-459.

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Janssen, J., Weyens, N., Croes, S., Beckers, B., Meiresonne, L., Van Peteghem, P., Carleer, R., Vangronsveld, J. (2015) Phytoremediation of Metal Contaminated Soil Using Willow: Exploiting Plant-Associated Bacteria to Improve Biomass Production and Metal Uptake. Int J Phytoremediation 17, 1123-1136.

Ji, R., L. Zhou, J. Liu, Y. Wang, L. Yang, Q. Zheng, C. Zhang, B. Zhang, H. Ge, Y. Yang, F. Zhao, S. Luan, and W. Lan. (2017. 'Calcium). Calcium-dependent protein kinase CPK31 interacts with arsenic transporter AtNIP1;1 and regulates arsenite uptake in Arabidopsis thaliana'thaliana, *PLoS One*, 12: e0173681.

Jiang, Y., Lei, M., Duan, L., Longhurst, P. (2015) Integrating phytoremediation with biomass valorisation and critical element recovery: A UK contaminated land perspective. Biomass and Bioenergy 83, 328-339.

Kabata-Pendias, (2010), Trace Elements in Soils and Plants, Fourth Edition. CRC Press, pp. 407-505.

Kang, J., Park, J., Choi, H., Burla, B., Kretzschmar, T., Lee, Y., Martinoia, E. (2011) Plant

ABC Transporters. The Arabidopsis Book / American Society of Plant Biologists 9, e0153.

Kertulis, G.M., Ma, L.Q., MacDonald, G.E., Chen, R., Winefordner, J.D., Cai, Y. (2005)

Arsenic speciation and transport in Pteris vittata L. and the effects on phosphorus in the xylem sap. Environmental and Experimental Botany 54, 239-247.

Kidd, P., Mench, M., Álvarez-López, V., Bert, V., Dimitriou, I., Friesl-Hanl, W., Herzig, R.,

Olga Janssen, J., Kolbas, A., Müller, I., Neu, S., Renella, G., Ruttens, A., Vangronsveld, J.,

Puschenreiter, M. (2015) Agronomic Practices for Improving Gentle Remediation of Trace ElementContaminatedElement-Contaminated Soils. International Journal of Phytoremediation 17, 1005-1037.

Konlechner, C., M. Türktaş, I. Langer, M. Vaculík, W. W. Wenzel, M. Puschenreiter and M. -T. Hauser (2013). "Expression of zinc and cadmium responsive genes in leaves of willow (Salix caprea L.) genotypes with different accumulation characteristics.", Environmental pollution (Barking, Essex : 1987) 178: 121-127.

Kuzovkina, Y.A., Quigley, M.F. (2005) Willows Beyond Wetlands: Uses of Salix L. Species for Environmental Projects. Water, Air, and Soil Pollution 162, 183-204.

Le Gall, H., F. Philippe, J. M. Domon, F. Gillet, J. Pelloux and C. Rayon (2015). "Cell Wall Metabolism in Response to Abiotic Stress." Plants (Basel) 4(1): 112-166.

LeBlanc, M.S., McKinney, E.C., Meagher, R.B., Smith, A.P. (2013) Hijacking membrane transporters for arsenic phytoextraction. Journal of Biotechnology 163, 1-9.

Levinson, W., Oppermann, H., Jackson, J. (1980) Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. Biochim Biophys Acta 606, 170-180.

Li, J., Jia, H., Han, X., Zhang, J., Sun, P., Lu, M., Hu, J. (2016) Selection of Reliable Reference Genes for Gene Expression Analysis under Abiotic Stresses in the Desert Biomass Willow, Salix psammophila. Front Plant Sci 7, 1505.

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Li, R.-Y., Ago, Y., Liu, W.-J., Mitani, N., Feldmann, J., McGrath, S.P., Ma, J.F., Zhao, F.-J. (2009) The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. Plant Physiol 150, 2071-2080.

Li, Y., O. P. Dhankher, L. Carreira, D. Lee, A. Chen, J. I. Schroeder, R. S. Balish and R. B. Meagher (2004). "Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity." Plant Cell Physiol 45(12): 1787-1797.

~~Li, J., Jia, H., Han, X., Zhang, J., Sun, P., Lu, M., Hu, J. (2016) Selection of Reliable Reference Genes for Gene Expression Analysis under Abiotic Stresses in the Desert Biomass Willow, Salix psammophila. Front Plant Sci 7, 1505.~~

~~Li, R.-Y., Ago, Y., Liu, W.-J., Mitani, N., Feldmann, J., McGrath, S.P., Ma, J.F., Zhao, F.-J. (2009) The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. Plant Physiol 150, 2071-2080.~~

Lindsay, E.R., Maathuis, F.J.M. (2016) Arabidopsis thaliana NIP7;1 is involved in tissue arsenic distribution and tolerance in response to arsenate. FEBS Letters 590, 779-786.

Lloyd, G.a.M., B.H. (1981) Woody Plant Medium (WPM)—A Mineral Nutrient Formulation for Microculture of Woody Plant Species. HortScience 16, 453.

Lou, L.Q., Ye, Z.H., Lin, A.J., Wong, M.H. (2010) Interaction of arsenic and phosphate on their uptake and accumulation in Chinese brake fern. Int J Phytoremediation 12, 487-502.

Ma, J.F., Yamaji, N., Mitani, N., Xu, X.-Y., Su, Y.-H., McGrath, S.P., Zhao, F.-J. (2008) Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proceedings of the National Academy of Sciences 105, 9931-9935.

~~National Academy of Sciences 105, 9931-9935.~~

Maciaszczyk-Dziubinska, E., Wawrzycka, D., Wysocki, R. (2012) Arsenic and Antimony Transporters in Eukaryotes. International Journal of Molecular Sciences 13, 3527-3548.

Mandal, B.K., Suzuki, K.T. (2002) Arsenic round the world: a review. Talanta 58, 201-235.

Matschullat, J. (2000) Arsenic in the geosphere — a review. Science of The Total Environment 249, 297-312.

Meers, E., Vandecasteele, B., Ruttens, A., Vangronsveld, J., Tack, F.M.G. (2007) Potential of five willow species (Salix spp.) for phytoextraction of heavy metals. Environmental and Experimental Botany 60, 57-68.

Meharg, A.A., Hartley-Whitaker, J. (2002) Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. New Phytologist 154, 29-43.

~~Meharg, A.A., Macnair, M.R. (1992) Suppression of the High Affinity Phosphate Uptake System: A Mechanism of Arsenate Tolerance in Holcus lanatus L. Journal of Experimental Botany 43, 519-524.~~

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~~Meharg, A.A., Macnair, M.R. (1994) Relationship between plant phosphorus status and the kinetics of arsenate influx in clones ofdeschampsia cespitosa (L.) beauv. that differ in their tolerance to arsenate. Plant and Soil 162, 99-106.~~

Mitani-Ueno, N., Yamaji, N., Zhao, F.J., Ma, J.F. (2011) The aromatic/arginine selectivity filter of NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic. J Exp Bot 62, 4391-4398.

Moreno-Jimenez, E., Esteban, E., Fresno, T., de Egea, C.L., Penalosa, J.M. (2010) Hydroponics as a valid tool to assess arsenic availability in mine soils. Chemosphere 79, 513-517.

Morimoto, R.I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12, ~~37883796.~~ 3788-3796.

Naidu, R., Smith, E., Owens, G., Bhattacharya, P., Nadebaum, P. (2006) Managing arsenic in the environment: from soil to human health. CSIRO Publishing, Collingwood.

Nie, L., Shah, S., Rashid, A., I. Burd, G., Dixon, D.G., Glick, B. (2002) Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth-promoting bacterium Enterobacter cloacae CAL2. Plant Physiology and Biochemistry 40, 335-361.

O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., et al. (2016) Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res 44, D733-745.

~~Park, J., Song, W.Y., Ko, D., Eom, Y., Hansen, T.H., Schiller, M., Lee, T.G., Martinoia, E., Lee, Y. (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Plant J 69, 278-288.~~

Park, J.H., Han, Y.S., Seong, H.J., Ahn, J.S., Nam, I.H. (2016) Arsenic uptake and speciation in Arabidopsis thaliana under hydroponic conditions. Chemosphere 154, 283-288.

~~Patra, M., Bhowmik, N., Bandopadhyay, B., Sharma, A. (2004) Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. Environmental and Experimental Botany 52, 199-223.~~

Paul, D.S., Hernández-Zavala, A., Walton, F.S., Adair, B.M., Dina, J.D., Matoušek, T., Stýblo, M. (2007) Examination of the Effects of Arsenic on Glucose Homeostasis in Cell Culture and Animal Studies: Development of a Mouse Model for Arsenic-Induced Diabetes. Toxicology and applied pharmacology 222, 305-314.

Pilon-Smits, E. (2005) Phytoremediation. Annu Rev Plant Biol 56, 15-39.

Puckett, E.E., Serapiglia, M.J., DeLeon, A.M., Long, S., Minocha, R., Smart, L.B. (2012) Differential expression of genes encoding phosphate transporters contributes to arsenic tolerance and accumulation in shrub willow (Salix spp.). Environmental and Experimental Botany 75, 248-257.

Purdy, J.J., Smart, L.B. (2008) Hydroponic Screening of Shrub Willow (Salix Spp.) for Arsenic

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Tolerance and Uptake. International Journal of Phytoremediation 10, 515-528.

Quaghebeur, M., Rengel, Z. (2003) The Distribution of Arsenate and Arsenite in Shoots and Roots of Holcus lanatus is Influenced by Arsenic Tolerance and Arsenate and Phosphate Supply. Plant Physiol. 132(3):1600-1609.

Raab, A., Schat, H., Meharg, A.A., Feldmann, J. (2005) Uptake, translocation and transformation of arsenate and arsenite in sunflower (Helianthus annuus): formation of arsenic–phytochelatin complexes during exposure to high arsenic concentrations. New Phytologist 168, ~~551558.~~551-558.

Rahman, A., M. G. Mostofa, M. M. Alam, K. Nahar, M. Hasanuzzaman and M. Fujita (2015). Calcium Mitigates Arsenic Toxicity in Rice Seedlings by Reducing Arsenic Uptake and Modulating the Antioxidant Defense and Glyoxalase Systems and Stress Markers. BioMed research international 2015: 340812-340812.

Raskin, I., Kumar, P.B.A.N., Dushenkov, S., Salt, D.E. (1994) Bioconcentration of heavy metals by plants. Current Opinion in Biotechnology 5, 285-290.

~~Rauret, G., F. López-Sánchez, J., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Quevauviller, P. (1999) Improvement of the BCR three step sequential extraction procedure prior to the certification of new sediment and soil reference materials. Journal of Environmental Monitoring 1, 57-61.~~

Rea, P. A., O. K. Vatamaniuk and D. J. Rigden (2004). Weeds, Worms, and More. Papain's Long-Lost Cousin, Phytochelatin Synthase. Plant Physiology 136(1): 2463-2474.

Reichard, J.F., Puga, A. (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2, 87-104.

Remans, T., Keunen, E., Bex, G.J., Smeets, K., Vangronsveld, J., Cuypers, A. (2014) Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. Plant Cell 26, 3829-3837.

~~Rousseau, M.-C., Straif, K., Siemiatycki, J. (2005) IARC Carcinogen Update. Environmental Health Perspectives 113, A580-A581.~~

Ruttens, A., Boulet, J., Weyens, N., Smeets, K., Adriaensen, K., Meers, E., Van Slycken, S., Tack, F., Meiresonne, L., Thewys, T., Witters, N., Carleer, R., Dupae, J., Vangronsveld, J. (2011) Short Rotation Coppice Culture of Willows and Poplars as Energy Crops on Metal Contaminated Agricultural Soils. International Journal of Phytoremediation 13, 194-207.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., Bleeker, P.M. (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and ~~nonhyperaccumulator~~non-hyperaccumulator metallophytes. J Exp Bot 53, 2381-2392.

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~~Schellingen K.,Van Der Straeten D., Vandenbussche F., Prinsen E., Remans T., Vangronsveld J., Cuypers, A. (2014) Cadmium-induced ethylene production and responses in Arabidopsis thaliana rely on ACS2 and ACS6 gene expression. BMC Plant Biol 14: 214.~~

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc 3(6): 1101-1108.

Schmöger, M.E.V., Oven, M., Grill, E. (2000) Detoxification of Arsenic by Phytochelatins in Plants. Plant Physiol 122, 793-802.

Shaibur, M. R., N. Kitajima, R. Sugawara, T. Kondo, S. M. Imamul Huq and S. Kawai (2008). Physiological and Mineralogical Properties of Arsenic-Induced Chlorosis in Barley Seedlings Grown Hydroponically. Journal of Plant Nutrition 31(2): 333-353.

Sharma, R., R. Bhardwaj, N. Handa, V. Gautam, S. K. Kohli, S. Bali, P. Kaur, A. K. Thukral, S. Arora, P. Ohri and A. P. Vig (2016). ~~Chapter 10 -~~ Responses of Phytochelatins and Metallothioneins in Alleviation of Heavy Metal~~-~~ Stress in Plants: An~~-~~ Overview. Plant Metal Interaction. P. Ahmad, Elsevier: 263-283.

Shelmerdine, P.A., Black, C.R., McGrath, S.P., Young, S.D. (2009) Modelling phytoremediation by the hyperaccumulating fern, Pteris vittata, of soils historically contaminated with arsenic. Environmental Pollution 157, 1589-1596.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J 39, 629-642.

Singh, A.P., Goel, R.K., Kaur, T. (2011) Mechanisms Pertaining to Arsenic Toxicity. Toxicology International 18, 87-93.

~~Smedley, P. L. (2007). Managing Arsenic in the Environment: from Soil to Human Health - Edited by R. Naidu, E. Smith, G. Owens, P. Bhattacharya & P Nadebaum. European Journal of Soil Science 58(2): 519-520.~~

Smith, A. H., A. Ercumen, Y. Yuan and C. M. Steinmaus (2009). "Increased lung cancer risks are similar whether arsenic is ingested or inhaled." Journal of exposure science & environmental epidemiology 19(4): 343-348.

Sok, J., Calfon, M., Lu, J., Lichtlen, P., Clark, S.G., Ron, D. (2001) Arsenite-inducible ~~RNAassociated~~RNA-associated protein (AIRAP) protects cells from arsenite toxicity. Cell Stress Chaperones 6, 6-15.

Song, W.Y., Park, J., Mendoza-Cozatl, D.G., Suter-Grotemeyer, M., Shim, D., Hortensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E. (2010) Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proc Natl Acad Sci U S A 107, 21187-21192.

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Spratlen, M.J., Gamble, M.V., Grau-Perez, M., Kuo, C.-C., Best, L.G., Yracheta, J., Francesconi, K., Goessler, W., Mossavar-Rahmani, Y., Hall, M., Umans, J.G., Fretts, A., Navas-Acien, A. (2017) Arsenic metabolism and one-carbon metabolism at low-moderate arsenic exposure: Evidence from the Strong Heart Study. Food and Chemical Toxicology 105, 387-397.

~~Evidence from the Strong Heart Study. Food and Chemical Toxicology 105, 387-397.~~

Srivastava, S. and M. Shrivastava (2017). Zinc supplementation imparts tolerance to arsenite stress in Hydrilla verticillata (L.f.) Royle. Int J Phytoremediation 19(4): 353-359.

~~Srivastava, S., A. K. Srivastava, G. Sablok, T. U. Deshpande and P. Suprasanna (2015). Transcriptomics profiling of Indian mustard (Brassica juncea) under arsenate stress identifies key candidate genes and regulatory pathways. Front Plant Sci 6: 646.~~

Stanhill, A., Haynes, C.M., Zhang, Y., Min, G., Steele, M.C., Kalinina, J., Martinez, E., Pickart, C.M., Kong, X.-P., Ron, D. (2006) An Arsenite-Inducible 19S Regulatory Particle-Associated Protein Adapts Proteasomes to Proteotoxicity. Molecular Cell 23, 875-885.

Sylvain, B., Mikael, M.-H., Florie, M., Emmanuel, J., Marilyne, S., Sylvain, B., Domenico, M. (2016) Phytostabilization of As, Sb and Pb by two willow species (S. viminalis and S. purpurea) on former mine technosols. CATENA 136, 44-52.

Tlustoš, P., Száková, J.i., Vysloužilová, M., Pavlíková, D., Weger, J., Javorská, H. (2007) Variation in the uptake of Arsenic, Cadmium, Lead, and Zinc by different species of willows Salix spp. grown in contaminated soils. Central European Journal of Biology 2, 254-275.

~~Ud, F., Ahmad, D., Ahmad, N., Rass Masood, K., Hussain, M., Faheem Malik, M., Qayyum, A. (2018) Phytoremediation of arsenic-contaminated soils by Eucalyptus camaldulensis, Terminalia arjuna and Salix tetrasperma.~~

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. (2012) Primer3--new capabilities and interfaces. Nucleic Acids Res 40, e115.

Wang, J., Zhao, F.J., Meharg, A.A., Raab, A., Feldmann, J., McGrath, S.P. (2002) Mechanisms of arsenic hyperaccumulation in Pteris vittata. Uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiol 130, 1552-1561.

Wang, X., Mu, X., Zhang, J., Huang, Q., Alamdar, A., Tian, M., Liu, L., Shen, H. (2015) Serum metabolomics reveals that arsenic exposure disrupted lipid and amino acid metabolism in rats: a step forward in understanding chronic arsenic toxicity. Metallomics 7, 544-552.

White, P. J. and P. H. Brown (2010). Plant nutrition for sustainable development and global health. Annals of botany 105(7): 1073-1080.

Witters, N., Slycken, S., Ruttens, A., Adriaensen, K., Meers, E., Meiresonne, L., Tack, F.M.G., Thewys, T., Laes, E., Vangronsveld, J. (2009) Short-rotation coppice of willow for phytoremediation of a metal-contaminated agricultural area: a sustainability assessment. BioEnergy Res 2.

Xu, W., Dai, W., Yan, H., Li, S., Shen, H., Chen, Y., Xu, H., Sun, Y., He, Z., Ma, M. (2015) Arabidopsis NIP3;1 Plays an Important Role in Arsenic Uptake and Root-to-Shoot Translocation under Arsenite Stress Conditions. Molecular Plant 8, 722-733.

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Xu, X.Y., McGrath, S.P., Zhao, F.J. (2007) Rapid reduction of arsenate in the medium mediated by plant roots. New Phytologist 176, 590-599.

Yan, X.L., Lin, L.Y., Liao, X.Y., Zhang, W.B. (2012) Arsenic accumulation and resistance mechanism in Panax notoginseng, a traditional rare medicinal herb. Chemosphere 87, 31-36.

Yang, Q., Tu, S., Wang, G., Liao, X., Yan, X. (2012) Effectiveness of Applying Arsenate Reducing Bacteria to Enhance Arsenic Removal From Polluted Soils by Pteris Vittata L. International Journal of Phytoremediation 14, 89-99.

Yanitch, A., Brereton, N.J.B., Gonzalez, E., Labrecque, M., Joly, S., Pitre, F.E. (2017) Transcriptomic Response of Purple Willow (Salix purpurea) to Arsenic Stress. Front Plant Sci 8, 1115.

Zhang, W., Cai, Y., Tu, C., Ma, L.Q. (2002) Arsenic speciation and distribution in an arsenic hyperaccumulating plant. Science of The Total Environment 300, 167-177.

Zhang, X., Zhao, F.J., Huang, Q., Williams, P.N., Sun, G.X., Zhu, Y.G. (2009) Arsenic uptake and speciation in the rootless duckweed Wolffia globosa. New Phytol 182, 421-428.

Zhang, Y., Han, X., Chen, S., Zheng, L., He, X., Liu, M., Qiao, G., Wang, Y., Zhuo, R. (2017) Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in Salix matsudana under different abiotic stresses. Scientific Reports 7, 40290.

~~Zhao, F. J., J. F. Ma, A. A. Meharg, and S. P. McGrath. 2009. 'Arsenic uptake and metabolism in plants', *New Phytol*, 181: 777-94.~~

Zhao, F.J., Ma, J.F., Meharg, A.A., McGrath, S.P. (2009) Arsenic uptake and metabolism in plants. New Phytol 181, 777-794.

~~Zimeri, A.M., Dhankher, O.P., McCaig, B., Meagher, R.B. (2005) The plant MT1 metallothioneins are stabilized by binding cadmiums and are required for cadmium tolerance and accumulation. Plant Mol Biol 58, 839-855~~

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Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

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Supplementary Table 1. Primer sequences used for the real time RT-PCR analyses.

Gene	Gene description	*S. purpurea* ortholog locus or NCBI annotation	Primer sequence F/R (5′ -3′)	Product size (bp)	Efficiency
Reference genes (1: used to normalize gene expression data in roots, 2: used to normalize gene expression data in leaves)					
OTU[1,2]	OTU-like cysteine protease family protein	SapurV1A.0615s020 0.1	GGCAGTGGTTC CTCTTCGAA ATCCCCATCTTT	114	91.2
ACT7[2]	Actin 7	SapurV1A.0231s032 0.1	CGCAGTCG CTGTCCTTTCCC TGTATGCCA GTCACGACCAG	140	90.6
α-TUB2[2]	Alpha-tubulin 2	SapurV1A.0598s003 0.1	CAAGATCCA CCAAGCGAGCA TTTGTCCAC CCCTCGTCATCA	133	97.6
DNAJ	Chaperone protein DnaJ 49	SapurV1A.0212s011 0.1	CCACCTTC GCTCCCGGTTCT TCTTATTTTCC AAATTAACCCC	117	81.3
EF1α[1]	Elongation factor 1-alpha	SapurV1A.0023s030 0.1	TCTCTGCGTAGT ACCAGATTTCC GAGCCCAAG TTGGCCCAAAA	150	90.4
ARF2[1]	ADP-ribosylation factor 2	SapurV1A.0014s016 0.1	GTGCAAACC TGGGGCTGTCTT TCACCAAG GGTCACAATCT CACCGAGCT	131	96.9
Arsenate transport					
HAPO4	High-affinity phosphate transporter 4	HQ228362.1	GAACGACGAGC ACCTGGTT ACGGGTTCTATT CGCCTTGA	108	86.1
NA-DPHOT	Sodium-dependent phosphate transporter	SapurV1A.0139s026 0.1	CAGCCACTTATC CCCAGCAA TCAAGGCGAAT AGAACCCGT	134	96.6
PHO1	Phosphate transporter PHO1-like protein	SapurV1A.0063s055 0.1	AGAGGCTGCGA TGTTGAACA GTCTGAAGCAA GGCGAGTCA	115	81.3
Arsenite transport					
BORON	Boron transporter	SapurV1A.0014s020 0.1	TCATTCGGGGA ACAACTGGAG ACTGTCGGCTCT GCAACTC	143	97.3
NIP1	Aquaporin NIP1.1	SapurV1A.0029s017 0.1	CAAGGTTGTGA CTCTTCCAGGA GACAGCAGGGT	106	93.0
SIP1	Aquaporin SIP.1	SapurV1A.1058s006 0.1	TGAAATGGG GCCAGTTCAGT ACAAGCACATG TGCAGCAGAGG	147	93.3
SILICON	Silicon 1	SapurV1A.1225s008 0.1	GTTTCGAG GGTAGCAGTCT CAGCAGGTG TGAAAGGTTCC CAGCAACTGT	94	95.6

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

~~PHO1~~

~~Phosphate transporter PHO1-like protein~~ ~~SapurV1A.0063s0550.1~~ ~~AGAGGCTGGCGATGTTGAACA~~ ~~115~~ ~~91.2%~~ ~~0.9938~~

~~NA-DPHOT~~ ~~Sodium-dependent phosphate transporter~~

Arsenate reductases			~~GGTCACAATCT CACCGAGCT~~		
CDC25-1	Tyrosine phosphatase	SapurV1A.0142s0310.1	ACGGCATCTTTAGGTCTGGTT TACGGCTCGGGACATAGACA	97	92.9%
CDC25-2	Tyrosine phosphatase	SapurV1A.0243s0430.1	TCAACTTTCACCACAGAAGACCT CACTAGTTGACGAGCCAGGA	147	89.9%
Thiol ~~metabolism~~ chelating response					
GR	Glutathione reductase	SapurV1A.0056s0770.1	ACGAAATGAGG GCTGTGGTT CCTCTCCATGAT CTGTGCGA	126	93.9 %
GS	Glutathione synthetase	SapurV1A.1124s0080.1	GCTGTCAAGTG CCCATCCAT CAGACTCCATA AGCCAGCGA	91	116. %
~~Flavonoid Synthesis~~					
PCS	Phytochelatin synthase	SapurV1A.0160s0210.1	~~GT~~GGAAGGGTA TTGCTGTAAGG A TGAGATGAAGG AACCAGCACA	137	98.5 %
GST	Glutathion S-transferase	SapurV1A.0016s1070.1	CGGTTCTTGGCT GGAGATGA CCTCCCCACATT TTCCCTGG	120	90.0
MT1A	Metallothionein~~-like protein~~	*S. matsudana* EF157299.1	CTTCGGTGCTGA GAATGGCT CTGCTTTGTTGG GACCATGC	97	90.5
~~Arsenic metabolism~~ Vacuolar transporters			~~CTGCTTTGTTGG GACCATGC~~		
ABCG	ABC transporter G	SapurV1A.0258s0220.1	AGGCTTGGATT CTACAACTGCT TGGCTGGTGGA TTGTTGTCA	94	84.3
~~CDC25-1 Tyrosine phosphatase~~ *CAX2-1*	Vacuolar cation/proton exchanger 2	SapurV1A.~~0142s0310~~1071s0020.1	~~ACGGCATCTTTA GGTCTGGTT TACGGCTCGGG ACATAGACA~~ TCTTGCAATCGT CGTCCACA ACCTAAACGCT CAGCCAAGG	~~97~~ 94	92.9
~~CDC25-2 Tyrosine phosphatase~~ *CAX2-2*	Vacuolar cation/proton exchanger 2	SapurV1A.~~0243s0430~~0338s0120.1	~~TCAACTTTCACC ACAGAAGACCT~~ TTGTTGGTGCTT GGATGTGC GCAGGACAGCA GGAAAGAG	~~147~~ 142	103. % ~~89 %~~
WBABCT **~~Arsenic transport related~~**	White-brown-complex ABC transporter	~~CACTAGTTGACG AGCCAGGA~~ SapurV1A.0084s0020.1	GCAAGAGGTGG TAGGACTGT ACACCCATCCG ACAAAACCA	96	97.3

~~HAPO4~~ ~~High-affinity phosphate transporter 4~~

CHS1	Chalcone synthase	~~CAX2-1~~ SapurV1A.0820s0070.1	CATTCCGTGGCCCTAGTGAC ~~Vacuolar cation/proton exchanger 2~~	90	96.9%	0.9974

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

*CHS2*CAX2-2	Chalcone synthase 2Vacuolar cation/proton exchanger 2	SapurV1A.0056s0660.1	CGGAGCCTACAATGAGAGCA AACTGCGAGCCACTAGACAC	145	91.5%	0.9992
*CHS3*NIP1	Chalcone synthase 3	SapurV1A.0820s0080.1 Aquaporin NIP1.1	AAAAGCACACCCCACTCCAA GCGGCCCAGACTATTCTACC	135	87.7%	0.9999
F3H	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	AGCCTCGGTCAGACTCTTCT TCTTGTCGGAGGCTATGGGA	136	96.7%	0.999
FLH	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGTATGGCGTTTGAGTCC TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT	114	88.6%	0.9949
2HFLR	Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCATCGA	96	92.7%	0.9661
FLS	Flavonol synthase	SapurV1A.1087s0040.1	TCCCAACCCAGATTGTGTCG CAAATAGGCCCCACTGCGAA	94	90.5%	0.9976
ANR	Anthocyanidin reductase	SapurV1A.0028s0410.1	TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT	129	93.8%	0.9930
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG	127	95.8%	0.9823
Stress related						
*ACCS*SIP1	1-aminocyclopropane-1-carboxylate synthaseAquaporin SIP1.1	SapurV1A.2160s0020.1	GCAGCACCAACTTTTGTCTCA	115	102.3%	0.9982
WBABCT	White-brown-complex ABC transporter		GGGGTTGTTCGTAGGGTGAA			
*AIP-1*ABCG	Arsenite-inducible RNA-associatedABC transporter G family protein AIP-1-related	SapurV1A.0229s0030.1	CTTGCCAGTTGAAGGTGTGC	140	93.2%	0.9940
SILICON *ER*	Ethylene receptor	SapurV1A.0052s0240.1 Silicon 1	ACAATCTTTTCCGTTCCTCAAGG TACCATACACGTGCCCACTG	90	120.0%	0.9870
BORON	Boron transporter		GTAGTAGAGGTACACGAACAGCA			
CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA TCCAGCAACAACTCCAACGA	125	90.5%	0.9919
			CTCTGAAGCAAGGCGAGTCA CAGCCACTTATCCCCAGCAA			
		SapurV1A.0139s0260.1	TCAAGGCGAATAGAACCCGT	134	94.5%	0.9873
		HQ228362.1	GAACGACGAGCACCTGGTT ACGGGTTCTATTCGCCTTGA	108	86.1%	0.9951
		SapurV1A.1071s0020.1	TCTTGCAATCGTCGTCCACA ACCTAAACGCTCAGCCAAGG	94	92.9%	0.9954
		SapurV1A.0338s0120.1	TTGTTGGTGCTTGGATGTGC GCAGGACAGCAGGAAAGAG	142	103.4%	0.9826
		SapurV1A.0029s0170.1	CAAGGTTGTGACTCTTCCAGGA GACAGCAGGGTTGAAATGGG	106	89.9%	0.9912
		SapurV1A.1058s0060.1	GCCAGTTCAGTACAAGCACATG TGCAGCAGAGGGTTTCGAG	147	103.9%	0.9911
		SapurV1A.0084s0020.1	GCAAGAGGTGGTAGGACTGT ACACCCATCCGACAAAACCA	96	97.3%	0.9979
		SapurV1A.0258s0220.1	AGGCTTGGATTCTACAACTGCT TGGCTGGTGGATTGTTGTCA	94	84.3%	0.9778

		SapurV1A.1225s0080.1	GGTAGCAGTCTCAGCAGGTG TGAAAGGTTCCCAGCAACTGT	94	85.2%	0.9977
		SapurV1A.0014s0200.1	TCATTCGGGGAACAACTGGAG ACTGTCGGCTCTGCAACTC	143	93.3%	0.9805

Table 1. Continued.

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Flavonoid Synthesis						
CHS1	Chalcone synthase	SapurV1A.0820s0070.1	CATTCCGTGGCCCTAGTGAC CGGAGCCTACAATGAGAGCA	90	96.9%	0.9974
CHS2	Chalcone synthase 2	SapurV1A.0056s0660.1	AACTGCGAGCCACTAGACAC AAAAGCACACCCCACTCCAA	145	91.5%	0.9992
CHS3	Chalcone synthase 3	SapurV1A.0820s0080.1	GCGGCCCAGACTATTCTACC AGCCTCGGTCAGACTCTTCT	135	87.7%	0.9999
F3H	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	TCTTGTCGGAGGCTATGGGA TCGGTATGGCGTTTGAGTCC	136	96.7%	0.999

FLH	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT	114	88.6%	0.9949
2HFLR	Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCATCGA	96	92.7%	0.9661
FLS	Flavonol synthase	SapurV1A.1087s0040.1	TCCCAACCCAGATTGTGTCG CAAATAGGCCCCACTGCGAA	94	90.5%	0.9976
ANR	Anthocyanidin reductase	SapurV1A.0028s0410.1	TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT	129	93.8%	0.9930
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG	127	95.8%	0.9823
As stress related						
ACCS	1-aminocyclopropane-1-carboxylate synthase	SapurV1A.2160s0020.1	GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA	115	102.3%	0.9982
AIP-1	Arsenite-inducible RNA-associated protein AIP-1-related	SapurV1A.0229s0030.1	CTTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCGTCAAGG	140	93.2%	0.9940
ER	*Ethy*lene receptor	SapurV1A.0052s0240.1	TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA	90	120.0%	0.9870
Cell wall synthesis						
CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA TCCAGCAACAACTCCAACGA	125	90.5%	0.9919

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Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

Table 21. Nutrients (mg Kgkg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters within each column and tissue indicate significant differences among treatments and time points on HSD test at $p < 0.05$.

Organ	Time (d	Treatment	Nutrient (mg Kg^{-1} DW) P	Ca	B	Zn	Fe
Roots	1	Control	5757.89 ± 430	4322.53 ± 207.32 b	19.62 ± 1.12 a	512.35 ± 53.	1955.93 ± 98.36 b
		As	5720.65 ± 379	4929.41 ± 242.98 a	21.98 ± 1.23 a	625.22 ± 29.	2364.78 ± 115.93 a
	3	Control	5791.72 ± 456.56 a	3271.75 ± 245.56 b	15.83 ± 0.89 c	439.97 ± 23.	1219.43 ± 62.34 d
		As	5507.21 ± 412.42 a	4556.62 ± 342.45 a	19.46 ± 0.93 a	469.75 ± 30.	1354.59 ± 49.45 c
	10	Control	3772.06 ± 235	3041.54 ± 289.87 c	15.53 ± 0.92 b	481.45 ± 32.	1087.50 ± 83.12 e
		As	2883.34 ± 176	4257.52 ± 458.96 b	17.53 ± 1.09 ab	432.36 ± 23.56 c	1287.73 ± 69.32 c
	30	Control	2729.45 ± 278	3093.95 ± 334.56 c	13.26 ± 1.01 d	330.76 ± 22.	943.27 ± 34.23 f
		As	1633.24 ± 99.8	4154.46 ± 354.98 a	14.38 ± 0.89 d	332.44 ± 15.69 d	1082.25± 50.54 e

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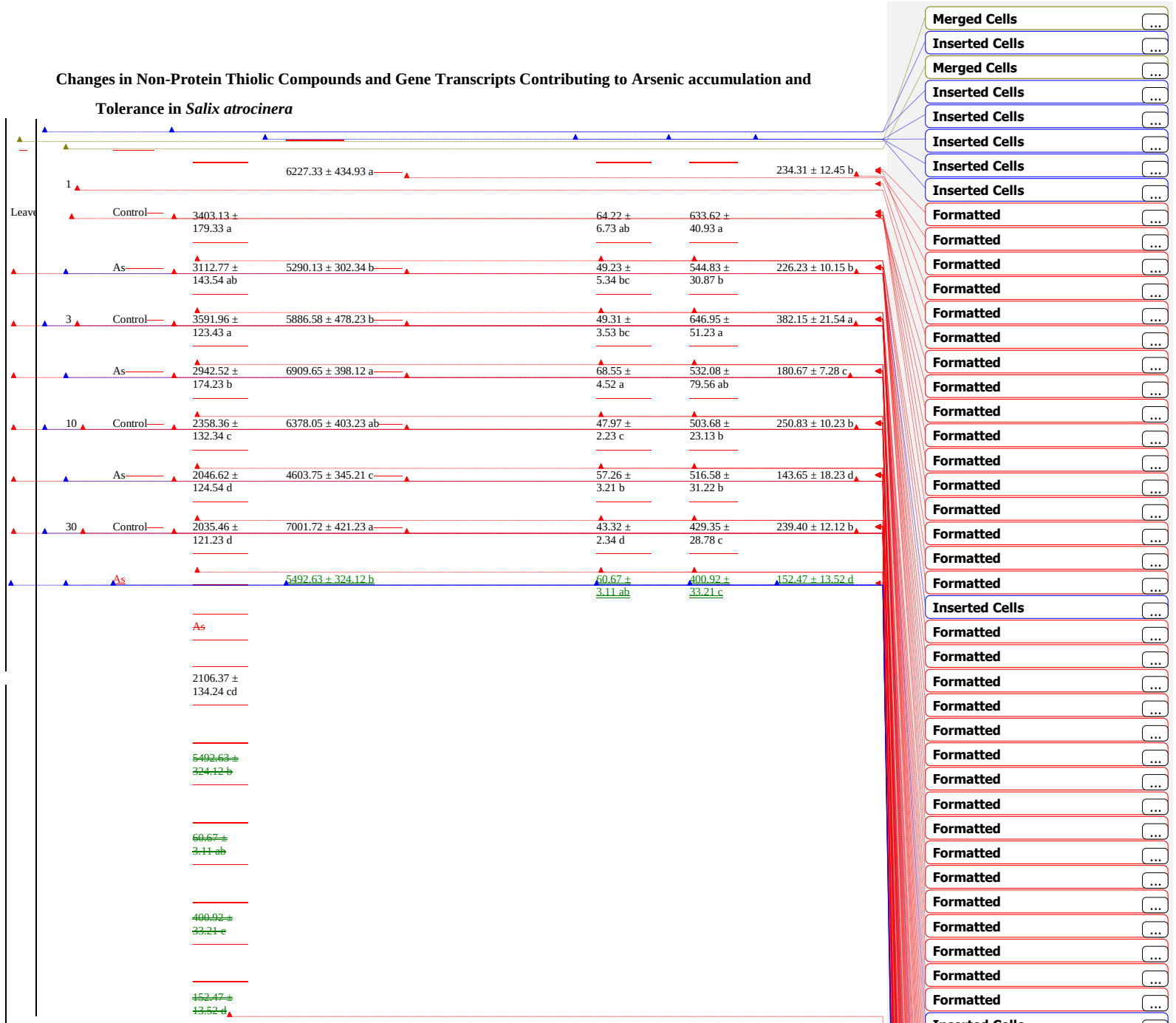
Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*



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Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

Table 2. Arsenic accumulation (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to arsenic for 30 days. Different letters within each column and plant tissue indicate significant differences among time points on HSD test at $p < 0.05$. nd: not detected.

Organ	Time point (d)	Arsenic		
		III	V	Total
Roots	1	16.14 ± 2.34 d	164.88 ± 149.33 d	182.43 ± 20.10 d
	3	33.45 ± 4.78 c	318.86 ± 21.95 c	353.65 ± 23.98 c
	10	929 ± 80.21 b	542.35 ± 41.29 b	1471.92 ± 123.87 b

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

	30	1688 ± 148.43 a	734.90 ± 65.20 a	2448 ± 178.32 a
Leaves	1	nd	2.78 ± 0.24 d	2.78 ± 0.24 d
	3	1.30 ± 0.08 e	5.76 ± 0.45 c	7.23 ± 0.39 c
	10	nd	18.75 ± 1.14 b	18.75 ± 1.14 b
	30	nd	25.45 ± 2.57 a	25.45 ± 2.57 a

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

**Table ~~4. Arsenic accumulation (mg Kg3~~. Non-protein thiolic peptides (nmol GSH g^{-1} ~~DW~~FW) in roots and leaves of *S. atrocinerea* exposed

~~1031~~ to control and arsenic

~~1032~~ conditions for 30 days. Different letters within each ~~column~~row and plant tissue indicate significant differences among ~~1033~~treatments and time points on HSD test at $p < 0.05$. nd: not detected.

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Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

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Organ	Thiol	1 d		3 d		10 d		30 d	
		Control	As	Control	As	Control	As	Control	As
Roots	Cys	nd	5.85 ± 1.73 a	nd	19.38 ± 0.18 b	nd	7.99 ± 2.15 a	Nd	4.06 ± 0.15 c
	GSH	13.73 ± 0.93 a	7.90 ± 0.66 b	11.68 ± 1.59 a	6.91 ± 0.55 bc	15.15 ± 1.83 a	6.15 ± 0.74 c	12.80 ± 1.40 a	6.05 ± 0.52 c
	TC_1	nd	14.69 ± 1.11 a	nd	10.62 ± 0.74 b	nd	6.72 ± 1.25 c	Nd	nd
	PC_2	nd	13.63 ± 1.85 b	nd	16.72 ± 1.78 ab	nd	19.10 ± 1.03 a	Nd	18.42 ± 1.75
	$Cys\text{-}PC_2$	nd	10.37 ± 0.76 d	nd	13.46 ± 1.51 c	nd	23.13 ± 0.02 b	Nd	34.35 ± 1.48
	TC_2	nd	6.78 ± 0.14 d	nd	10.17 ± 0.32 c	nd	17.12 ± 0.45 b	Nd	21.79 ± 0.29
	PC_3	nd	20.32 ± 1.40 d	nd	33.64 ± 1.09 c	nd	47.01 ± 9.54 b	Nd	65.38 ± 1.06
	$desGly\text{-}PC_3$	nd	8.88 ± 0.53 d	nd	34.35 ± 2.27 c	nd	73.86 ± 4.27 b	Nd	150.19 ± 12
	$Cys\text{-}PC_3$	nd	10.91 ± 0.71 d	nd	61.14 ± 1.74 c	nd	74.34 ± 3.73 b	Nd	169.27 ± 11
	Total ΣNPTs	13.73 ± 0.93 e	99.34 ± 2.71 d	11.68 ± 1.59 e	174.85 ± 18.70 c	15.15 ± 1.83 e	267.57 ± 12.93 b	12.80 ± 1.40 e	469.52 ± 21
Leaves	GSH	43.09 ± 2.53 b	49.35 ± 1.80 a	40.30 ± 3.45 bc	49.87 ± 3.33 a	44.70 ± 2.78 ab	37.74 ± 1.88 c	45.63 ± 2.45 ab	41.41 ± 1.86
	$desGly\text{-}PC_2$	nd	2.22 ± 0.31 d	nd	5.18 ± 1.03 c	nd	7.65 ± 0.53 b	Nd	9.39 ± 0.88 a

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

desGly-PC_4	2.50 ± 0.11 d	2.52 ± 0.10 d	2.46 ± 0.14 d	3.06 ± 0.36 c	2.74 ±0.12 cd	2.87 ± 0.14 c	4.09 ± 0.52 b	6.46 ± 0.48 a
TC_3	4.48 ± 0.38 b	5.45 ± 0.23 a	4.04 ± 0.49 b	6.25 ± 0.64 a	4.04 ± 0.34 b	5.08 ± 0.70 ab	3.03 ± 0.18 c	3.95 ± 0.41 b
Total ΣNPTs	55.40 ± 1.89 bc	67.60 ± 1.45 a	50.54 ± 3.45 c	69.58 ± 5.70 a	53.56 ± 4.05 bc	58.16 ± 2.50 b	54.70 ± 4.11 bc	62.51 ± 5.94 ab

Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*

Table 5. Relative gene expression levels in roots and leaves of *S. atrocinerea* exposed to arsenic for 30 days of genes with a fold regulation higher than 2. Values are mean normalized expression relative to the non-exposed accession at each time point (set at 1.00) ± S.D. of at least three biological replicates, each containing at least one individual plant. Statistically significant at $p < 0.05$ As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (= upregulation; = downregulation). Different letters within each column and plant tissue indicate significant differences among time points on HSD test at $p < 0.05$.

Organ	Gene	Description	Annotation *S. purpurea* V1.0	Time point (d) 1	3	10	30
Roots	*NIP1*	Aquaporins	SapurV1A.0029s0170.1	11.95 ± 0.27 a	5.60 ± 0.48 b	4.09 ± 0.81 b	3.69 ± 0.23 c
	SIP1		SapurV1A.1058s0060.1	6.64 ± 0.53 a	2.09 ± 0.44 b	1.87 ± 0.10 d	1.30 ± 0.19 d
	BORON	Boron transporter	SapurV1A.0014s0200.1	[redacted]	2.74 ± 0.16 a	2.79 ± 0.30 a	2.68 ± 0.17 a
	CDC25-1	Arsenate reductase	SapurV1A.0142s0310.1	2.22 ± 0.44 b	3.39 ± 0.24 b	2.46 ± 0.35 a	2.92 ± 0.17 a
	AIP-1	Chaperone	SapurV1A.0229s0030.1	41.06 ± 2.67 a	7.66 ± 0.90 d	15.85 ± 0.74 b	11.30 ± 0.64 c
	ACCS	ACC synthase	SapurV1A.2160s0020.1	33.86 ± 0.45 a	30.25 ± 2.62 b	9.21 ± 0.70 c	7.87 ± 0.93 c
	GS	Glutathione synthetase	SapurV1A.1124s0080.1	17.28 ± 0.69 a	5.16 ± 0.16 b	5.01 ± 0.47 b	3.26 ± 0.20 c
	WBABCT	Vacuolar transporter	SapurV1A.0084s0020.1	1.44 ± 1.01 b	2.01 ± 0.13 a	4.34 ± 2.35 a	2.27 ± 0.53 a
Leaves	*ACCS*	ACC synthase	SapurV1A.2160s0020.1	3.16 ± 0.26 a	0.68 ± 0.05 c	1.80 ± 0.12 b	3.81 ± 0.30 a

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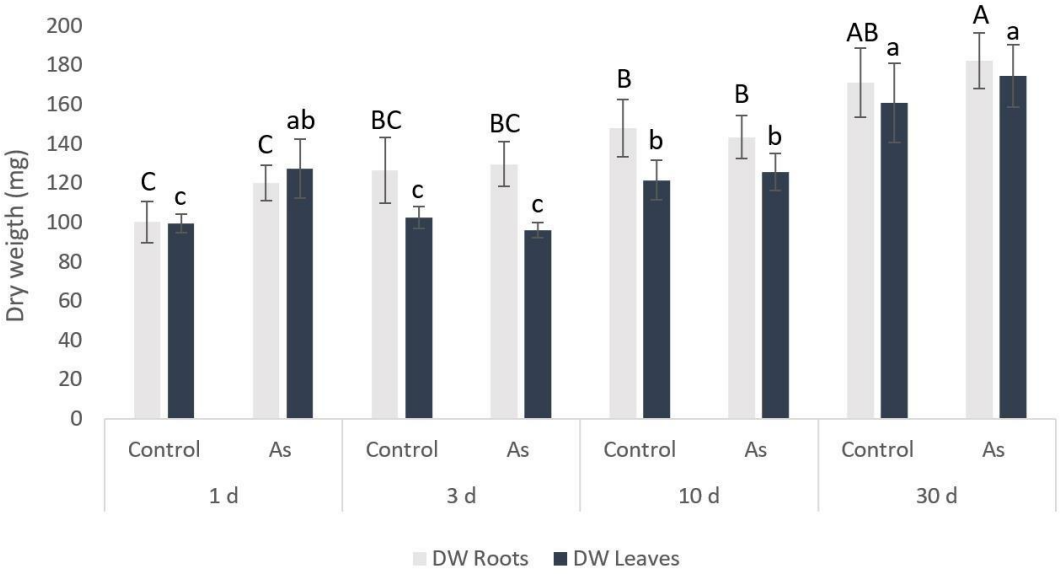

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~~Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*~~

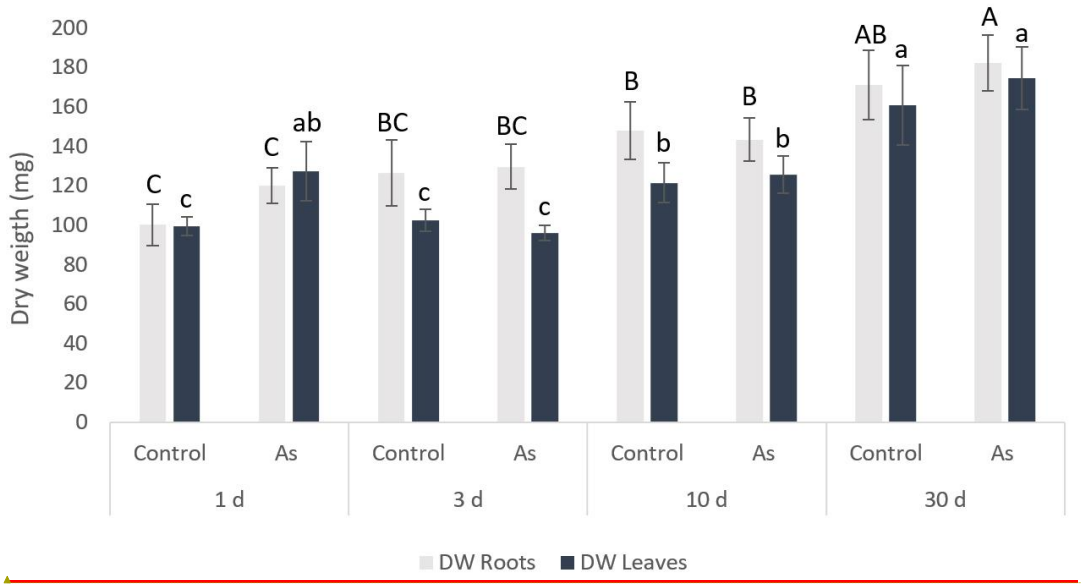

Fig. 1. Dry weight (mg DW) of roots and leaves of *S. atrocinerea* exposed to control and ~~arsenic~~As conditions for 30 days. Different letters (upper case for comparison within roots and lower case for comparisons within leaves) denote significant differences on HSD test at $p < 0.05$.

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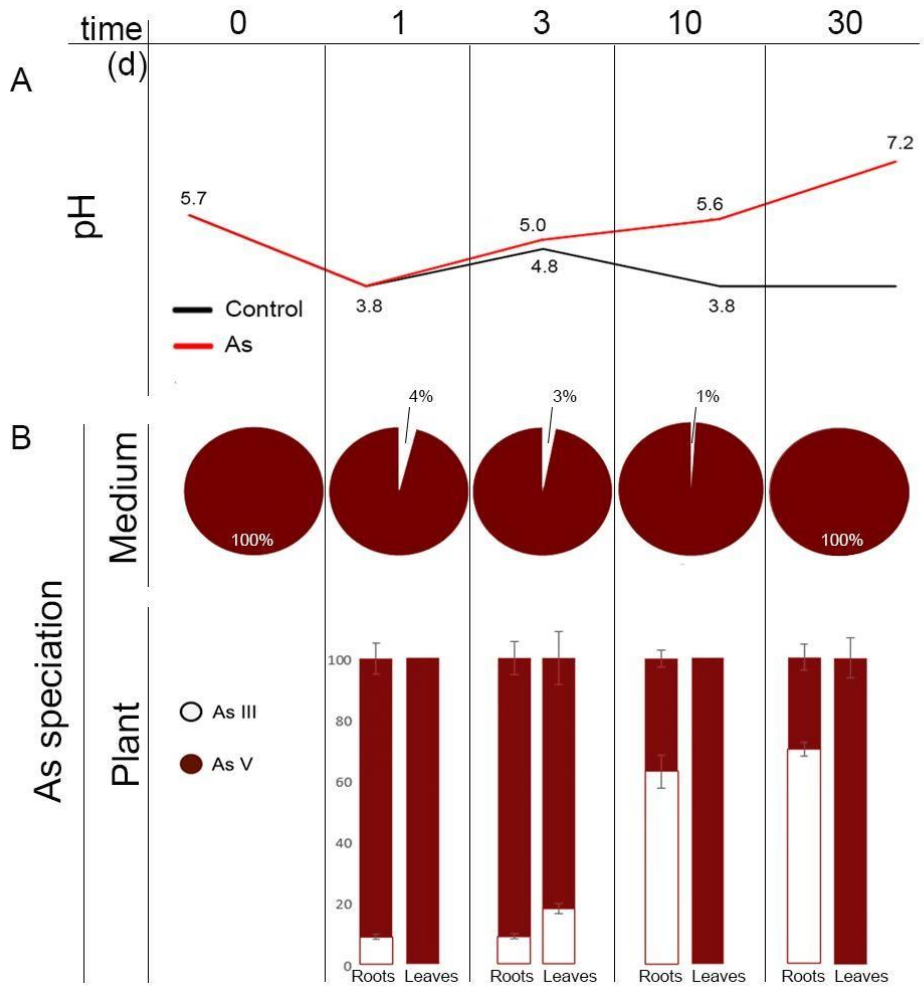

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~~Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*~~

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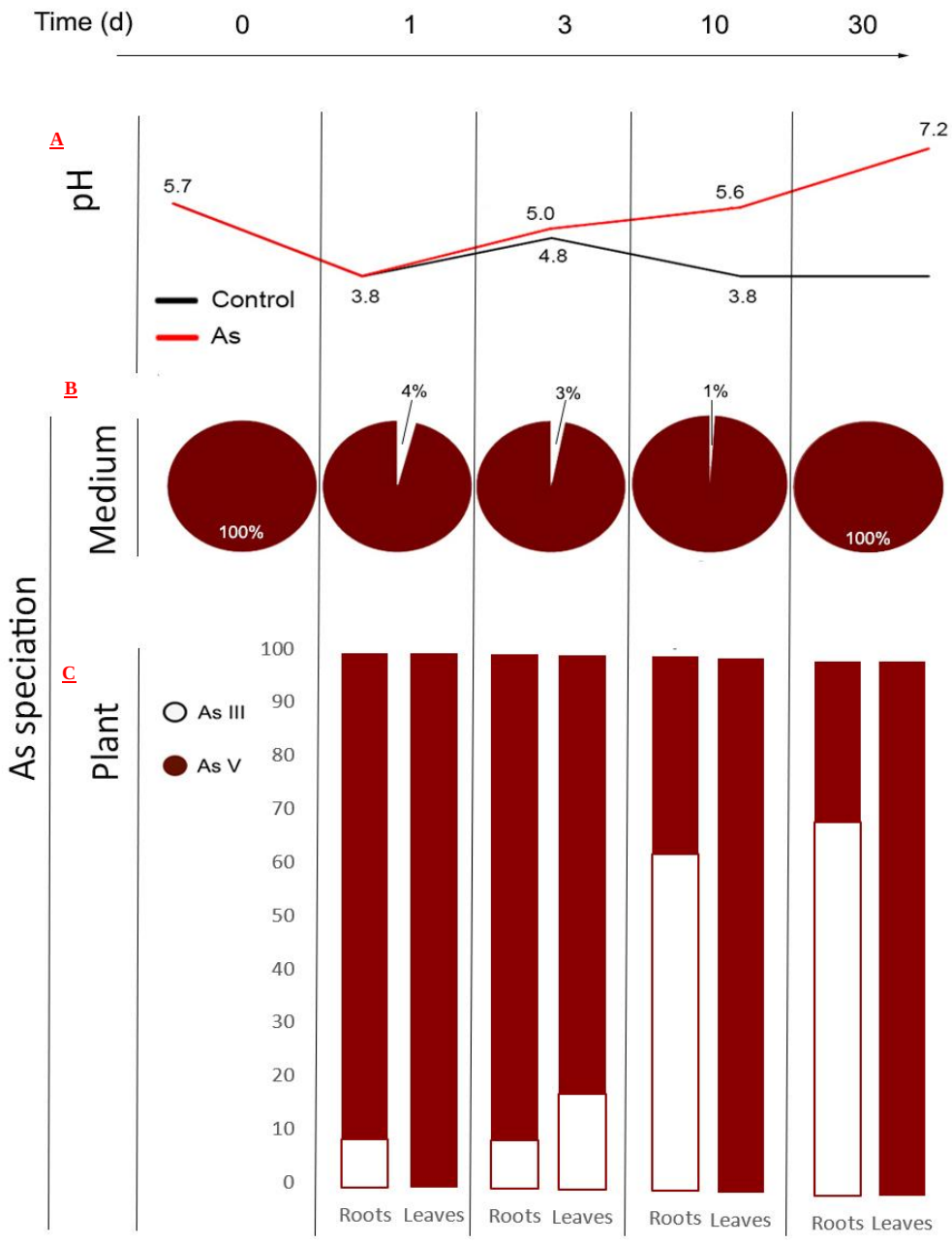

Fig. 2. pH in the culture medium **(A~~). Percentage~~)** and percentage of arsenic speciation in the culture

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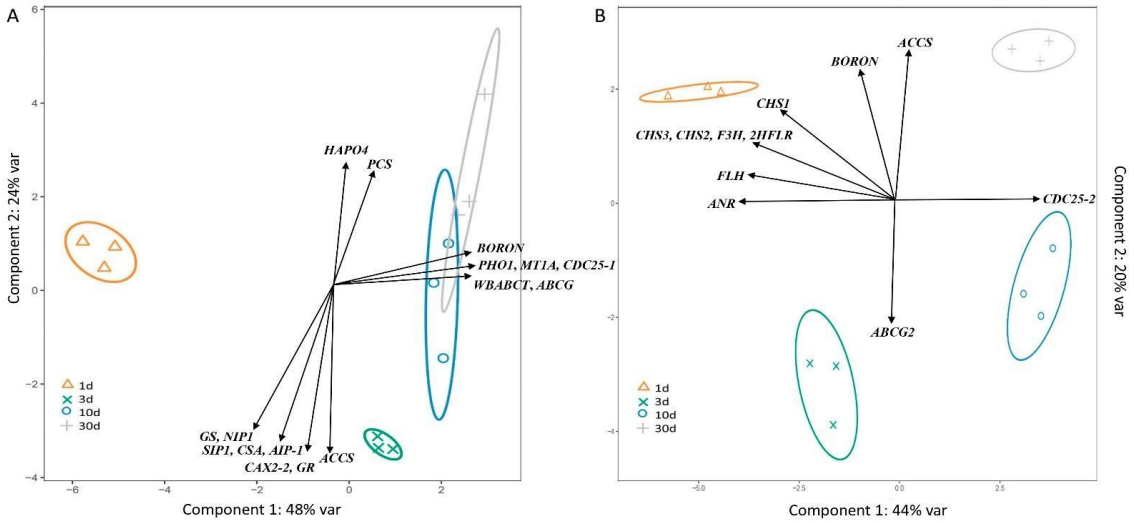
~~Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera*~~

medium (B) and roots and leaves (~~B~~C) of *S. atrocinerea* exposed to arsenic for 30 days (Red: As V, white: As III). ~~1071~~



~~Fig 3.~~ ~~Biplots of the principal component analysis~~

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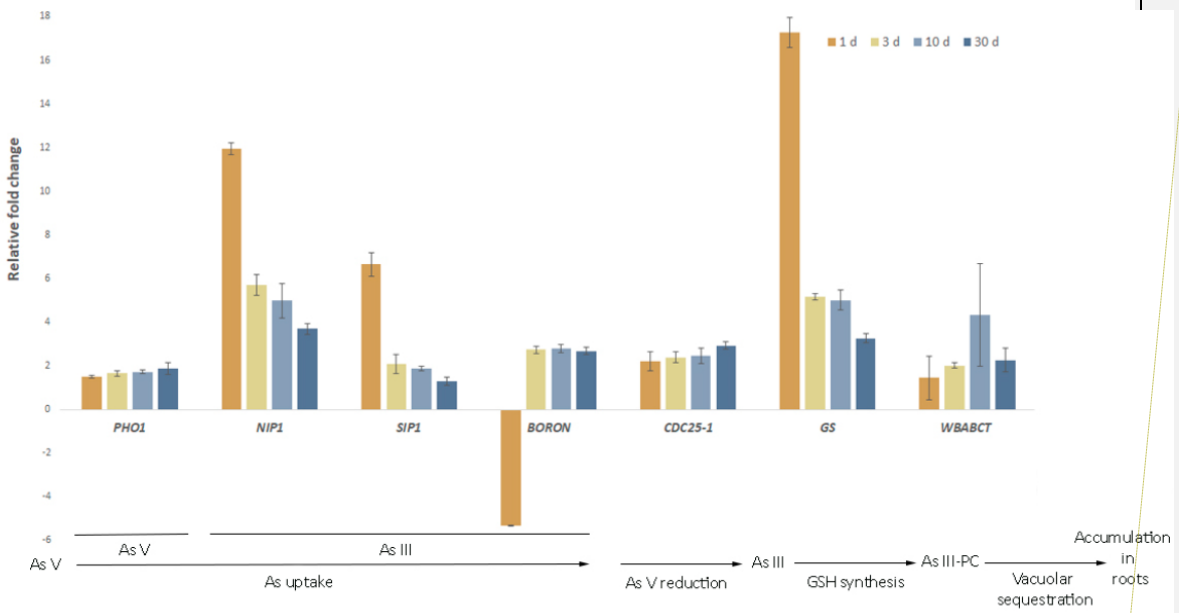

Fig. 3. Relative fold change of the gene expression levels in roots of *S. atrocinerea* exposed to As regarding those genes involved in As uptake and reduction, thiol synthesis and vacuolar sequestration, that showed the most markedly regulation along the 30 days. Values represented are the fold change (± S.D.) of mean normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *BORON*, boron transporter; *CDC25-1*, tyrosine phosphatase 1, *GS*, glutathione synthetase; *NIP1*, aquaporin NIP1.1; *PCS*, phytochelatin synthase; *PHO1*, phosphate transporter PHO1; *SIP1*, aquaporin SIP.1; *WBABCT*, white-brown-complex ABC transporter.

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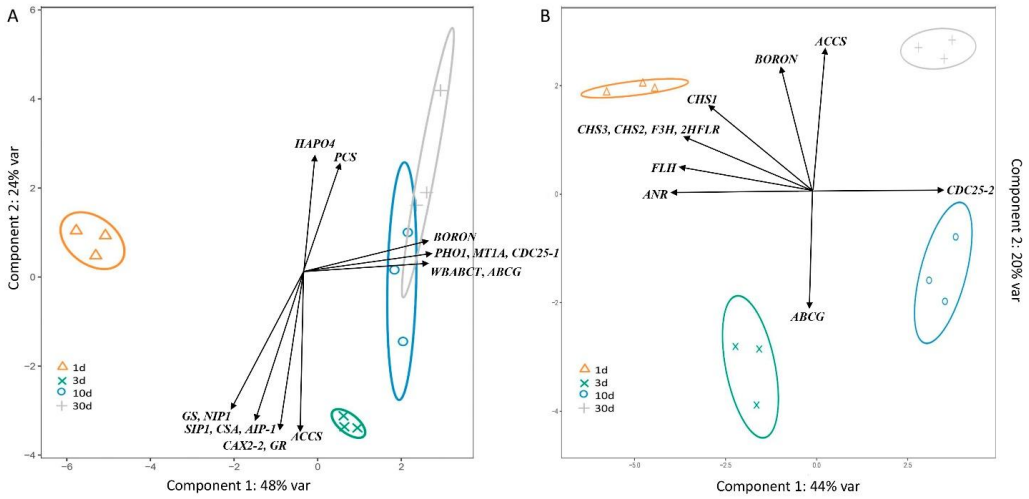

Fig 4. Biplots of the principal component analysis (PCA) in samples of roots **(A)** and leaves **(B)** of *S. atrocinerea* exposed to arsenic for 30 days calculated with the normalized gene expression levels relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant ~~of each measured gene in roots (left) and leaves (right) of *S.*~~. *2HFLR*, dihydroflavonol 4-reductase; *ABCG*: ABC transporter G; *ACCS*, aminocyclopropane-1-carboxylate synthase; *AIP-1*, arsenite-inducible RNA-associated protein AIP-1-related; *ANR*, anthocyanidin reductase; *BORON*, boron transporter; *CAX2-2*, vacuolar cation/proton exchanger 2; *CDC25-1*, *2*, tyrosine phosphatase 1, 2; *CHS1*,*3*, chalcone synthase 1,3; *CSA*, cellulose synthase A; *F3H*, flavanone 3-hydroxylase; *FLH*, Flavonoid 3'-hydroxylase; *GR*, glutathione reductase; *GS*, glutathione synthetase; *HAPO4*, high-affinity phosphate transporter 4; *MT1A*, Metallothionein; *NIP1*,

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aquaporin NIP1.1; *PCS*, phytochelatin synthase; *PHO1*, phosphate transporter PHO1; *SIP1*, aquaporin SIP.1; *WBABCT*, white-brown-complex ABC transporter.

~~*atrocinerea* exposed to arsenic for 30 days. Values are the normalized expression relative.~~

~~Abbreviations: see **Table 1.**~~

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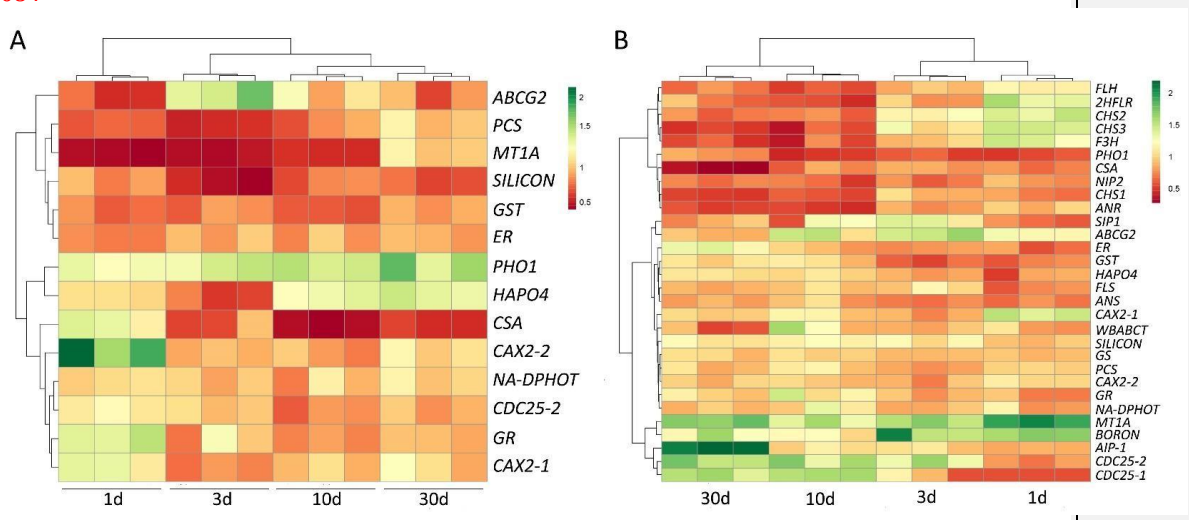

Fig 4.

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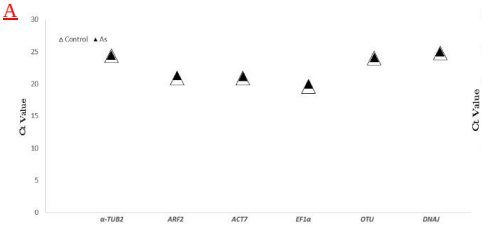

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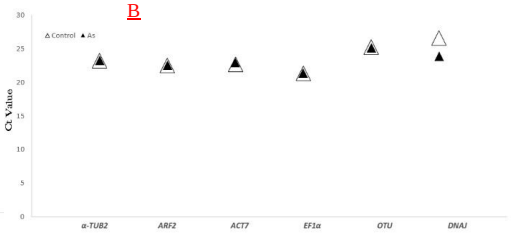

Supplementary Fig. 1. qRT-PCR Ct values of the 6 candidate RGs in control and As conditions. Mean values.

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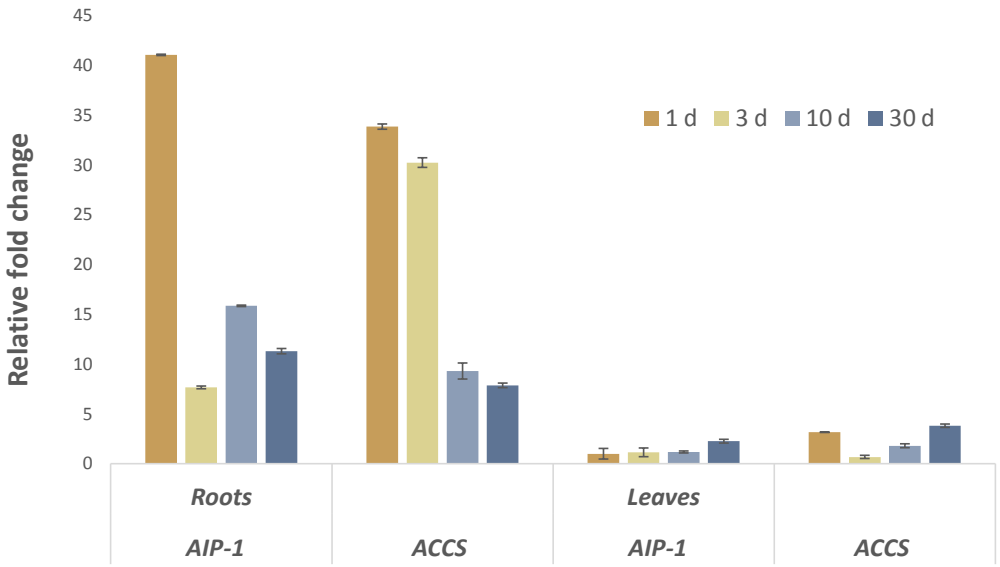

Supplementary Fig. 2. Relative fold change of the gene expression levels in roots of *S. atrocinerea* exposed to As regarding those genes involved in stress response that showed the most markedly regulation along the 30 days. Values represented are the fold change (± S.D.) of mean normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *ACCS*, aminocyclopropane-1-carboxylate synthase; *AIP-1*, arsenite-inducible RNA-associated protein AIP-1-related.

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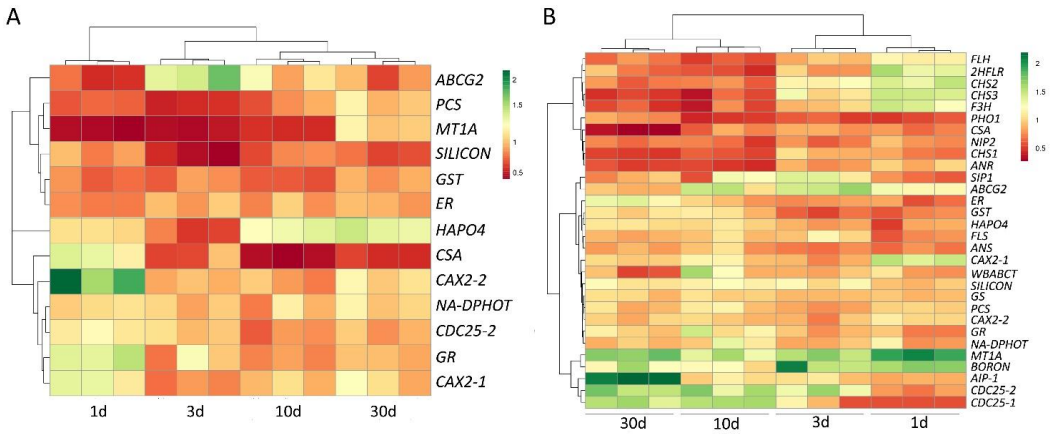

Supplementary Fig. 3. Heat map representations of the gene expression data obtained in samples of roots **(A)** and leaves **(B)** of *S. atrocinerea* exposed to arsenic for 30 days and hierarchical clustering based on the most differentially expressed genes with a fold regulation equal or lower than 2. Gene expression ~~levels~~level values are the normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. ~~Abbreviations:~~For gene abbreviations see ***Supplementary Table 1***.

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~~**Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in *Salix atrocinera***~~

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*Graphical Abstract

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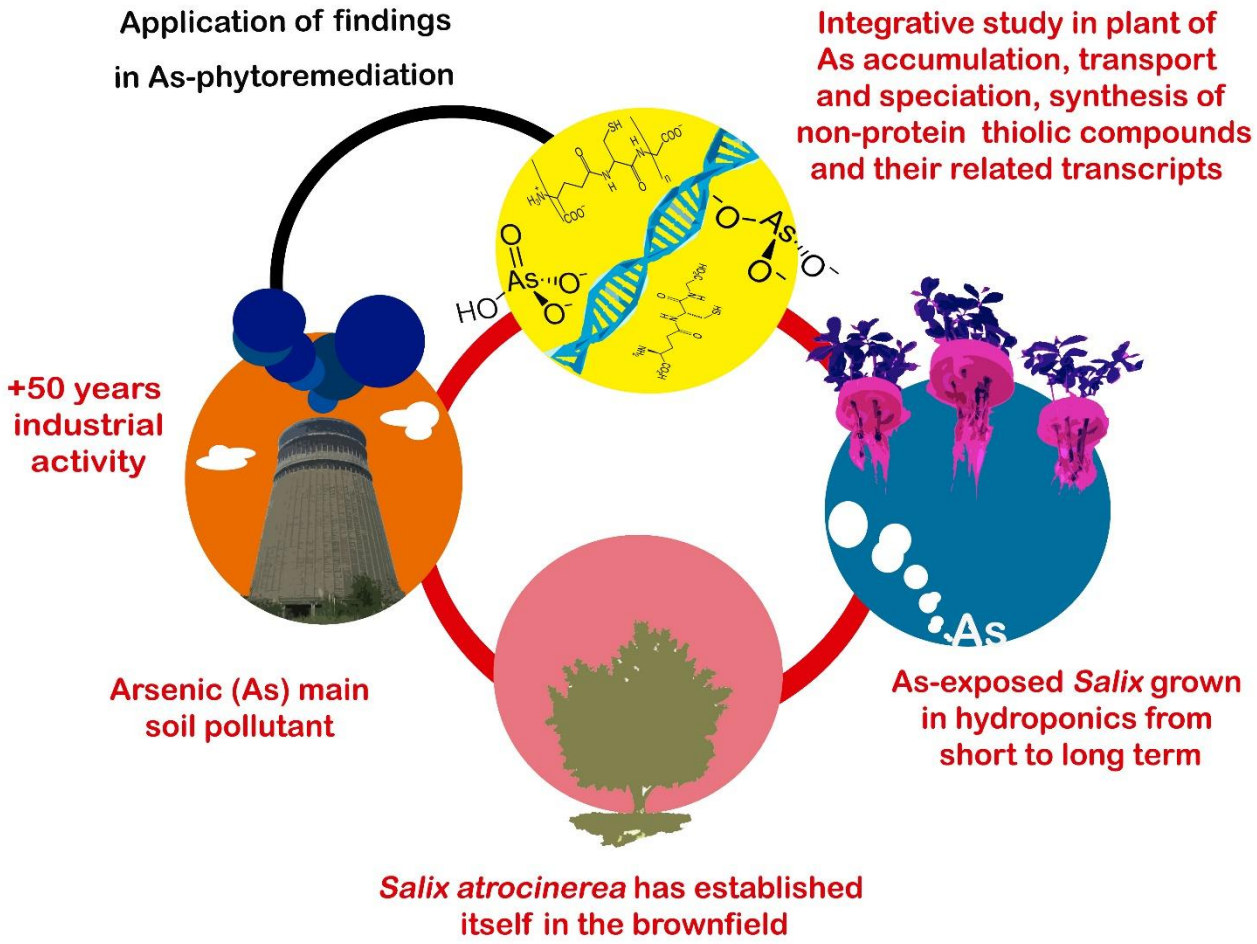

***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

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- *Salix atrocinerea* accumulates and tolerates high As concentrations in its tissues.
- Inside the roots As V rapidly reduces to As III and accumulates.
- As exposure decreased P and increased Ca and Fe concentrations in roots.
- Transcriptional regulation of As transporters and reductases are key for tolerance.
- *De novo* synthesis and accumulation of thiols occurs in As-exposed plants.

Integrative response of Arsenic Uptake, Speciation and Detoxification by *Salix atrocinerea*

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Abstract

Despite arsenic (As) being very toxic with deleterious effects on metabolism, it can be tolerated and accumulated by some plants. General genetic mechanisms responsible for As tolerance in plants, including *Salix* species, have been described in transcriptomic analysis, but further experimental verification of the significance of particular transcripts is needed. In this study, a *Salix atrocinerea* clone, able to thrive in an As-contaminated brownfield, was grown hydroponically in controlled conditions under an As concentration similar to the bioavailable fraction of the contaminated area (18 mg kg^{-1}) for 30 days. At different time points, i.e. short-term and long-term exposure, biometric data, As accumulation, phytochelatin synthesis, non-protein thiol production and expression of target genes related to these processes were studied. Results showed that *S. atrocinerea* presents a great tolerance to As and accumulates up to 2,400 mg As kg^{-1} dry weight in roots and 25 mg As kg^{-1} dry weight in leaves. Roots reduce As V to As III rapidly, with As III being the predominant form of As accumulated in root tissues, whereas in the leaves it is As V. After 1 d of As exposure, roots and leaves show *de novo* synthesis and an increase in non-protein thiols as compared to the control. Integrating these data on As accumulation in the plant and its speciation, non-protein thiol production and the kinetic gene expression of related target genes, a fundamental role is highlighted for these processes in As accumulation and tolerance in *S. atrocinerea*. As such, this study offers new insights in the plant tolerance mechanisms to As, which provides important knowledge for future application of high-biomass willow plants in phytoremediation of As-polluted soils.

Keywords: *Salix*, arsenic, non-protein thiols, speciation, phytochelatins, gene expression

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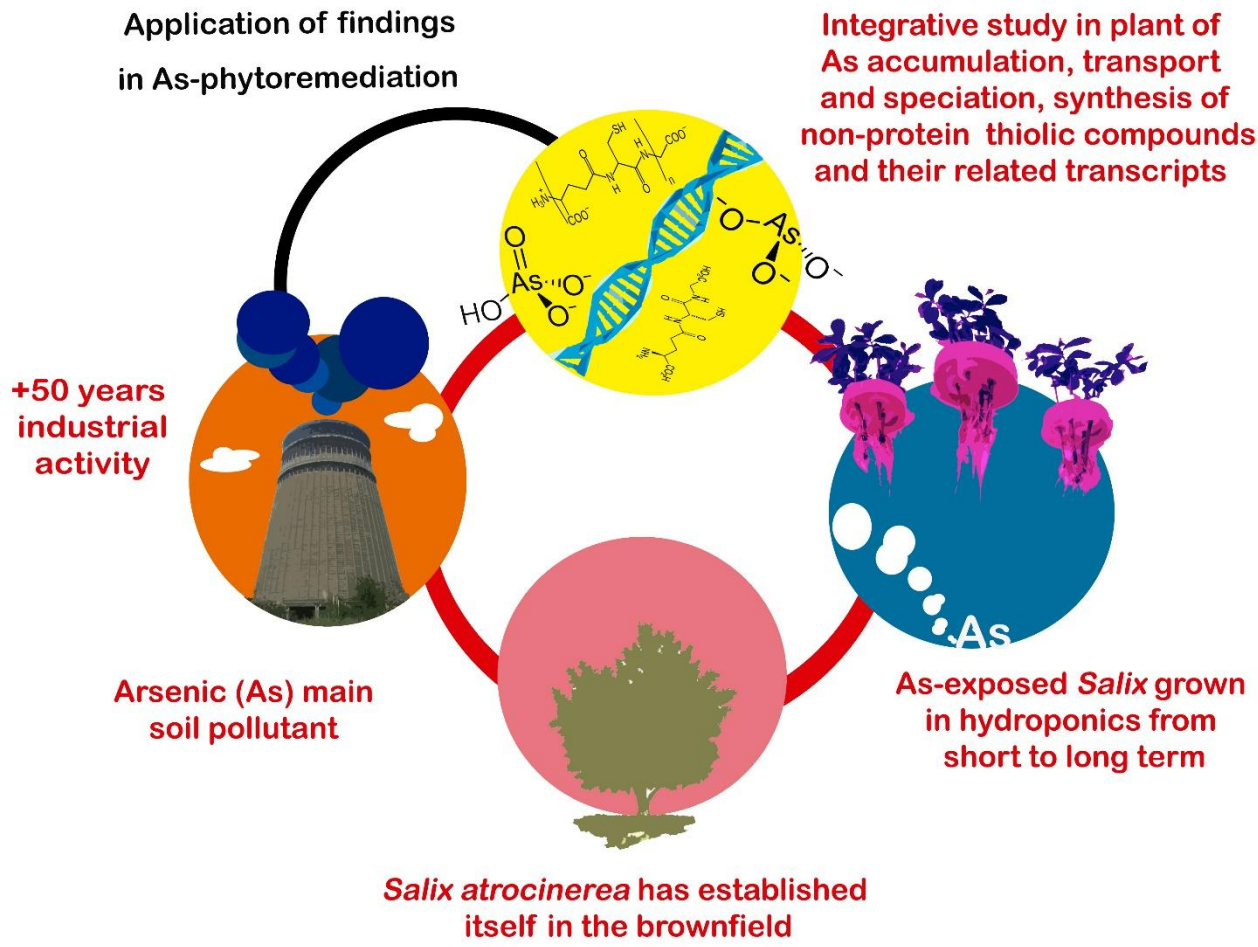

1 Introduction

Arsenic (As) is a metalloid widely spread in the upper Earth's crust although at very low concentrations. The overall mean value of the total As for different soils is estimated as 6.83 mg kg^{-1} soil. However, As soil concentrations may range from 0.1 to more than 1,000 mg kg^{-1} in some locations due to both anthropological and geological factors (Kabata-Pendias, 2010). Concerning its toxicity, As is the only known human carcinogen for which there is adequate evidence of carcinogenic risk for both exposure routes, inhalation and ingestion (Smith et al., 2009). Therefore, As has been defined as a group 1 carcinogen and is placed in the highest health hazard category by the international agency for research on cancer (Naidu et al., 2006). By the use of natural resources, humans release As into the air, water and soil (Mandal and Suzuki, 2002). Sixty percent of the anthropogenic As emissions can be accounted to only two sources: Cu-smelting and coal combustion. Nevertheless, the application of herbicides, Pb and Zn smelting, glass production, wood preservation, waste incineration and steel production are also responsible for As emissions (Matschullat, 2000). According to the European Commission (2000), air contributes less than 1% of the total As exposure since most of this emitted As ends up retained in the water and soils, making these the major sources of As exposure to humans.

Once inside the cell, As toxicity depends on its speciation state. Arsenite (As III) has a high affinity for sulfhydryl groups found in the amino acid cysteine. As such, it inactivates a wide range of enzymes by disrupting protein structure and impairs the metabolism by preventing protein–protein interactions (Ehlrich, 1990). This affects many key metabolic processes in the cell such as fatty acid metabolism, glucose uptake and glutathione production (Paul et al., 2007; Ahsan et al., 2008; Wang et al., 2015). Arsenate (As V) is a phosphate analogue and can substitute inorganic phosphate affecting ATP synthesis and therefore interrupting the production of energy, carbon metabolism and nucleic acid synthesis (Singh et al., 2011; Spratlen et al., 2017). This can also negatively affect DNA

repair and methylation and thus impact on gene expression (Reichard and Puga, 2010). Therefore, removal or lowering of As concentrations from highly As-polluted soil and water is an environmental priority. Among the most eco-friendly cleanup technologies and opposite to traditional excavation and disposal in landfills, phytoremediation emerges. This green technology, already described more than two decades ago by Raskin et al. (1994), exploits the ability of certain plants species to accumulate metal(loid)s in their tissues, thus reducing their concentrations or attenuating their mobility in the environment, and therefore offering a solution to the above-mentioned pollution challenge (Pilon-Smits, 2005; Kidd et al., 2015).

It is well known that toxic metal(loid)s induce loss of plant biomass, among other deleterious effects, mainly associated with growth inhibition (Gill et al., 2015). Plants differ in As tolerance, from sensitive plant species like all major crops, to tolerant plants such as certain ecotypes of the grass *Holcus lanatus* (Quaghebeur and Rengel, 2003), as well as hyperaccumulators like *Pteris vittata* (Chinese break fern), which can accumulate 2% of its dry weight as As (Wang et al., 2002). However, hyperaccumulator species are usually limited by a low biomass production, which may pose serious restrictions to this cleaning procedure (Shelmerdine et al., 2009, Fernández et al., 2010). Some plant species and soil biota populations, usually autochthonous to polluted soils, are able to colonize and thrive in highly polluted environments, even when high concentrations of metals are found in their cells and tissues. This is the case of *Salix atrocinerea* (grey willow). So far, about 450 species of *Salix* worldwide have been described (Argus, 1995), with some of them reported as suitable in phytoremediation processes because of their high growth rate and deep-rooting traits (Kuzovkina and Quigley, 2005; Janssen et al., 2015). Nevertheless, the focus on the use of *Salix* for As uptake is still low because it is not a metal(loid) hyperaccumulating species. However, some investigations have highlighted its phytoremediation potential for As (Purdy and Smart, 2008; Puckett et al., 2012; Yanitch et al., 2017). In addition, complementary studies exploring the

feasibility of high biomass plants to extract metals from polluted soils such as willow, concluded that the high biomass compensates for the moderate metal concentrations found in the aboveground tissues (Hammer et al., 2003; Ruttens et al., 2011).

Understanding As tolerance in plants is useful to know whether plants avoid As uptake and, thus, reduce the As intake by humans and the As-associated health problems (Song et al., 2010), or enhance As uptake and its removal by phytoremediation (Yang et al., 2012). To achieve this, it is necessary to study the As behavior from the soil to its accumulation in the aboveground plant tissues. Although As is toxic and not essential for plants it is effectively absorbed through various transporters into the roots, mainly as As V, the most thermodynamically stable and hence dominant species in aerobic environments (Quaghebeur and Rengel, 2003). As such, As transporters include the high affinity phosphate uptake systems for As V (Shin et al., 2004; Catarecha et al., 2007; LeBlanc et al., 2013), while As III uses the silicon transporters (Xu et al., 2015; Lindsay and Maathuis, 2016). Once inside the plant cells, a small amount may be transported to the xylem but the majority is reduced to As III by arsenate reductases (Ellis et al., 2006; Duan et al., 2007; Zhao et al., 2009). In this form, As can be exported back into soil, transported via the xylem to stem and leaves, or complexed with thiol-rich molecules like metallothioneins (MTs), glutathione (GSH) or, more stably, by phytochelatins (PCs) (Schmöger et al., 2000; Hartley-Whitaker et al., 2001; Dave et al., 2013; Batista et al., 2014). Then these As-PCs complexes can subsequently be transferred from the cytosol into the vacuole by ABC transporters for storage in order to prevent cell damage (Song et al., 2010). Therefore, this suggests that non-protein thiols (NPTs) compounds play an important role in decreasing As toxicity in plants and preventing its transport from roots to shoots.

Apart from the works on arsenic with *Salix* of Purdy and Smart (2008), Puckett et al. (2012), and more recently the extensive transcriptomic study by Yanitch et al. (2017) that have provided unequivocal useful information to understand the tolerance of *Salix* to As, still an integrative

approach concerning the tolerance mechanisms of *Salix* to As is needed. Besides, special attention needs to be paid to the speciation state of As, since this determines its uptake and also its tolerance by the plant. In the current study, a *S. atrocinerea* clone, previously selected for its As accumulation (unpublished data), was grown hydroponically in the presence of As V. Samples were harvested at different time points to kinetically study As accumulation and its chemical speciation in roots and shoots. In addition, the production of NPTs as well as the expression of the main transcripts involved in the genetic response behind As tolerance were also measured. Therefore, this study aims to describe the As uptake and accumulation in *S. atrocinerea*, together with the changes in the mechanisms involved in As tolerance at different biological organization levels.

2 Material and Methods

2.1 Plant material and hydroponic culture conditions

Salix atrocinerea plants were selected from an *in vitro* willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Stem cuttings of 15 cm length were placed on cellulose plugs in a hydroponic system containing 50 mL of 1/10 Woody Plant Medium (pH 5.7) (Lloyd, 1981) with an aeration system to prevent lack of oxygen (Moreno-Jimenez et al., 2010). After 3 weeks of growth, 48 cuttings were exposed to 0 and 18 mg L^{-1} As. This As concentration was similar to that found at the exchangeable fraction of the Nitrastur brownfield soil. The As was added as sodium heptahydrate arsenate, $Na_2HAsO_4.7H_2O$, since in this form the As is freely soluble.

Plants were cultured under a 12 h light photoperiod and 22 °C/18 °C with 65% relative humidity. Light was provided by a combination of blue, red and far-red Philips Green-Power LED

155 modules, simulating the photosynthetically active radiation (PAR) of sunlight. The PAR level
156 reached 170 μmol m^{-2} s^{-1} at the plant apex level.

157 After 1, 3, 10 and 30 days (d), plants were carefully removed from beakers and roots
158 exhaustively rinsed with tap water first, and 3 times with double de-ionized water at 4 °C. Leaves
159 were rinsed only once in distilled water. To determine the influence of the treatments on plant
160 growth, fresh and dry weights and lengths of roots and leaves were measured. Leaves and root
161 samples of at least 3 different plants were analyzed individually for each treatment. Plant material
162 was homogenized with liquid nitrogen and stored at −80 °C until further use.

163

164 **2.2 Analysis of essential elements, arsenic and arsenic speciation**

165 Nutrients, such as boron (B), calcium (Ca), iron (Fe), phosphorus (P), and zinc (Zn), together
166 with As, were determined in leaves and roots of *S. atrocinerea*. For this, 100 mg of dry powdered
167 samples were dissolved in 8 mL of 50% nitric acid solution (Sigma, Aldrich, USA) using a
168 microwave at 800 W during 15 min (Multiwave3000, Anton Paar). The solutions were diluted up to
169 50 mL with ultrapure water and filtered through a 0.45 μm polytetrafluorethylene (PTFE) filter prior
170 to their analysis. Plant samples were analyzed by ICP-MS (Agilent Technologies 7700 ICP-MS)
171 using isotopic dilution analysis (IDA) as previously described (Gallego et al., 2015).

172 To determine the As speciation in leaves and roots, 100 mg of dry powdered samples were
173 extracted in 2.5 mL of 0.3 M nitric acid solution at 95 °C for 90 min (Huang et al., 2012). The
174 extracts were centrifuged at 3000 *g* during 15 min and the supernatants were filtered through a 0.45
175 μm PTFE membrane filter. The solutions were neutralized by the addition of NaOH. The As species
176 were separated through a mobile phase of 0.2 M EDTA dissolved in 2 M PBS (Phosphate Buffered
177 Saline; pH 6.0) in a separation column with a 1260 Infinity HPLC coupled to a 7700 ICPMS (both

178 from Agilent Technologies). Identification of As species was confirmed by spiking real extracts with
179 a mixture of standard solutions: As III, As V, monomethylarsenic acid (MMA), and dimethylarsenic
180 acid (DMA).

181 **2.3 Analysis of non-protein thiolic compounds**

182 The extraction and analysis of non-protein thiols (NPTs) were carried out from 150 mg of fresh
183 weight leaves and roots of *S. atrocinerea* following the protocol described by Fernández et al. (2012).
184 The high-performance liquid chromatography (HPLC) separation was performed using a
185 chromatograph Waters 600 (Waters Corporation) with a post-column derivatization with Ellman's
186 reagent (Ellman, 1959). The sample (100 µL) was injected into a Kromasil 100 C18 5 µm
187 (250 × 4.6 mm) column (Scharlau) and eluted with solvent A (acetonitrile: H_2O, 2: 98 (v/v) to which
188 0.05% trifluoroacetic acid (TFA) was added) and solvent B (acetonitrile: H_2O, 98: 2 (v/v) also with
189 0.05% TFA). Samples were separated using a linear gradient (0–25% in 25 min and 25–50% in
190 5 min) of solvent B at 1.5 mL min^{-1} flow for 30 min. The derivatized thiols were detected at 412 nm
191 using a Waters 996 photodiode array detector and the obtained peaks were identified by comparison
192 with the standards of GSH and a mix of PCs. The quantitative changes in the thiol compounds
193 observed were calculated by the integration of their peak areas at 412 nm converted into nmol and
194 quantified as GSH equivalents.
195

196 **2.4 Gene expression analysis**

197 Isolation of RNA was carried out using the protocol described by Chang et al. (1993) with
198 slight modifications. Frozen leaves or roots (100 mg) were homogenized with 550 µL of buffer
199 containing 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP),
200 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermidine and 2% β-

mercaptoethanol. Then, it was extracted twice by adding 550 µL of chloroform:isoamyl alcohol (24:1) and centrifuged at 14,000 *g* for 20 min at 4 °C. After addition of 10 µL LiCl (10 M), RNA was precipitated overnight at 4 °C and harvested by centrifugation at 14,000 *g* for 20 min at 4 °C. The pellet obtained was washed with 75% ethanol and resuspended in RNase free water. The concentration of RNA was determined spectrophotometrically at 260 nm using Nanodrop equipment (Isogen Life Science) and the RNA quality was tested using the Experion™ automated electrophoresis system (Bio-Rad). DNA was removed using a TURBO DNA-free Kit (Ambion) and the cDNA synthesis was performed using PrimerScript RT reagent Kit (Takara) with equal amounts of RNA input (1 µg). Finally, the cDNA was ten-fold diluted using a 1/10 dilution of TE (Tris-EDTA) buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at −20 °C.

Reverse Transcription quantitative PCR (RT-qPCR) was performed with an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. Gene forward and reverse primers (***Supplementary Table 1***) were designed using Primer 3 (Untergasser et al., 2012), according to sequences of genes obtained in the *Phytozome* nucleotide database of the closely related species *Salix purpurea* v1.0, for which the whole genome has been sequenced (Goodstein et al., 2012), only for three genes, high-affinity phosphate transporter (*HAPO4*), arsenite-inducible RNA-associated protein (*AIP-1*) and metallothioneins (*MT1A*), their sequences were obtained from willow reference sequences annotated at the NCBI (National Center for Biotechnology Information) (O'Leary et al., 2016). Their specificity was verified *in silico* using BLAST (http://www.arabidopsis.org/Blast/index.jsp). The genes measured (***Supplementary Table 1***) were selected based on different genetic aspects behind As tolerance (Konlechner et al., 2013; Puckett et al., 2012; Yanitch et al., 2017). The qPCR efficiency of the primers was determined using a standard curve consisting of a two-fold dilution series of a pooled sample. Only primers with an efficiency between 90 and 110% were used for qPCR analysis and their amplification specificity was

validated by melting curves. PCR amplifications were done in a total volume of 10 μL containing 2 μL cDNA sample, 5 μL SYBR Green, 0.6 μL of primers (300 nM) and 2.4 μL RNase free water. The reaction cycle was as follows: 20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C. Gene expression was calculated relatively as $2^{-\Delta Cq}$, in which ΔCq represents each corresponding quantification cycle (Cq) value minus the minimum Cq value observed (Schmittgen, 2008). Gene expression was normalized with a normalization factor based on the expression of six reference genes from *Salix* selected from literature under As and other abiotic stresses (Li et al., 2016; Zhang et al., 2017). The 6 selected candidate reference genes, *α-TUB2, Alpha-tubulin 2; ACT7, Actin 7; ARF2, ADP-ribosylation factor 2; DNAJ, Chaperone protein DnaJ 49; EF1α, Elongation factor 1-alpha* and *OTU; OTU-like cysteine protease* (***Supplementary Table 1***) are also orthologs of genes in *S. purpurea*. The primer sequences, amplicon length, PCR amplification efficiency and correlation coefficient are shown in ***Supplementary Table 1***. To evaluate the stability of the 6 candidate reference genes (RG) at the transcript level under As exposure, the gene expression levels were determined by the average Cq values (***Supplementary Fig. 1***). In order to detect the stabilities of 6 candidate RGs, the best combination of RG for normalization of our transcripts of interest was suggested by the Graynorm algorithm (Remans et al., 2014). In roots *AFR2, OTU* and *EF1α* were the three most stable reference genes in all the sample sets according to the *GrayNorm* algorithm and the combination of the three was used for normalization (***Supplementary Fig. 1A***). In leaves a different combination of genes than that obtained in roots showed the most stable pattern in all the sample sets, and therefore a combination of *α-TUB2, OTU* and *ACT7* was used for normalization (***Supplementary Fig. 1B***). In both roots and leaves the suggestion selected by *GrayNorm* corresponded to the genes less affected by As exposure.

A principal component analysis and heat maps were constructed to compare expression levels between different genes and samples at different time points.

2.5 Statistical analysis

To evaluate the effects of As toxicity in *S. atrocinerea* over the different time points on the measured variables, depending on the number of variables to compare a one-way or a two-way Analysis of variance (ANOVA) was performed. Log transformation was applied to approximate when it was necessary (e.g. to determine statistical significance of gene expression data, datasets were first log-transformed). Data normality was tested using the Shapiro-Wilk test, while homoscedasticity was verified via Bartlett's and Levene's tests. If data did not meet the normality assumption, a non-parametric Kruskall-Wallis test was used, followed by the Wilcoxon rank sum test. When the F ratio was significant ($p \leq 0.05$), Tukey's least significant difference test (HSD, $p \leq 0.05$) was employed to compare between individual means of different data groups (e.g. different treatments). In the gene analysis the previous was performed on both the normalized and the non-normalized data, although only the first are presented both were taking into account to establish the significance of the results. Results are expressed as the mean ± standard deviation of at least three independent replicates. All data were analyzed using R (version 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae (version 1.2e4, http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level $p \leq 0.05$.

3 Results

3.1 Plant growth and nutrient analysis

After 30 d of exposure, no external symptoms of phytotoxicity (data not shown) nor growth reduction, measured as dry weight, were observed between plants grown on control or As-containing medium (**Fig. 1**).

With regard to nutrient concentrations, total P concentration significantly decreased in roots from 10 d onwards in As-exposed plants as compared to controls, whereas in leaves the P concentration was lower in As-exposed plants as compared to controls at 3 and 10 d. However, P concentration in leaves was similar in both treatments after 30 d of exposure (**Table 1**). Accumulation of Ca increased in As-exposed roots along the exposure time when compared to control conditions. However, in leaves a Ca decrease was observed in As-exposed plants, except at 3 d (**Table 1**). Although the B concentration was slightly higher in roots of As-exposed plants, this increase was only significant at 3 d. In leaves, an increase in B concentration at 3 d was observed and this increase was maintained in As-exposed plants till the end of the experiment. For Zn concentrations, there was an increase in roots and a decrease in leaves as compared to control conditions at 1 d of As exposure. However, no differences were observed in for this elements at other time periods. Fe concentrations were higher in roots of As-exposed plants throughout the experiment, whereas the opposite trend was observed in leaves (**Table 1**).

With regard to the pH of the culture medium, we generally observed a decrease during the first 3 days of the experiment. However, an increase from 10 d onwards was observed under As exposure as compared to control medium (**Fig. 2A**).

3.2 Arsenic accumulation and speciation

We observed that roots of *S. atrocinerea* accumulated As concentrations ranging from 180 mg As kg^{-1} dry weight at 1 d to more than 2,400 mg As kg^{-1} dry weight after 30 d of exposure (**Table 2**). In leaves, As accumulation was much lower, although after 30 d of exposure, it reached an As

295 concentration higher than that present in the culture medium (**Table 2**). Although only As V was
296 added to the culture medium, 4% of As III was observed in the medium after 1 d of exposure and it
297 decreased to 0% by the end of the experiment (**Fig. 2B**). Total As concentration in the medium
298 decreased 14 % due to plant uptake and no spontaneous As speciation was detected in the medium
299 when *S. atrocinerea* was not present (data not shown).

300 In plant tissues, the As was detected as As III or As V, but no As methylated species were
301 observed (**Table 2**). In roots, As V was more abundant (91%) during the first 3 d of exposure but
302 after 10 d, As III was the predominant As form (**Fig. 2C**). In leaves, As V was the predominant
303 speciation form observed throughout the experiment and As III was only detected at 3 d of exposure
304 in low quantity (18%) (**Fig. 2C**).

305

306 **3.3 Analysis of non-protein thiolic compounds**

307 In roots of control plants, only GSH was observed and present at a 2-fold higher concentration
308 than that observed in the As-exposed roots (**Table 3**). However, in roots and leaves of *S. atrocinerea*
309 plants exposed to As, changes in the concentrations of non-protein thiols (NPTs) were already
310 observed after 1 d and this trend was maintained over time (**Table 3**). Besides, in the roots of As-
311 exposed plants the total concentration of NPTs increased over time (up to 4.5-fold higher after 30 d
312 compared to 1 d) and it was always higher than that observed in leaves. This NPTs increase in roots
313 under As exposure was mainly due to an increment of *de novo* synthesized compounds such as PC_2,
314 Cys-PC_2, PC_3, desGly-PC_3, Cys-PC_3 and also two unidentified thiolic compounds that were named
315 TC_1 and TC_2 (**Table 3**).

316 In leaves of control plants, the thiolic compounds GSH, desGly-PC_4, and TC_3 were detected
317 (Table 3), whereas under As exposure we observed *de novo* synthesis of desGly-PC_2 at increasing

concentrations over time. In both control and As-exposed plants, GSH concentrations in leaves were always higher than those observed in roots and were initially higher in As-exposed plants than in control plants (**Table 3**). This increase in GSH, together with *de novo* synthesis of desGly-PC_2, accounted for a higher NPTs concentration at 1 d and 3 d in leaves of As-exposed plants. However, after 10 and 30 d of exposure, the total NPTs concentration in leaves of plants exposed to As did not significantly differ from that observed in leaves of plants grown under control conditions (**Table 3**).

3.4 Gene expression

In general, the gene expression pattern in roots between control and As-treated samples differed due to the prominent regulation of transcripts related to As transport, As V reduction to As III, thiol metabolism and vacuolar transports. In this way changes were observed in transcripts coding for the phosphate transporter (PHO1), aquaporins (NIP1, SIP1 and SILICON), boron transporter (BORON), As V reductase CDC25-like tyrosine phosphatase (CDC25-1), glutathione synthase (GS) and ABC transporter (WBABCT) (**Fig. 3**). This differential regulation was also accompanied by changes in transcripts for As stress-related proteins like cellulose synthase (*CSA*), arsenite inducible protein (*AIP-1*) and aminocyclopropane-1-carboxylate synthase (*ACCS*). In leaves, regulation of the transcripts measured was not so noticeable as in roots and differences in gene expression between control and As-exposed plant were due mainly to the overexpression of ACCS (***Supplementary Fig. 2***). A heat map representation of the other transcripts measured in this study with a fold regulation lower than two can be found for both roots (***Supplementary Fig. 3A***) and leaves (***Supplementary Fig. 3B***).

To establish the kinetic gene expression of related target genes, a principal component analysis (PCA) was performed using the gene expression data obtained in leaves and roots of *S. atrocinerea* plants collected at 1, 3, 10 and 30 d. According to PCA component 1, roots of plants exposed to As

342 for 1 d showed the highest gene expression for *GS*, *NIP1, SIP1, CSA, AIP-1,* vacuolar transporter
343 (*CAX2-2*), glutathione reductase (*GR*) and *ACCS*, whereas the expression of *BORON*, *PHO1*, *MT1A*,
344 *CDC25-2, WBABCT*, vacuolar transporter (*ABCG*) was higher at later time points. On the other hand,
345 PCA component 2 in roots (24% of the total variation) indicated that the increased expression of
346 *ABCG*, clustered samples at 3 d, whereas the decrease in expression for phytochelatin synthase (*PCS*)
347 and transcripts for a high-affinity phosphate transporter (*HAPO4*) at 3 d separated this group from the
348 rest (**Fig. 4A**).

349 In leaves, according to PCA component 1, the differential gene expression collected from
350 plants growing under As exposure at 1 and 3 d, had a more similar pattern than that observed at 10
351 and 30 d. Main differences were attributed to the up-regulation of genes involved in the flavonoid
352 pathway *CHS3*, *CHS2*, *ANR*, *F3H*, *FLH*, *2HFLR*, *BORON* and *CHS1* expression at 1 and 3 d and of
353 *CDC25-2* at 10 and 30 d. Component 2 (20% of the total variation), however, indicated a separation
354 between the initial (1 d) and the last time point (30 d) from the intermediate points (3 and 10 d) as a
355 consequence of lower *ACCS* and higher *ABCG2* expression in these intermediate points (**Fig. 4B**).
356

357 **4 Discussion**

358 The total pollutant concentration of a certain element in the soil is not a representation of the
359 amount that is available (exchangeable) for the plant uptake, neither a good indicator to establish
360 plant toxicity limits. Therefore, when phytoremediation processes will rely on the use of certain plant
361 species that tolerate and accumulate high concentrations of metal(loid)s, it is very important that the
362 studies conducted in the laboratory under controlled conditions, on which the basic physiological
363 knowledge is set, are based on well reflected pollutant concentrations. Many hydroponic studies have
364 used higher As concentrations than those found in soil solution, and their environmental relevance
365 has been questioned (Fitz and Wenzel, 2002). According to this, some authors propose that

hydroponic cultures should include As doses in the range of 0 – 10 μM to allow the extrapolation of the results to As-polluted soils (Moreno-Jimenez et al., 2010). Nonetheless, the fact that *S. atrocinerea* plants used in this study already grow on a brownfield with an As exchangeable fraction of 18 mg kg^{-1} (data not shown), suggests that the As dose could be increased for this hydroponic assay. Furthermore, this As concentration matches that recommended in previous hydroponic studies with willow (Purdy and Smart, 2008) and it has already been used in analyzing differential As gene expression under hydroponic conditions (Puckett et al., 2012). Although some authors have reported that willows have the capability to translocate As from roots to aboveground tissues (Tlustoš et al., 2007; Puckett et al., 2012; Sylvain et al., 2016) the As accumulation in leaves does not reach those quantities present in hyperaccumulating species like *Pteris vittata* (Caille et al., 2004), as it was our case, but the phytoremediation potential is compensated with a higher biomass (Meers et al., 2007, Witters, 2009). Furthermore, after 30 d of As exposure, *S. atrocinerea* did not show any phytotoxic symptoms and was capable of accumulating a higher As concentration than that present in the culture medium, showing therefore a bioaccumulation factor higher than 1, which is an added value for the phytoremediation of As.

It has been reported that exposure to toxic metalloids, such as As, can disturb the nutrient profile of the plant and hence lead to toxicity (Lou et al., 2010), and also that As V uptake and tolerance to its induced toxicity is intimately linked to phosphate nutrition. In the soil, As is mainly present in its As V form (Cordos et al., 2006) and once it is in contact with the roots, As V can enter via phosphate transporters (Maciaszczyk-Dziubinska et al., 2012). Therefore, changes in transcripts encoding for As V-related transporter proteins could be expected and, in our case, the As V added to the hydroponic solution caused a differential regulation of transcripts for phosphate transporters. The up-regulation of *PHO1* in roots of *S. atrocinerea* from the onset of the As exposure (**Fig. 3**) and that observed at 10 and 30 d of transcripts encoding for a high-affinity phosphate transporter protein (*HAPO4*) (***Supplementary Fig. 3A***), relate to the first lower and then similar P concentrations in

391 roots of As-exposed *S. atrocinerea* as compared to non-exposed plants (**Table 1**). It has been
392 suggested that reduced uptake of As V is a well-known mechanism of As V resistance employed by
393 many plant species, which is achieved through a reduction of the phosphate/arsenate uptake system
394 in resistant plants (Meharg and Hartley-Whitaker, 2002). Moreover, it is thought that this reduction
395 decreases As V influx to a level at which the plant can detoxify As, presumably by constitutive
396 mechanisms (Catarecha et al., 2007). However, according to our results of As accumulation and a
397 lower concentration of P under As exposure as compared to the control condition, it can be suggested
398 that the transcript upregulation of phosphate-related transcripts in roots is based on preventing As V
399 competition and avoiding P deprivation. Therefore, since As does accumulate at high concentrations
400 in roots of willow, a more effective detoxification mechanism than inhibition of phosphate
401 transporters as seen in other plants would be necessary in *S. atrocinerea*. After 30 d, As
402 concentration in leaves of *S. atrocinerea* reached levels higher than toxicity levels established for
403 non-tolerant plants (1-20 mg As kg^{-1} dry weight; White and Brown, 2010). Under these conditions, a
404 differential regulation of As V-related transporters in leaves of *S. atrocinerea* was observed. The
405 decrease in *PHO1* transcripts at 1, 3 and 10 d (***Supplementary Fig. 3*B**), is a similar response to that
406 of As resistant species, where avoiding As uptake in leaves by reducing phosphate uptake constitutes
407 a tolerance mechanism (Meharg and Hartley-Whitaker, 2002). However, at 30 d the down-regulation
408 ceased and there were no differences in transcript levels of *PHO1* compared to those observed in
409 leaves of control plants and it matched with a similar P concentration in leaves of both treatments
410 (**Table 1**). According to the Ca concentrations observed in plants of *S. atrocinerea* (**Table 1**), it can
411 be suggested that Ca accumulation in the roots and its reduced translocation to the leaves is a
412 response to As accumulation. Ca is an essential plant macronutrient and it plays an important role in
413 cell wall and membrane stabilization and regulates nutrient uptake as well as different stress
414 responses (Ahmad et al., 2015), including an increase of the antioxidant defense under As exposure

and reducing As uptake (Rahman et al., 2015). In multiple studies, it has been shown that micronutrient accumulation is affected by As exposure, but it can also have an impact on As uptake and hence As toxicity (Srivastava et al., 2017). It has been proposed that B channels might have a role in As transport into the cell (Yanitch et al., 2017), which is also reflected in our results with increased *BORON* transcript levels in roots (**Fig. 3**) and leaves (***Supplementary Fig. 3*B**). Furthermore, boric acid transporter NIP5.1 from *Arabidopsis* is also permeable to As III (Mitani-Ueno et al., 2011), and our data showed that B accumulation in plant tissues changes along the time of exposure to As (**Table 1**), with *BORON* transcripts 5-fold down-regulated at 1 d (**Fig. 3**), when As III concentration in the medium was the highest (**Fig. 2B**). In leaves, *BORON* transcripts are induced at 1 and 3 d in response to As (***Supplementary Fig. 3*B**), coinciding at 3 d with the highest B concentration (**Table 1**). Whereas Zn is described as an indispensible micronutrient, which mitigates As toxicity by modulating ROS and the antioxidant function in plants (Das et al. 2016) or by improving the thiol metabolism (Srivastava and Srivastava, 2017), no major changes were detected in Zn concentration apart from the increase at 1 d in As-exposed plants. With regard to Fe, our data showed that Fe translocation to leaves was more affected by As than any of the other elements, with an increased Fe concentration in roots exposed to As, whereas in leaves it decreased (**Table 1**). Shaibur et al. (2008) described that one of the symptoms of As toxicity is the formation of Fe plaques in roots and, as also seen in our case, Fe:P ratios in the roots of the As-exposed plants were higher than those observed in the control roots. This suggests that, in the liquid culture medium, As may have been adsorbed with Fe on the surface of the roots, forming Fe-As plaques. Thus, the iron plaque formed on the root surface will act as a natural As barrier and reduce As uptake by the plant and its translocation to shoots.

Besides the impact of other elements in the medium on As uptake, the speciation of As also plays an important role in the accumulation of As and tolerance by the plant (Moreno-Jimenez et al., 2010). The As was added to the culture medium as As V and after 24 h a 4% reduction to As III was

observed (**Fig. 2B**). This chemical reduction can be attributed to metabolic activities of *S. atrocinerea* since no speciation was detected when the plant cuttings were not present in the culture medium. For this observation, two possible explanations can be given. On one side, plants might induce changes in the pH and in the redox potential of the culture medium as it was observed in this study (**Fig. 2A**), and those changes might affect the speciation of As. For an example, it has been proposed that protons released from organic acids (R-COOH) and excreted by plant roots may contribute to the reduction of As V to As III, while increasing the pH as the process consumes H^+ (Park et al., 2016). Interestingly, only As V was detected after 30 d and it matched with the highest pH value in the medium; whereas the highest As III concentration was detected at 1 d, when the pH was the lowest (**Fig. 2A**). Therefore, another possible explanation for the presence of As III in the medium is a direct efflux of As III from the plant to the medium that can be linked to the proton gradient across the plasma membranes or dependent of the plant metabolism (e.g. direct As III from plant cells to the medium) (Xu et al., 2007; Park et al., 2016). Taking transcriptional regulations into account, since willow plants were able to induce the occurrence of As III in the medium, differences in transcript levels of genes encoding for As III transport were expected in roots of As-exposed plants.

In our case, we observed a noticeable up-regulation of the transcripts encoding the aquaporin NIP1.1, reported for As III uptake into the roots (Ma et al., 2008), and in transcripts for SIP1 at 1 d of As exposure (**Fig. 3**). This up-regulation diminishes over time, probably as a consequence of a very active As V reduction to As III during the first days of exposure, with a lot of free As III initially in the cytoplasm. This transcript up-regulation for As III transporters in roots, suggests that As V reduction has occurred even before its entry into the roots, which is supported by the presence of 4% of As III in the medium at 1 d of exposure (**Fig. 2B**), and which coincides with the highest up-regulation of *NIP1* and *SIP1*. In leaves, where As was mainly present as As V, no changes in transcript levels for the aquaporin transcripts were observed (***Supplementary Fig. 3B***).

Once inside the cell, since As V has no affinity for the "-SH" groups in the PCs, the first step in As detoxification is As reduction (Finnegan and Chen, 2012). The main mechanism for As V reduction is the presence of As V reductases where GSH acts as electron donor (Dhankher et al., 2002). Arsenate reductases are believed to have evolved from the CDC-25 (cell division cycle) dual-specificity tyrosine phosphatases (Duan et al., 2007). Based on homology with the yeast As V reductase, ACR2P, Bleeker et al. (2006) identified a CDC25-like plant candidate and showed that it had arsenate reductase activity like it was also observed in other assays (Dhankher et al., 2006). In our study, As V reduction to As III was observed in roots right after As uptake with an increasing As III concentration in root tissues from 9% to 70% by the end of the study (**Fig. 2C**). This coincides with the *CDC25-1* up-regulation in roots of plants exposed to As (**Fig. 3**), whereas no changes were observed for *CDC25-2* (***Supplementary Fig. 3A***).

Another mechanism to reduce As V in the plant is through a non-enzymatic reduction, where GSH is implied, but this process is relatively slow, so according to the NPT data we can attribute the large up-regulation observed in *GS* transcripts along the As-exposure time to PC production as a detoxification mechanism. This was reflected by the increased NPT concentrations in *S. atrocinerea* roots after As uptake (**Table 3**). Our results showed that although there was a clear up-regulation of *GS* transcripts in roots (**Fig. 3**), GSH concentrations of As-exposed plants remained constant over time and lower than those in the roots of control plants (**Table 3**). However, since PCs use GSH as a building substrate, the decreasing concentrations of GSH are consistent with its use in PCs or other NPTs. This fast increase in NPT concentrations and the As III presence in the roots, support our observations of As speciation in the medium; where it seems that As III efflux by roots occurs right after As V uptake and that this efflux diminishes once the As III is complexed with thiols and stored in the vacuoles. Therefore, an increase in NPTs under increased As III presence points towards an As-PC complex formation possibly leading to less As III efflux. In support to this explanation, Raab et al. (2005) found that in sunflower roots the amount of As not complexed by thiols fell from 90% of

489 total As after 1 h exposure, to 43% after 4 d of exposure. In addition, although PCs were synthesized
490 in *S. atrocinerea* in response to As exposure and their concentration increased over time in roots
491 (**Table 3**), there was only a slight increase, in transcripts coding for PCS at 1 d, similar to the
492 behavior observed in transcripts of *GR* (***Supplementary Fig. 3A***). This suggests that the induction of
493 *PCS* expression is unlikely to play a significant role in regulating PC biosynthesis (Cobbett 2000).
494 This agrees with Rea el al. (2004), who reported that PCS enzymes are expressed constitutively at
495 relatively high levels and are generally unaffected by exposure of cell cultures or plants to heavy
496 metal(loid)s. As described in other plant species, PC-based sequestration is considered to be essential
497 for As tolerance, where hypertolerant ecotypes present higher PC concentrations under As exposure
498 compared to non-tolerant ecotypes (Meharg and Hartley-Whitaker, 2002; Schat et al., 2002;
499 Fernández et al., 2013). The 7-fold increase in NPT concentration observed in our case in roots of
500 willow after 1 d of As exposure, it is then related to a fast As V reduction to As III and to the need to
501 synthetize longer-chain PCs to chelate the increasing concentrations of As III, and therefore
502 maintaining cellular stability. As it has been reported by Sharma et al. (2016), longer chain PCs
503 contribute to a more effective cellular detoxification due to a higher metal-binding capacity and
504 formation of more stable As-complexes that will prevent the interaction with sulfhydryl groups of
505 other proteins and hence affect the metabolism.
506 We also observed that in the roots, the organ where more As was accumulated, a greater PCs
507 synthesis was present than in leaves where As accumulation is lower. Another interesting observation
508 of our study is the presence of many unknown thiol products. This is in accordance with the results of
509 Li et al. (2004) in *Arabidopsis*, where As exposure resulted in the expression of many unknown thiol
510 products, whereas cadmium induced higher increases in traditional PCs (PC_2, PC_3, PC_4).
511 Most of the As speciation experiments described in literature propose As III as being the
512 predominant As form in leaves (Kertulis et al., 2005; Zhang et al., 2009; Yan et al., 2012; Park et al.,

513 2016). However, in our case As V was the main As species observed in leaves throughout the
514 experiment (**Fig. 2C**). Despite As exposure caused *de novo* synthesis of desGly-PC_2 and the increase
515 of desGly-PC_4 in leaves of As-exposed plants, a possible explanation for the lack of As III observed
516 in leaves, with the exception at 3 d, could be attributed to the relatively low concentration of As in
517 leaves as compared to roots (**Table 2)** , which might require a less effective NPT response. Another
518 explanation could be related to the stability of the As-thiol complexes present in leaves, where As III
519 could be mainly bound to GSH which was present at higher concentrations than in roots, and
520 represent the main NPT in leaves (**Table 3**). Since As III – GSH complexes are less stable that As III
521 – PCs, a dissociation of these complexes could take place with the consequent re-oxidation of As III
522 to As V (Bluemlein et al., 2009; Zhao et al., 2009). In relation to this, the As V presence in leaves of
523 *S. atrocinerea* might explain the need for the up-regulation of the CDC25-like tyrosine phosphatases
524 pathway observed at 10 and 30 d, when As increased in shoots (***Supplementary Fig. 3B***), and
525 exceeded plant toxic limits (White and Brown, 2010). In contrast to PCs that rely on enzymatic
526 synthesis, MTs, which are also important metal chelators in plant cells, are direct products of mRNA
527 translation (Anjum et al., 2015). Examples of MTs induction under metal exposure in *Salix* have been
528 described by Konlechner et al. (2013), and it is known that metals like Zn or Fe bind to MTs with the
529 highest affinity (Blindauer et al. 2010). Therefore, in this study according to the differential
530 transcription pattern of *MT1A* between roots and leaves, due to the Fe accumulation in roots and its
531 reduction in leaves, it could be that *MT1A* induction in leaves (***Supplementary Fig. 3*B**) corresponds
532 to the need of supplying enough Fe and that this up-regulation is not involved in direct As chelation.
533 However, its induced expression in leaves forms part of the response to the As-induced stress.

534 Once As V is reduced to As III and complexed to NPTs to limit its toxicity, these complexes
535 are taken up by ABC transporters and stored in the vacuole. ABC transporters constitute one of the
536 largest protein families, present in organisms ranging from bacteria to humans, and have been
537 identified as transporters involved in detoxification processes by transporting metal(loid)-PC

538 complexes (Kang et al., 2011). It is known that As III-PCs complexes have a low stability and their
539 storage into the acidic environment of vacuoles can limit its dissociation and As release back into the
540 cytosol (Schmöger et al., 2000). Song et al. (2010) already emphasized that engineering of vacuolar
541 PC transporters in plants may be of potential use in phytoremediation. According to this, in our
542 study, the up-regulation observed of *WBABCT* transcripts (**Fig. 3**) highlights its role in metal(loid)-
543 PC complexes transportation and constitutes an interesting target gene to increase accumulation for
544 phytoremediation purposes. Interestingly, in leaves, since only As V was present and at low
545 concentration compared to roots, no differential up-regulation of *WBACT* transcripts was observed as
546 compared to control (***Supplementary Fig. 3*B**).

547 Taking into account that speciation and distribution of As in the plant can provide important
548 information and help to understand the mechanisms for As accumulation, translocation, and
549 transformation as noted by Zhang et al. (2002), our results suggest that the As tolerance mechanism
550 of *S. atrocinerea* relies on As V reduction in roots but not in leaves. Therefore, limited As V
551 translocation by an effective As V reduction to As III and its complexation to NPT compounds and
552 further sequestration into the root vacuoles, as supported by the gene expression, seems to be the
553 reason for the tolerance of *S. atrocinerea* to As.

554 Under As accumulation, stress is induced in plant cells. Although in leaves As detoxification
555 processes are not really activated as seen in roots, *S. atrocinerea* plants respond to As in both roots
556 and leaves by altering gene expression related to general stress. This response includes alterations at
557 the transcript level of genes related to (1) cell wall synthesis, as a down-regulation of the cellulose
558 synthase like A (*CSA*) (***Supplementary Fig. 3*A,B**) is observed; (2) ethylene biosynthesis, with *ACCS*
559 notably up-regulated in roots and to a lesser extent in leaves, probably explained a low As
560 translocation (***Supplementary Fig. 2***); and (3) transcripts related to the synthesis of heat shock
561 proteins, like *AIP-1*, a highly conserved gene selectively activated by As III in many cell types (Sok

et al., 2001). In our experiment the highest up-regulation was observed in roots at 1 d after exposure and it decreased over time (***Supplementary Fig. 2***). Since this protein is As III-induced, and As III concentrations increase over time in roots, this suggests an effective complexation of As III from 1 d on with NPTs to prevent its toxicity. Another As-related response of willow is the biosynthesis of phenylpropanoids that may culminate with the increased production of tannins (Yanitch et al., 2017) and might be an important stress defense mechanism in leaves. In our study, we observed an early up-regulation of selected genes (*CHS1, CHS2, 2HFLR* and *F3H*) in the flavonoid pathway at 1 d after As exposure (**Fig. 4 B** and ***Supplementary Fig. 3*B**), whereas no major changes in the expression pattern or a down-regulation were noticed later on as compared to control conditions. Therefore, by the information provided by the transcript levels, we suggest that *S. atrocinerea* relies on the phenylpropanoid pathway to cope with As toxicity during the early times of exposure, but further investigation at metabolic level is essential.

5 Conclusions

The selected *S. atrocinerea* clone naturally growing in an As-contaminated brownfield showed great tolerance when grown in the presence of a high concentration of As and accumulated more than 2,400 mg As kg^{-1} dry weight in its roots without showing phytotoxicity symptoms. Our findings reveal that under the presence of As V in hydroponic conditions, willow plants show a transcriptional regulation of genes involved in nutrient transporters, As V reduction, glutathione synthesis and sequestration of As into the vacuoles, together with genes involved in stress responses, which coincides with a rapid As III presence and accumulation in root tissues, altered nutrient profile and *de novo* synthesis and increase of NPT compounds, all of which contribute to the tolerance to the metalloid by *S. atrocinerea*.

The high As accumulation together with a high biomass yield makes this willow species a potential tool for its use in As phytoremediation. Overall, a better understanding of the physiological mechanisms of tolerance to arsenic toxicity in *S. atrocinerea* was achieved through this study by experimental verification of the significance of particular transcripts complemented by an integrative analysis of nutrient profile, As accumulation and speciation, as well as NPT compounds synthesis. However, according to our observations, further research should also focus on what happens in real polluted soils where, apart from As, there are usually other metal(loid)s at high concentrations that can affect the plant detoxification responses.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

AN: Conceptualization, Investigation, Original draft; SH: Review; AC: Methodology, Formal analysis, Validation, Resources, Review and Editing; AG: Resources, Review and Editing.

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10 References

Ahmad, P., M. Sarwat, N. A. Bhat, M. R. Wani, A. G. Kazi and L. S. Tran (2015). Alleviation of cadmium toxicity in Brassica juncea L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. PLoS One 10(1): e0114571.

Ahsan, N., Lee, D.G., Alam, I., Kim, P.J., Lee, J.J., Ahn, Y.-O., Kwak, S.S., Lee, I.J., Bahk, J.D., Kang, K.Y., Renaut, J., Komatsu, S., Lee, B.H. (2008) Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. Proteomics. 8, 3561-3576.

Anjum, N. A., M. Hasanuzzaman, M. A. Hossain, P. Thangavel, A. Roychoudhury, S. S. Gill, M. A. M. Rodrigo, V. Adam, M. Fujita, R. Kizek, A. C. Duarte, E. Pereira and I. Ahmad (2015). "Jacks of metal/metalloid chelation trade in plants—an overview." Frontiers in Plant Science 6(192)

Argus, G.W. (1995) Salicaceae Willow Family: Part Two: Salix L. Willow. Journal of the Arizona-Nevada Academy of Science 29, 39-62.

Batista, B.L., Nigar, M., Mestrot, A., Rocha, B.A., Barbosa Junior, F., Price, A.H., Raab, A., Feldmann, J. (2014) Identification and quantification of phytochelatins in roots of rice to long-term exposure: evidence of individual role on arsenic accumulation and translocation. J Exp Bot 65, 1467-1479.

Bleeker, P.M., Hakvoort, H.W., Bliek, M., Souer, E., Schat, H. (2006) Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant Holcus lanatus. Plant J 45, 917-929.

Blindauer, C. A. and O. I. Leszczyszyn (2010). "Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more." Nat Prod Rep 27(5): 720-741.

Bluemlein, K., Raab, A., Feldmann, J. (2009) Stability of arsenic peptides in plant extracts: off-line versus on-line parallel elemental and molecular mass spectrometric detection for liquid chromatographic separation. Anal Bioanal Chem 393, 357-366.

Caille, N., Swanwick, S., Zhao, F.J., McGrath, S.P. (2004) Arsenic hyperaccumulation by Pteris vittata from arsenic contaminated soils and the effect of liming and phosphate fertilisation. Environmental Pollution 132, 113-120.

Catarecha, P., Segura, M.D., Franco-Zorrilla, J.M., García-Ponce, B., Lanza, M., Solano, R., Paz-Ares, J., Leyva, A. (2007) A Mutant of the Arabidopsis Phosphate Transporter PHT1;1 Displays Enhanced Arsenic Accumulation. The Plant Cell 19, 1123-1133.

Chang, S., Puryear, J., Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113-116.

Cobbett, C. S. (2000). Phytochelatins and Their Roles in Heavy Metal Detoxification. Plant Physiology 123(3): 825-832.

Commission, European. 2000. Ambient Air Pollution by AS, CD and NI Compounds (Position Paper—Final), Luxembourg: Office for Official Publications of the European Communities.

Cordos, E. A., T. Frentiua, M. Pontaa, l. Marginean, B. Abrahamb and C. Roman (2006). Distribution study of inorganic arsenic (III) and (V) species in soil and their mobility in the area of Baia-Mare, Romania. Chemical Speciation & Bioavailability 18(1): 11-25

Das, I., Sanyal, S. K., Koushik G., and Das, D. K.. (2016). Arsenic mitigation in soil-plant system through zinc application in West Bengal soils. Bioremediation journal, 20: 24-37.

Dave, R., Singh, P.K., Tripathi, P., Shri, M., Dixit, G., Dwivedi, S., Chakrabarty, D., Trivedi, P.K., Sharma, Y.K., Dhankher, O.P., Corpas, F.J., Barroso, J.B., Tripathi, R.D. (2013) Arsenite tolerance is related to proportional thiolic metabolite synthesis in rice (Oryza sativa L.). Arch Environ Contam Toxicol 64, 235-242.

Dhankher, O. P., Y. Li, B. P. Rosen, J. Shi, D. Salt, J. F. Senecoff, N. A. Sashti and R. B. Meagher (2002). Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat Biotechnol 20(11): 1140-1145.

Dhankher, O.P., Rosen, B.P., McKinney, E.C., Meagher, R.B. (2006) Hyperaccumulation of arsenic in the shoots of Arabidopsis silenced for arsenate reductase (ACR2). Proceedings of the National Academy of Sciences of the United States of America 103, 5413-5418.

Duan, G.L., Zhou, Y., Tong, Y.P., Mukhopadhyay, R., Rosen, B.P., Zhu, Y.G. (2007) A CDC25 homologue from rice functions as an arsenate reductase. New Phytol 174, 311-321.

Ehlrich, H.L. (1990) Geomicrobiology. Marcel Dekker New York.

Ellis, D.R., Gumaelius, L., Indriolo, E., Pickering, I.J., Banks, J.A., Salt, D.E. (2006) A novel arsenate reductase from the arsenic hyperaccumulating fern Pteris vittata. Plant Physiol 141, 1544-1554.

Ellman, G.L. (1959) Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82, 70-77.

Fernandez, R., A. Bertrand, R. Reis, M. P. Mourato, L. L. Martins and A. Gonzalez (2013). Growth and physiological responses to cadmium stress of two populations of Dittrichia viscosa (L.) Greuter. J Hazard Mater 244-245: 555-562.

Fernández, R., Bertrand, A., García, J.I., Tamés, R.S., González, A. (2012) Lead accumulation and synthesis of non-protein thiolic peptides in selected clones of Melilotus alba and Melilotus officinalis. Environmental and Experimental Botany 78, 18-24.

Fernández, R., Carballo, I., Nava, H., Sánchez-Tamés, R., Bertrand, A., González, A. (2010) Looking for native hyperacumulator species useful in phytoremediation. In: Handbook of Phytoremediation. Golubev, I.A (ed), Nova Science Publishers, Inc.New York, pp.297-330.

Finnegan, P. M. and W. Chen (2012). Arsenic Toxicity: The Effects on Plant Metabolism. Frontiers in Physiology 3: 182.

Fitz, W.J., Wenzel, W.W. (2002) Arsenic transformations in the soil–rhizosphere–plant system: fundamentals and potential application to phytoremediation. Journal of Biotechnology 99, 259-278.

Fu, S.F., Chen, P.Y., Nguyen, Q.T., Huang, L.Y., Zeng, G.R., Huang, T.L., Lin, C.Y., Huang, H.J. (2014) Transcriptome profiling of genes and pathways associated with arsenic toxicity and tolerance in Arabidopsis. BMC Plant Biol 14, 94.

Gallego, J.R., Esquinas, N., Rodríguez-Valdés, E., Menéndez-Aguado, J.M., Sierra, C. (2015) Comprehensive waste characterization and organic pollution co-occurrence in a Hg and As mining and metallurgy brownfield. Journal of Hazardous Materials 300, 561-571.

Gill, R.A., Zang, L., Ali, B., Farooq, M.A., Cui, P., Yang, S., Ali, S., Zhou, W. (2015) Chromium-induced physio-chemical and ultrastructural changes in four cultivars of Brassica napus L. Chemosphere 120, 154-164.

694 Gong, J. M., D. A. Lee and J. I. Schroeder (2003). "Long-distance root-to-shoot transport of
695 phytochelatins and cadmium in Arabidopsis." Proc Natl Acad Sci U S A 100(17): 10118-
696 10123

697 Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W.,
698 Hellsten, U., Putnam, N., Rokhsar, D.S. (2012) Phytozome: a comparative platform for
699 green plant genomics. Nucleic Acids Res 40, D1178-D1186.
700 Hammer, D., Kayser, A., Keller, C. (2003) Phytoextraction of Cd and Zn with Salix viminalis in field
701 trials. Soil Use and Management 19, 187-192.
702 Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W.T., Schat, H., Meharg, A.A. (2001)
703 Phytochelatins Are Involved in Differential Arsenate Tolerance in Holcus lanatus. Plant
704 Physiol 126, 299-306.
705 Hasanuzzaman, M., K. Nahar, T. I. Anee and M. Fujita (2017). Glutathione in plants: biosynthesis
706 and physiological role in environmental stress tolerance. Physiology and molecular
707 biology of plants: an international journal of functional plant biology 23(2): 249-268.
708 Huang, J.H., Fecher, P., Ilgen, G., Hu, K.N., Yang, J. (2012) Speciation of arsenite and arsenate in
709 rice grain – Verification of nitric acid based extraction method and mass sample survey.
710 Food Chemistry 130, 453-459.
711 Janssen, J., Weyens, N., Croes, S., Beckers, B., Meiresonne, L., Van Peteghem, P., Carleer, R.,
712 Vangronsveld, J. (2015) Phytoremediation of Metal Contaminated Soil Using Willow:
713 Exploiting Plant-Associated Bacteria to Improve Biomass Production and Metal Uptake.
714 Int J Phytoremediation 17, 1123-1136.
715 Ji, R., L. Zhou, J. Liu, Y. Wang, L. Yang, Q. Zheng, C. Zhang, B. Zhang, H. Ge, Y. Yang, F. Zhao,
716 S. Luan, and W. Lan. (2017). Calcium-dependent protein kinase CPK31 interacts with
717 arsenic transporter AtNIP1;1 and regulates arsenite uptake in Arabidopsis thaliana, *PLoS*
718 *One*, 12: e0173681.
719 Jiang, Y., Lei, M., Duan, L., Longhurst, P. (2015) Integrating phytoremediation with biomass
720 valorisation and critical element recovery: A UK contaminated land perspective. Biomass
721 and Bioenergy 83, 328-339.
722 Kabata-Pendias, (2010), Trace Elements in Soils and Plants, Fourth Edition. CRC Press, pp. 407-505.
723 Kang, J., Park, J., Choi, H., Burla, B., Kretzschmar, T., Lee, Y., Martinoia, E. (2011) Plant ABC
724 Transporters. The Arabidopsis Book / American Society of Plant Biologists 9, e0153.
725 Kertulis, G.M., Ma, L.Q., MacDonald, G.E., Chen, R., Winefordner, J.D., Cai, Y. (2005) Arsenic
726 speciation and transport in Pteris vittata L. and the effects on phosphorus in the xylem
727 sap. Environmental and Experimental Botany 54, 239-247.
728 Kidd, P., Mench, M., Álvarez-López, V., Bert, V., Dimitriou, I., Friesl-Hanl, W., Herzig, R., Olga
729 Janssen, J., Kolbas, A., Müller, I., Neu, S., Renella, G., Ruttens, A., Vangronsveld, J.,
730 Puschenreiter, M. (2015) Agronomic Practices for Improving Gentle Remediation of
731 Trace Element-Contaminated Soils. International Journal of Phytoremediation 17, 1005-
732 1037.
733 Konlechner, C., M. Türktaş, I. Langer, M. Vaculík, W. W. Wenzel, M. Puschenreiter and M.-T.
734 Hauser (2013). Expression of zinc and cadmium responsive genes in leaves of willow
735 (Salix caprea L.) genotypes with different accumulation characteristics. Environmental
736 pollution (Barking, Essex : 1987) 178: 121-127.
737 Kuzovkina, Y.A., Quigley, M.F. (2005) Willows Beyond Wetlands: Uses of Salix L. Species for
738 Environmental Projects. Water, Air, and Soil Pollution 162, 183-204.
739 Le Gall, H., F. Philippe, J. M. Domon, F. Gillet, J. Pelloux and C. Rayon (2015). "Cell Wall
740 Metabolism in Response to Abiotic Stress." Plants (Basel) 4(1): 112-166.

741 LeBlanc, M.S., McKinney, E.C., Meagher, R.B., Smith, A.P. (2013) Hijacking membrane
742 transporters for arsenic phytoextraction. Journal of Biotechnology 163, 1-9.
743 Levinson, W., Oppermann, H., Jackson, J. (1980) Transition series metals and sulfhydryl reagents
744 induce the synthesis of four proteins in eukaryotic cells. Biochim Biophys Acta 606, 170-
745 180.
746 Li, J., Jia, H., Han, X., Zhang, J., Sun, P., Lu, M., Hu, J. (2016) Selection of Reliable Reference
747 Genes for Gene Expression Analysis under Abiotic Stresses in the Desert Biomass
748 Willow, Salix psammophila. Front Plant Sci 7, 1505.
749 Li, R.-Y., Ago, Y., Liu, W.-J., Mitani, N., Feldmann, J., McGrath, S.P., Ma, J.F., Zhao, F.-J. (2009)
750 The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. Plant Physiol
751 150, 2071-2080.
752 Li, Y., O. P. Dhankher, L. Carreira, D. Lee, A. Chen, J. I. Schroeder, R. S. Balish and R. B. Meagher
753 (2004). "Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced
754 arsenic tolerance and cadmium hypersensitivity." Plant Cell Physiol 45(12): 1787-1797.
755 Lindsay, E.R., Maathuis, F.J.M. (2016) Arabidopsis thaliana NIP7;1 is involved in tissue arsenic
756 distribution and tolerance in response to arsenate. FEBS Letters 590, 779-786.
757 Lloyd, G.a.M., B.H. (1981) Woody Plant Medium (WPM)—A Mineral Nutrient Formulation for
758 Microculture of Woody Plant Species. HortScience 16, 453.
759 Lou, L.Q., Ye, Z.H., Lin, A.J., Wong, M.H. (2010) Interaction of arsenic and phosphate on their
760 uptake and accumulation in Chinese brake fern. Int J Phytoremediation 12, 487-502.
761 Ma, J.F., Yamaji, N., Mitani, N., Xu, X.-Y., Su, Y.-H., McGrath, S.P., Zhao, F.-J. (2008)
762 Transporters of arsenite in rice and their role in arsenic accumulation in rice grain.
763 Proceedings of the National Academy of Sciences 105, 9931-9935.
764 Maciaszczyk-Dziubinska, E., Wawrzycka, D., Wysocki, R. (2012) Arsenic and Antimony
765 Transporters in Eukaryotes. International Journal of Molecular Sciences 13, 3527-3548.
766 Mandal, B.K., Suzuki, K.T. (2002) Arsenic round the world: a review. Talanta 58, 201-235.
767 Matschullat, J. (2000) Arsenic in the geosphere — a review. Science of The Total Environment 249,
768 297-312.
769 Meers, E., Vandecasteele, B., Ruttens, A., Vangronsveld, J., Tack, F.M.G. (2007) Potential of five
770 willow species (Salix spp.) for phytoextraction of heavy metals. Environmental and
771 Experimental Botany 60, 57-68.
772 Meharg, A.A., Hartley-Whitaker, J. (2002) Arsenic uptake and metabolism in arsenic resistant and
773 nonresistant plant species. New Phytologist 154, 29-43.
774 Mitani-Ueno, N., Yamaji, N., Zhao, F.J., Ma, J.F. (2011) The aromatic/arginine selectivity filter of
775 NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic.
776 J Exp Bot 62, 4391-4398.
777 Moreno-Jimenez, E., Esteban, E., Fresno, T., de Egea, C.L., Penalosa, J.M. (2010) Hydroponics as a
778 valid tool to assess arsenic availability in mine soils. Chemosphere 79, 513-517.
779 Morimoto, R.I. (1998) Regulation of the heat shock transcriptional response: cross talk between a
780 family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev
781 12, 3788-3796.
782 Naidu, R., Smith, E., Owens, G., Bhattacharya, P., Nadebaum, P. (2006) Managing arsenic in the
783 environment: from soil to human health. CSIRO Publishing, Collingwood.

784 Nie, L., Shah, S., Rashid, A., I. Burd, G., Dixon, D.G., Glick, B. (2002) Phytoremediation of arsenate
785 contaminated soil by transgenic canola and the plant growth-promoting bacterium
786 Enterobacter cloacae CAL2. Plant Physiology and Biochemistry 40, 335-361.

787 O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., et al. (2016)
788 Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and
789 functional annotation. Nucleic Acids Res 44, D733-745.
790 Park, J.H., Han, Y.S., Seong, H.J., Ahn, J.S., Nam, I.H. (2016) Arsenic uptake and speciation in
791 Arabidopsis thaliana under hydroponic conditions. Chemosphere 154, 283-288.
792 Paul, D.S., Hernández-Zavala, A., Walton, F.S., Adair, B.M., Dina, J.D., Matoušek, T., Stýblo, M.
793 (2007) Examination of the Effects of Arsenic on Glucose Homeostasis in Cell Culture
794 and Animal Studies: Development of a Mouse Model for Arsenic-Induced Diabetes.
795 Toxicology and applied pharmacology 222, 305-314.
796 Pilon-Smits, E. (2005) Phytoremediation. Annu Rev Plant Biol 56, 15-39.
797 Puckett, E.E., Serapiglia, M.J., DeLeon, A.M., Long, S., Minocha, R., Smart, L.B. (2012)
798 Differential expression of genes encoding phosphate transporters contributes to arsenic
799 tolerance and accumulation in shrub willow (Salix spp.). Environmental and
800 Experimental Botany 75, 248-257.
801 Purdy, J.J., Smart, L.B. (2008) Hydroponic Screening of Shrub Willow (Salix Spp.) for Arsenic
802 Tolerance and Uptake. International Journal of Phytoremediation 10, 515-528.
803 Quaghebeur, M., Rengel, Z. (2003) The Distribution of Arsenate and Arsenite in Shoots and Roots of
804 Holcus lanatus is Influenced by Arsenic Tolerance and Arsenate and Phosphate Supply.
805 Plant Physiol. 132(3):1600-1609.
806 Raab, A., Schat, H., Meharg, A.A., Feldmann, J. (2005) Uptake, translocation and transformation of
807 arsenate and arsenite in sunflower (Helianthus annuus): formation of arsenic–
808 phytochelatin complexes during exposure to high arsenic concentrations. New
809 Phytologist 168, 551-558.
810 Rahman, A., M. G. Mostofa, M. M. Alam, K. Nahar, M. Hasanuzzaman and M. Fujita (2015).
811 Calcium Mitigates Arsenic Toxicity in Rice Seedlings by Reducing Arsenic Uptake and
812 Modulating the Antioxidant Defense and Glyoxalase Systems and Stress Markers.
813 BioMed research international 2015: 340812-340812.
814 Raskin, I., Kumar, P.B.A.N., Dushenkov, S., Salt, D.E. (1994) Bioconcentration of heavy metals by
815 plants. Current Opinion in Biotechnology 5, 285-290.
816 Rea, P. A., O. K. Vatamaniuk and D. J. Rigden (2004). Weeds, Worms, and More. Papain's Long-
817 Lost Cousin, Phytochelatin Synthase. Plant Physiology 136(1): 2463-2474.
818 Reichard, J.F., Puga, A. (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene
819 regulation. Epigenomics 2, 87-104.
820 Remans, T., Keunen, E., Bex, G.J., Smeets, K., Vangronsveld, J., Cuypers, A. (2014) Reliable gene
821 expression analysis by reverse transcription-quantitative PCR: reporting and minimizing
822 the uncertainty in data accuracy. Plant Cell 26, 3829-3837.
823 Ruttens, A., Boulet, J., Weyens, N., Smeets, K., Adriaensen, K., Meers, E., Van Slycken, S., Tack,
824 F., Meiresonne, L., Thewys, T., Witters, N., Carleer, R., Dupae, J., Vangronsveld, J.
825 (2011) Short Rotation Coppice Culture of Willows and Poplars as Energy Crops on Metal
826 Contaminated Agricultural Soils. International Journal of Phytoremediation 13, 194-207.
827 Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., Bleeker, P.M. (2002) The role of
828 phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator
829 and non-hyperaccumulator metallophytes. J Exp Bot 53, 2381-2392.
830 Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T)
831 method." Nat Protoc 3(6): 1101-1108.

Schmöger, M.E.V., Oven, M., Grill, E. (2000) Detoxification of Arsenic by Phytochelatins in Plants. Plant Physiol 122, 793-802.

Shaibur, M. R., N. Kitajima, R. Sugawara, T. Kondo, S. M. Imamul Huq and S. Kawai (2008). Physiological and Mineralogical Properties of Arsenic-Induced Chlorosis in Barley Seedlings Grown Hydroponically. Journal of Plant Nutrition 31(2): 333-353.

Sharma, R., R. Bhardwaj, N. Handa, V. Gautam, S. K. Kohli, S. Bali, P. Kaur, A. K. Thukral, S. Arora, P. Ohri and A. P. Vig (2016). Responses of Phytochelatins and Metallothioneins in Alleviation of Heavy Metal Stress in Plants: An Overview. Plant Metal Interaction. P. Ahmad, Elsevier: 263-283.

Shelmerdine, P.A., Black, C.R., McGrath, S.P., Young, S.D. (2009) Modelling phytoremediation by the hyperaccumulating fern, Pteris vittata, of soils historically contaminated with arsenic. Environmental Pollution 157, 1589-1596.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J 39, 629-642.

Singh, A.P., Goel, R.K., Kaur, T. (2011) Mechanisms Pertaining to Arsenic Toxicity. Toxicology International 18, 87-93.

Smith, A. H., A. Ercumen, Y. Yuan and C. M. Steinmaus (2009). "Increased lung cancer risks are similar whether arsenic is ingested or inhaled." Journal of exposure science & environmental epidemiology 19(4): 343-348.

Sok, J., Calfon, M., Lu, J., Lichtlen, P., Clark, S.G., Ron, D. (2001) Arsenite-inducible RNA-associated protein (AIRAP) protects cells from arsenite toxicity. Cell Stress Chaperones 6, 6-15.

Song, W.Y., Park, J., Mendoza-Cozatl, D.G., Suter-Grotemeyer, M., Shim, D., Hortensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E. (2010) Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proc Natl Acad Sci U S A 107, 21187-21192.

Spratlen, M.J., Gamble, M.V., Grau-Perez, M., Kuo, C.-C., Best, L.G., Yracheta, J., Francesconi, K., Goessler, W., Mossavar-Rahmani, Y., Hall, M., Umans, J.G., Fretts, A., Navas-Acien, A. (2017) Arsenic metabolism and one-carbon metabolism at low-moderate arsenic exposure: Evidence from the Strong Heart Study. Food and Chemical Toxicology 105, 387-397.

Srivastava, S. and M. Shrivastava (2017). Zinc supplementation imparts tolerance to arsenite stress in Hydrilla verticillata (L.f.) Royle. Int J Phytoremediation 19(4): 353-359.

Stanhill, A., Haynes, C.M., Zhang, Y., Min, G., Steele, M.C., Kalinina, J., Martinez, E., Pickart, C.M., Kong, X.-P., Ron, D. (2006) An Arsenite-Inducible 19S Regulatory Particle-Associated Protein Adapts Proteasomes to Proteotoxicity. Molecular Cell 23, 875-885.

Sylvain, B., Mikael, M.-H., Florie, M., Emmanuel, J., Marilyne, S., Sylvain, B., Domenico, M. (2016) Phytostabilization of As, Sb and Pb by two willow species (S. viminalis and S. purpurea) on former mine technosols. CATENA 136, 44-52.

Tlustoš, P., Száková, J.i., Vysloužilová, M., Pavlíková, D., Weger, J., Javorská, H. (2007) Variation in the uptake of Arsenic, Cadmium, Lead, and Zinc by different species of willows Salix spp. grown in contaminated soils. Central European Journal of Biology 2, 254-275.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. (2012) Primer3--new capabilities and interfaces. Nucleic Acids Res 40, e115.

Wang, J., Zhao, F.J., Meharg, A.A., Raab, A., Feldmann, J., McGrath, S.P. (2002) Mechanisms of arsenic hyperaccumulation in Pteris vittata. Uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiol 130, 1552-1561.

Wang, X., Mu, X., Zhang, J., Huang, Q., Alamdar, A., Tian, M., Liu, L., Shen, H. (2015) Serum metabolomics reveals that arsenic exposure disrupted lipid and amino acid metabolism in rats: a step forward in understanding chronic arsenic toxicity. Metallomics 7, 544-552.
White, P. J. and P. H. Brown (2010). Plant nutrition for sustainable development and global health. Annals of botany 105(7): 1073-1080.
Witters, N., Slycken, S., Ruttens, A., Adriaensen, K., Meers, E., Meiresonne, L., Tack, F.M.G., Thewys, T., Laes, E., Vangronsveld, J. (2009) Short-rotation coppice of willow for phytoremediation of a metal-contaminated agricultural area: a sustainability assessment. BioEnergy Res 2.
Xu, W., Dai, W., Yan, H., Li, S., Shen, H., Chen, Y., Xu, H., Sun, Y., He, Z., Ma, M. (2015) Arabidopsis NIP3;1 Plays an Important Role in Arsenic Uptake and Root-to-Shoot Translocation under Arsenite Stress Conditions. Molecular Plant 8, 722-733.
Xu, X.Y., McGrath, S.P., Zhao, F.J. (2007) Rapid reduction of arsenate in the medium mediated by plant roots. New Phytologist 176, 590-599.
Yan, X.L., Lin, L.Y., Liao, X.Y., Zhang, W.B. (2012) Arsenic accumulation and resistance mechanism in Panax notoginseng, a traditional rare medicinal herb. Chemosphere 87, 31-36.
Yang, Q., Tu, S., Wang, G., Liao, X., Yan, X. (2012) Effectiveness of Applying Arsenate Reducing Bacteria to Enhance Arsenic Removal From Polluted Soils by Pteris Vittata L. International Journal of Phytoremediation 14, 89-99.
Yanitch, A., Brereton, N.J.B., Gonzalez, E., Labrecque, M., Joly, S., Pitre, F.E. (2017) Transcriptomic Response of Purple Willow (Salix purpurea) to Arsenic Stress. Front Plant Sci 8, 1115.
Zhang, W., Cai, Y., Tu, C., Ma, L.Q. (2002) Arsenic speciation and distribution in an arsenic hyperaccumulating plant. Science of The Total Environment 300, 167-177.
Zhang, X., Zhao, F.J., Huang, Q., Williams, P.N., Sun, G.X., Zhu, Y.G. (2009) Arsenic uptake and speciation in the rootless duckweed Wolffia globosa. New Phytol 182, 421-428.
Zhang, Y., Han, X., Chen, S., Zheng, L., He, X., Liu, M., Qiao, G., Wang, Y., Zhuo, R. (2017) Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in Salix matsudana under different abiotic stresses. Scientific Reports 7, 40290.
Zhao, F.J., Ma, J.F., Meharg, A.A., McGrath, S.P. (2009) Arsenic uptake and metabolism in plants. New Phytol 181, 777-794.

Supplementary Table 1. Primer sequences used for the real time RT-PCR analyses.

Gene	Gene description	*S. purpurea* ortholog locus or NCBI annotation	Primer sequence F/R (5′-3′)	Product size (bp)	Efficiency	R^2
Reference genes (1: used to normalize gene expression data in roots, 2: used to normalize gene expression data in leaves)						
OTU[1,2]	OTU-like cysteine protease family protein	SapurV1A.0615s0200.1	GGCAGTGGTTCCTCTTCGAA ATCCCCATCTTTCGCAGTCG	114	91.2%	0.9951
ACT7[2]	Actin 7	SapurV1A.0231s0320.1	CTGTCCTTTCCCTGTATGCCA GTCACGACCAGCAAGATCCA	140	90.6%	0.9939
α-TUB2[2]	Alpha-tubulin 2	SapurV1A.0598s0030.1	CCAAGCGAGCATTTGTCCAC CCCTCGTCATCACCACCTTC	133	97.6%	0.9917
DNAJ	Chaperone protein DnaJ 49	SapurV1A.0212s0110.1	GCTCCCGGTTCTTCTTATTTTCC AAATTAACCCCTCTCTGCGTAGT	117	81.3 %	0.9871
EF1α[1]	Elongation factor 1-alpha	SapurV1A.0023s0300.1	ACCAGATTTCCGAGCCCAAG TTGGCCCAAAAGTGCAAACC	150	90.1%	0.9932
ARF2[1]	ADP-ribosylation factor 2	SapurV1A.0014s0160.1	TGGGGCTGTCTTTCACCAAG GGTCACAATCTCACCGAGCT	131	96.9%	0.9992
Arsenate transport						
HAPO4	High-affinity phosphate transporter 4	HQ228362.1	GAACGACGAGCACCTGGTT ACGGGTTCTATTCGCCTTGA	108	86.1%	0.9951
NA-DPHOT	Sodium-dependent phosphate transporter	SapurV1A.0139s0260.1	CAGCCACTTATCCCCAGCAA TCAAGGCGAATAGAACCCGT	134	94.5%	0.9873
PHO1	Phosphate transporter PHO1-like protein	SapurV1A.0063s0550.1	AGAGGCTGCGATGTTGAACA GTCTGAAGCAAGGCGAGTCA	115	81.3%	0.9938
Arsenite transport						
BORON	Boron transporter	SapurV1A.0014s0200.1	TCATTCGGGGAACAACTGGAG ACTGTCGGCTCTGCAACTC	143	93.3%	0.9805
NIP1	Aquaporin NIP1.1	SapurV1A.0029s0170.1	CAAGGTTGTGACTCTTCCAGGA GACAGCAGGGTTGAAATGGG	106	89.9%	0.9912

SIP1	Aquaporin SIP.1	SapurV1A.1058s0060.1	GCCAGTTCAGTACAAGCACATG TGCAGCAGAGGGTTTCGAG	147	103.9%	0.9911
SILICON	Silicon 1	SapurV1A.1225s0080.1	GGTAGCAGTCTCAGCAGGTG TGAAAGGTTCCCAGCAACTGT	94	85.2%	0.9977
Arsenate reductases						
CDC25-1	Tyrosine phosphatase	SapurV1A.0142s0310.1	ACGGCATCTTTAGGTCTGGTT TACGGCTCGGGACATAGACA	97	92.9%	0.9851
CDC25-2	Tyrosine phosphatase	SapurV1A.0243s0430.1	TCAACTTTCACCACAGAAGACCT CACTAGTTGACGAGCCAGGA	147	89.9%	0.9960
Thiol chelating response						
GR	Glutathione reductase	SapurV1A.0056s0770.1	ACGAAATGAGGGCTGTGGTT CCTCTCCATGATCTGTGCGA	126	93.9 %	0.9624
GS	Glutathione synthetase	SapurV1A.1124s0080.1	GCTGTCAAGTGCCCATCCAT CAGACTCCATAAGCCAGCGA	91	116.4%	0.9889
PCS	Phytochelatin synthase	SapurV1A.0160s0210.1	GTGGAAGGGTATTGCTGTAAGGA TGAGATGAAGGAACCAGCACA	137	98.53%	0.9922
GST	Glutathion S-transferase	SapurV1A.0016s1070.1	CGGTTCTTGGCTGGAGATGA CCTCCCCACATTTTCCCTGG	120	90.0%	0.9935
MT1A	Metallothionein	*S. matsudana* EF157299.1	CTTCGGTGCTGAGAATGGCT CTGCTTTGTTGGGACCATGC	97	90.5%	0.9998
Vacuolar transporters						
ABCG	ABC transporter G	SapurV1A.0258s0220.1	AGGCTTGGATTCTACAACTGCT TGGCTGGTGGATTGTTGTCA	94	84.3%	0.9778
CAX2-1	Vacuolar cation/proton exchanger 2	SapurV1A.1071s0020.1	TCTTGCAATCGTCGTCCACA ACCTAAACGCTCAGCCAAGG	94	92.9%	0.9954
CAX2-2	Vacuolar cation/proton exchanger 2	SapurV1A.0338s0120.1	TTGTTGGTGCTTGGATGTGC GCAGGACAGCAGGAAAGAG	142	103.4%	0.9826
WBABCT	White-brown-complex ABC transporter	SapurV1A.0084s0020.1	GCAAGAGGTGGTAGGACTGT ACACCCATCCGACAAAACCA	96	97.3%	0.9979

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Flavonoid Synthesis						
CHS1	Chalcone synthase	SapurV1A.0820s0070.1	CATTCCGTGGCCCTAGTGAC CGGAGCCTACAATGAGAGCA	90	96.9%	0.9974
CHS2	Chalcone synthase 2	SapurV1A.0056s0660.1	AACTGCGAGCCACTAGACAC AAAAGCACACCCCACTCCAA	145	91.5%	0.9992
CHS3	Chalcone synthase 3	SapurV1A.0820s0080.1	GCGGCCCAGACTATTCTACC AGCCTCGGTCAGACTCTTCT	135	87.7%	0.9999
F3H	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	TCTTGTCGGAGGCTATGGGA TCGGTATGGCGTTTGAGTCC	136	96.7%	0.999
FLH	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT	114	88.6%	0.9949
2HFLR	Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCATCGA	96	92.7%	0.9661
FLS	Flavonol synthase	SapurV1A.1087s0040.1	TCCCAACCCAGATTGTGTCG CAAATAGGCCCCACTGCGAA	94	90.5%	0.9976
ANR	Anthocyanidin reductase	SapurV1A.0028s0410.1	TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT	129	93.8%	0.9930
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG	127	95.8%	0.9823
Stress related						
ACCS	1-aminocyclopropane-1-carboxylate synthase	SapurV1A.2160s0020.1	GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA	115	102.3%	0.9982
AIP-1	Arsenite-inducible RNA-associated protein AIP-1-related	SapurV1A.0229s0030.1	CTTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG	140	93.2%	0.9940
ER	Ethylene receptor	SapurV1A.0052s0240.1	TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA	90	120.0%	0.9870

CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA TCCAGCAACAACTCCAACGA	125	90.5%	0.9919

931 **Table 1. Continued**

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940 **Table 1**. Nutrients (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic
941 conditions for 30 days. Different letters within each column and tissue indicate significant differences
942 among treatments and time points on HSD test at $p < 0.05$.

Organ	Time (d)	Treatment	Nutrient				
			P	Ca	B	Zn	Fe
Roots	1	Control	5757.89 ± 430.12 a	4322.53 ± 207.32 b	19.62 ± 1.12 a	512.35 ± 53.12 b	1955.93 ± 98.36 b
		As	5720.65 ± 379.34 a	4929.41 ± 242.98 a	21.98 ± 1.23 a	625.22 ± 29.75 a	2364.78 ± 115.93 a
	3	Control	5791.72 ± 456.56 a	3271.75 ± 245.56 b	15.83 ± 0.89 c	439.97 ± 23.45 bc	1219.43 ± 62.34 d
		As	5507.21 ± 412.42 a	4556.62 ± 342.45 a	19.46 ± 0.93 a	469.75 ± 30.45 bc	1354.59 ± 49.45 c
	10	Control	3772.06 ± 235.67 b	3041.54 ± 289.87 c	15.53 ± 0.92 b	481.45 ± 32.34 bc	1087.50 ± 83.12 e
		As	2883.34 ± 176.34 c	4257.52 ± 458.96 b	17.53 ± 1.09 ab	432.36 ± 23.56 c	1287.73 ± 69.32 c
	30	Control	2729.45 ± 278.45 d	3093.95 ± 334.56 c	13.26 ± 1.01 d	330.76 ± 22.34 d	943.27 ± 34.23 f
		As	1633.24 ± 99.83 e	4154.46 ± 354.98 a	14.38 ± 0.89 d	332.44 ± 15.69 d	1082.25± 50.54 e
Leaves	1	Control	3403.13 ± 179.33 a	6227.33 ± 434.93 a	64.22 ± 6.73 ab	633.62 ± 40.93 a	234.31 ± 12.45 b
		As	3112.77 ± 143.54 ab	5290.13 ± 302.34 b	49.23 ± 5.34 bc	544.83 ± 30.87 b	226.23 ± 10.15 b
	3	Control	3591.96 ± 123.43 a	5886.58 ± 478.23 b	49.31 ± 3.53 bc	646.95 ± 51.23 a	382.15 ± 21.54 a

	As	2942.52 ± 174.23 b	6909.65 ± 398.12 a	68.55 ± 4.52 a	532.08 ± 79.56 ab	180.67 ± 7.28 c
10	Control	2358.36 ± 132.34 c	6378.05 ± 403.23 ab	47.97 ± 2.23 c	503.68 ± 23.13 b	250.83 ± 10.23 b
	As	2046.62 ± 124.54 d	4603.75 ± 345.21 c	57.26 ± 3.21 b	516.58 ± 31.22 b	143.65 ± 18.23 d
30	Control	2035.46 ± 121.23 d	7001.72 ± 421.23 a	43.32 ± 2.34 d	429.35 ± 28.78 c	239.40 ± 12.12 b
	As	2106.37 ± 134.24 cd	5492.63 ± 324.12 b	60.67 ± 3.11 ab	400.92 ± 33.21 c	152.47 ± 13.52 d

Table 2. Arsenic accumulation (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to arsenic for 30 days. Different letters within each column and plant tissue indicate significant differences among time points on HSD test at $p < 0.05$. nd: not detected.

Organ	Time point (d)	Arsenic		
		III	V	Total
Roots	1	16.14 ± 2.34 d	164.88 ± 149.33 d	182.43 ± 20.10 d
	3	33.45 ± 4.78 c	318.86 ± 21.95 c	353.65 ± 23.98 c
	10	929 ± 80.21 b	542.35 ± 41.29 b	1471.92 ± 123.87 b
	30	1688 ± 148.43 a	734.90 ± 65.20 a	2448 ± 178.32 a
Leaves	1	nd	2.78 ± 0.24 d	2.78 ± 0.24 d
	3	1.30 ± 0.08 e	5.76 ± 0.45 c	7.23 ± 0.39 c
	10	nd	18.75 ± 1.14 b	18.75 ± 1.14 b
	30	nd	25.45 ± 2.57 a	25.45 ± 2.57 a

Table 3. Non-protein thiolic peptides (nmol GSH g^{-1} FW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters within each row and plant tissue indicate significant differences among treatments and time points on HSD test at $p < 0.05$. nd: not detected.

		1 d		3 d		10 d		30 d	
Organ	Thiol	Control	As	Control	As	Control	As	Control	As

Roots	Cys	nd	5.85 ± 1.73 a	nd	19.38 ± 0.18 b	nd	7.99 ± 2.15 a	Nd	4.06 ± 0.15 c
	GSH	13.73 ± 0.93 a	7.90 ± 0.66 b	11.68 ± 1.59 a	6.91 ± 0.55 bc	15.15 ± 1.83 a	6.15 ± 0.74 c	12.80 ± 1.40 a	6.05 ± 0.52 c
	TC_1	nd	14.69 ± 1.11 a	nd	10.62 ± 0.74 b	nd	6.72 ± 1.25 c	Nd	nd
	PC_2	nd	13.63 ± 1.85 b	nd	16.72 ± 1.78 ab	nd	19.10 ± 1.03 a	Nd	18.42 ± 1.75 a
	Cys-PC_2	nd	10.37 ± 0.76 d	nd	13.46 ± 1.51 c	nd	23.13 ± 0.02 b	Nd	34.35 ± 1.48 a
	TC_2	nd	6.78 ± 0.14 d	nd	10.17 ± 0.32 c	nd	17.12 ± 0.45 b	Nd	21.79 ± 0.29 a
	PC_3	nd	20.32 ± 1.40 d	nd	33.64 ± 1.09 c	nd	47.01 ± 9.54 b	Nd	65.38 ± 1.06 a
	desGly-PC_3	nd	8.88 ± 0.53 d	nd	34.35 ± 2.27 c	nd	73.86 ± 4.27 b	Nd	150.19 ± 12.24 a
	Cys-PC_3	nd	10.91 ± 0.71 d	nd	61.14 ± 1.74 c	nd	74.34 ± 3.73 b	Nd	169.27 ± 11.71 a
	Total ΣNPTs	13.73 ± 0.93 e	99.34 ± 2.71 d	11.68 ± 1.59 e	174.85 ± 18.70 c	15.15 ± 1.83 e	267.57 ± 12.93 b	12.80 ± 1.40 e	469.52 ± 21.32 a
Leaves	GSH	43.09 ± 2.53 b	49.35 ± 1.80 a	40.30 ± 3.45 bc	49.87 ± 3.33 a	44.70 ± 2.78 ab	37.74 ± 1.88 c	45.63 ± 2.45 ab	41.41 ± 1.86 b
	desGly-PC_2	nd	2.22 ± 0.31 d	nd	5.18 ± 1.03 c	nd	7.65 ± 0.53 b	Nd	9.39 ± 0.88 a
	desGly-PC_4	2.50 ± 0.11 d	2.52 ± 0.10 d	2.46 ± 0.14 d	3.06 ± 0.36 c	2.74 ±0.12 cd	2.87 ± 0.14 c	4.09 ± 0.52 b	6.46 ± 0.48 a
	TC_3	4.48 ± 0.38 b	5.45 ± 0.23 a	4.04 ± 0.49 b	6.25 ± 0.64 a	4.04 ± 0.34 b	5.08 ± 0.70 ab	3.03 ± 0.18 c	3.95 ± 0.41 b
	Total ΣNPTs	55.40 ± 1.89 bc	67.60 ± 1.45 a	50.54 ± 3.45 c	69.58 ± 5.70 a	53.56 ± 4.05 bc	58.16 ± 2.50 b	54.70 ± 4.11 bc	62.51 ± 5.94 ab

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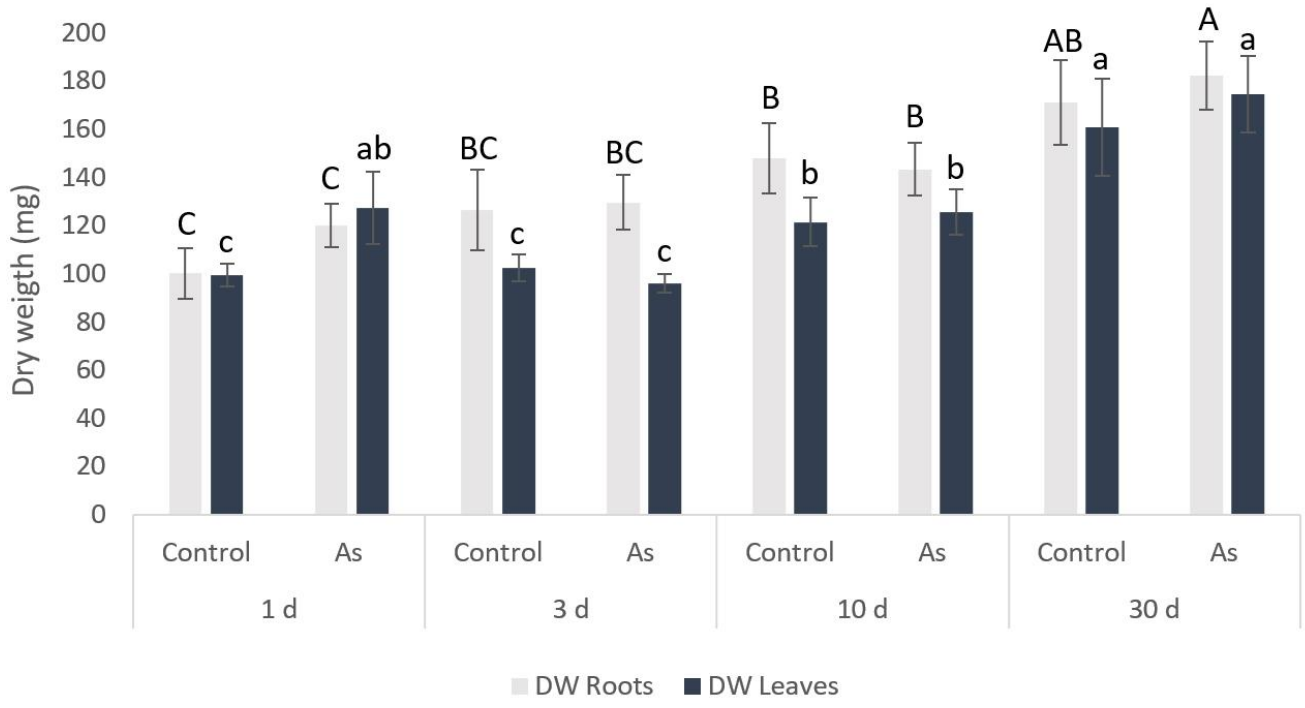

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985 **Fig. 1.** Dry weight (mg DW) of roots and leaves of *S. atrocinerea* exposed to control and As

986 conditions for 30 days. Different letters (upper case for comparison within roots and lower case for

987 comparisons within leaves) denote significant differences on HSD test at $p < 0.05$.

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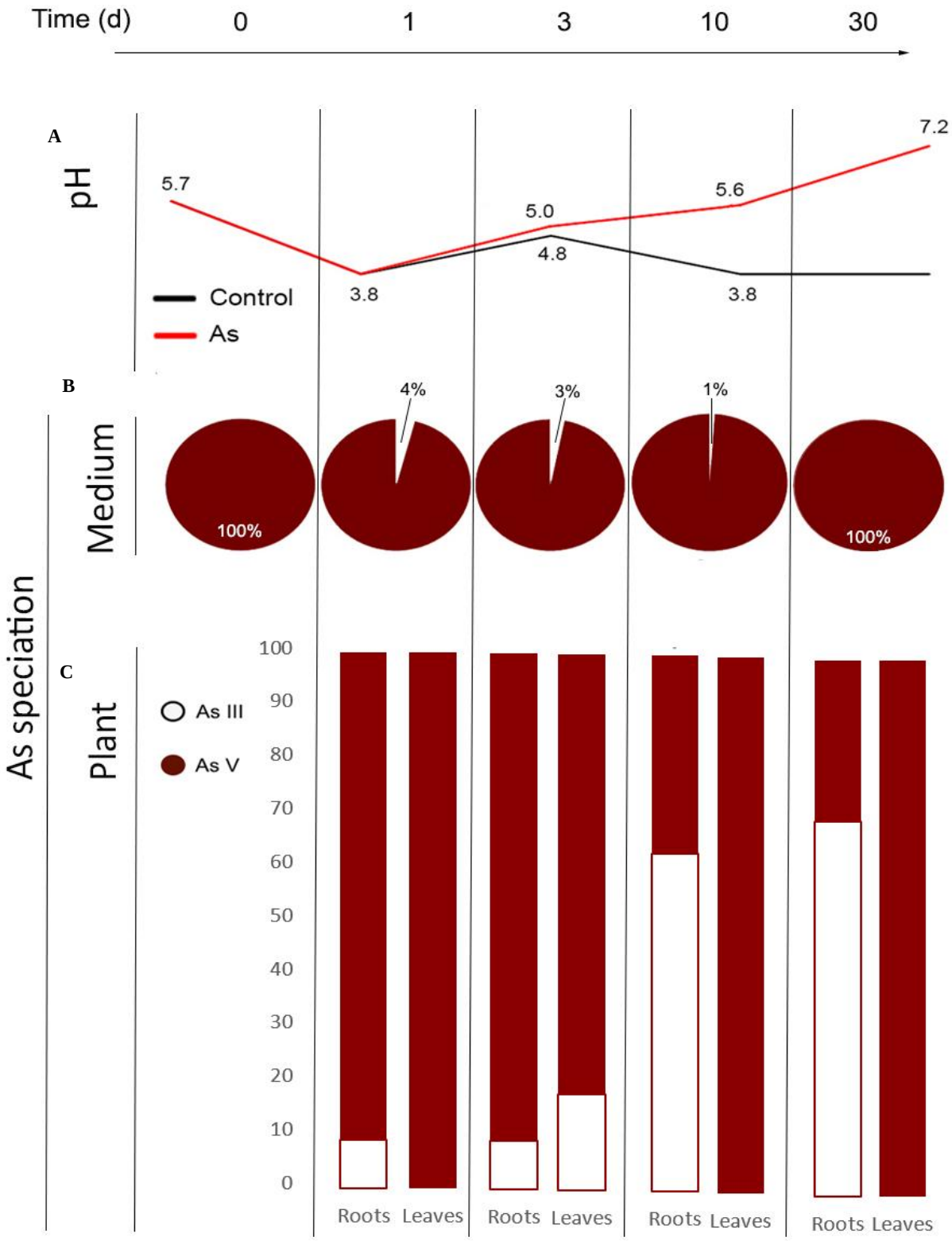


Fig. 2. pH in the culture medium **(A)** and percentage of arsenic speciation in the culture medium **(B)** and roots and leaves **(C)** of *S. atrocinerea* exposed to arsenic for 30 days (Red: As V, white: As III).

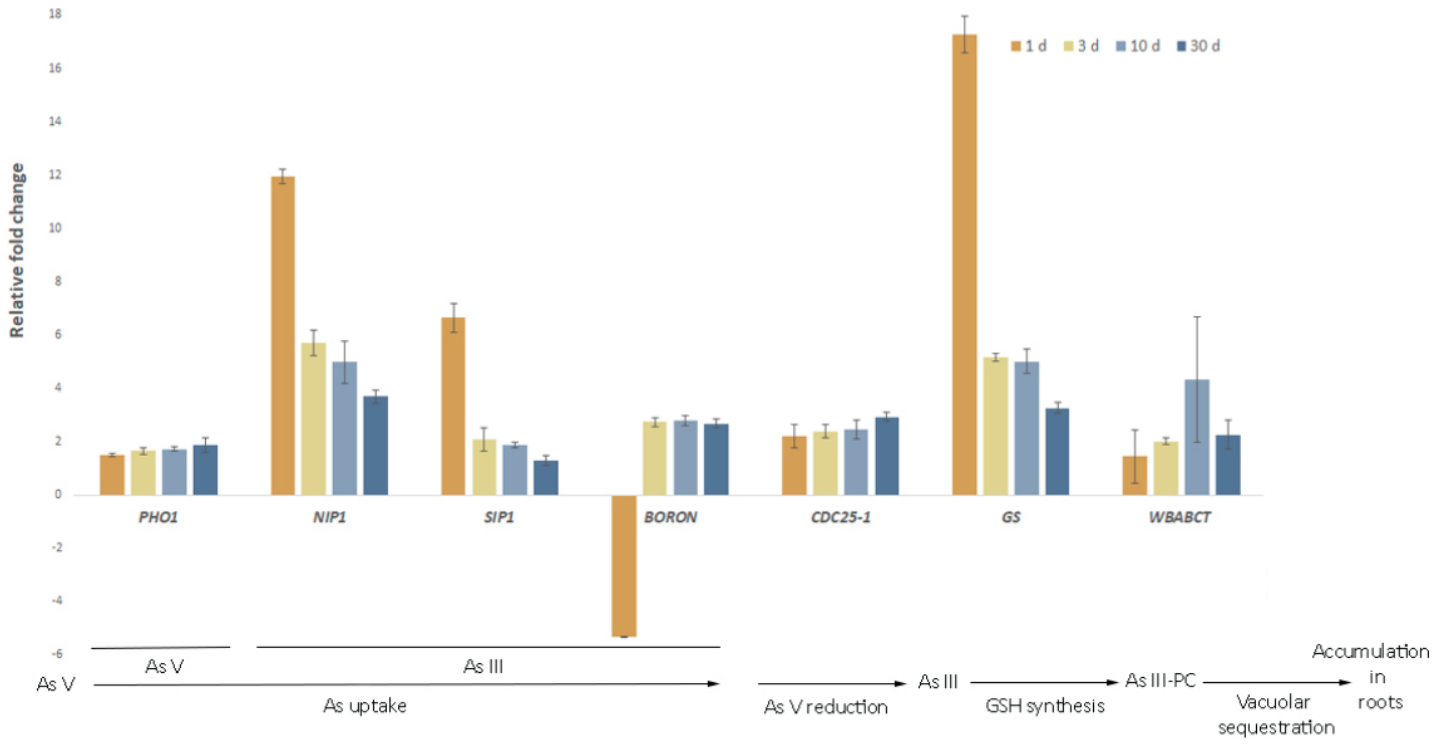


Fig. 3. Relative fold change of the gene expression levels in roots of *S. atrocinerea* exposed to As regarding those genes involved in As uptake and reduction, thiol synthesis and vacuolar sequestration, that showed the most markedly regulation along the 30 days. Values represented are the fold change (± S.D.) of mean normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *BORON*, boron transporter*; CDC25-1*, tyrosine phosphatase 1, *GS*, glutathione synthetase; *NIP1*, aquaporin NIP1.1; *PCS*, phytochelatin synthase; *PHO1*, phosphate transporter PHO1; *SIP1*, aquaporin SIP.1; *WBABCT*, white-brown-complex ABC transporter.

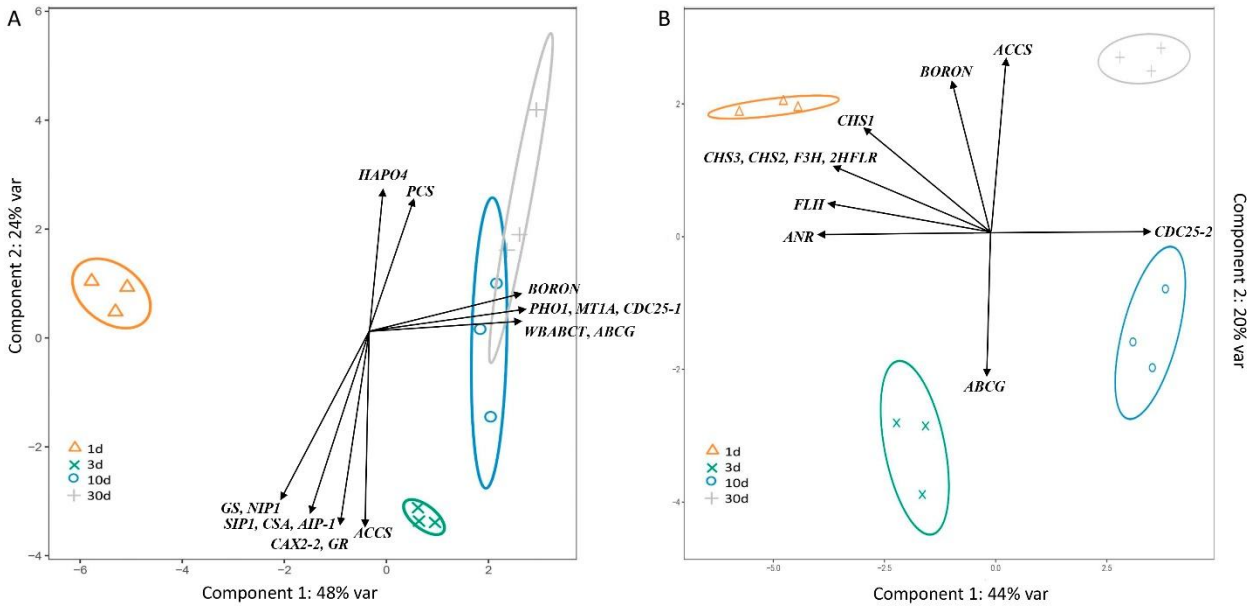
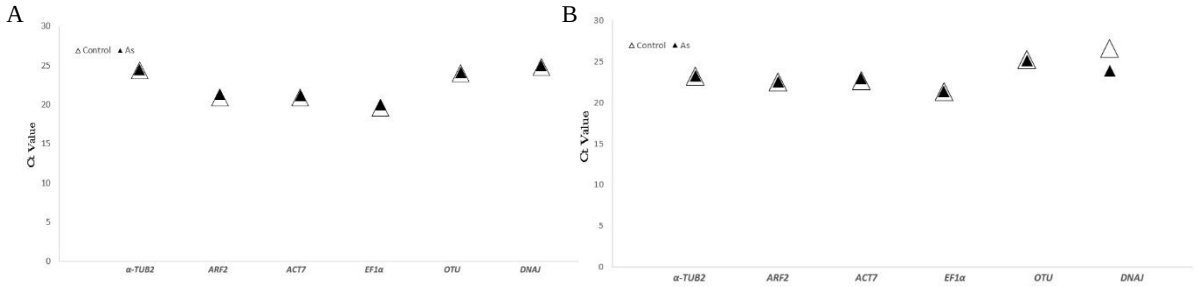
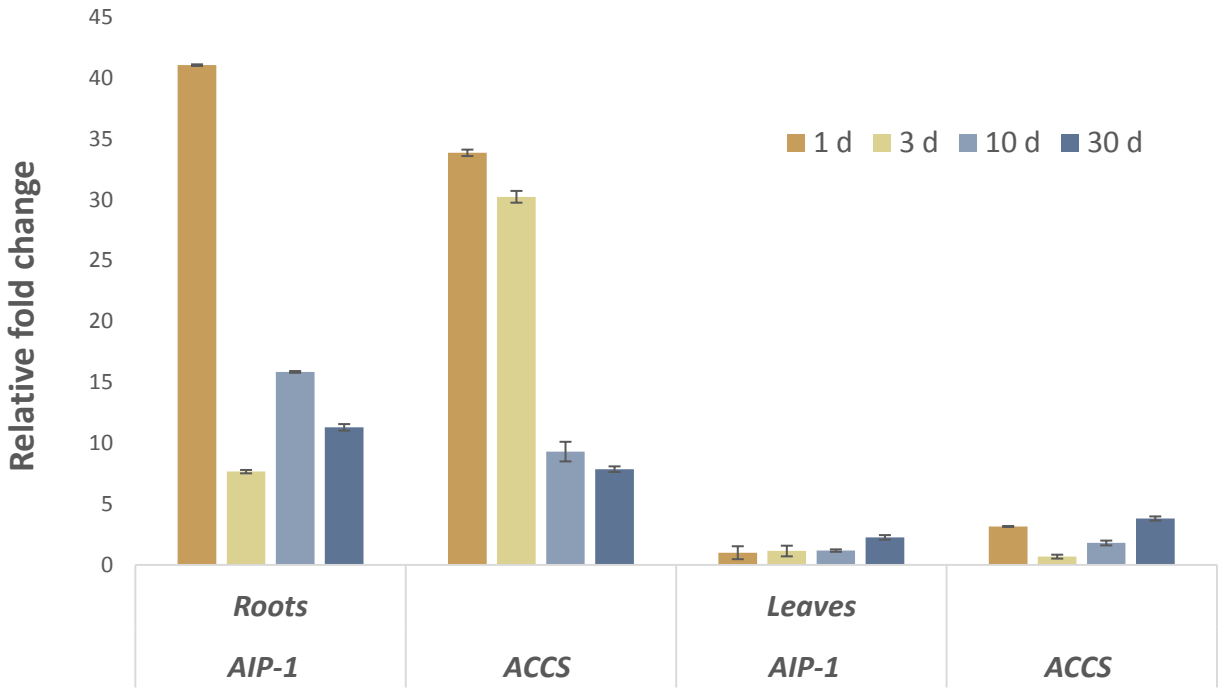


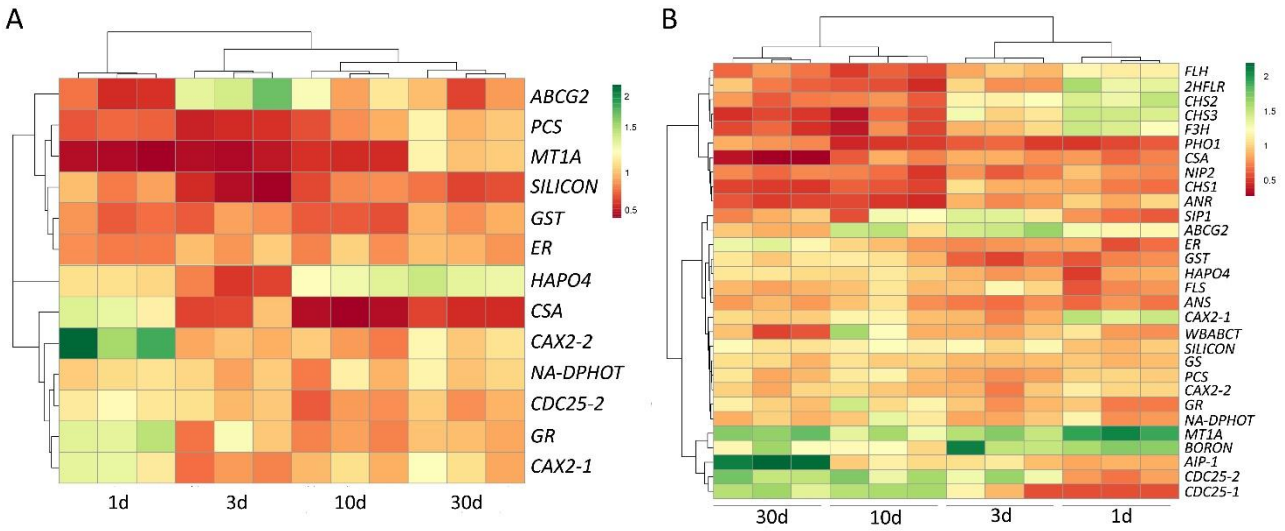
Fig 4. Biplots of the principal component analysis (PCA) in samples of roots **(A)** and leaves **(B)** of *S. atrocinerea* exposed to arsenic for 30 days calculated with the normalized gene expression levels relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *2HFLR*, dihydroflavonol 4-reductase; *ABCG*: ABC transporter G; *ACCS*, aminocyclopropane-1-carboxylate synthase; *AIP-1*, arsenite-inducible RNA-associated protein AIP-1-related; *ANR*, anthocyanidin reductase; *BORON*, boron transporter*; CAX2-2*, vacuolar cation/proton exchanger 2; *CDC25-1*, *2*, tyrosine phosphatase 1, 2; *CHS1,3*, chalcone synthase 1,3; *CSA*, cellulose synthase A; *F3H*, flavanone 3-hydroxylase; *FLH*, Flavonoid 3'-hydroxylase; *GR*, glutathione reductase; *GS*, glutathione synthetase; *HAPO4*, high-affinity phosphate transporter 4; *MT1A*, Metallothionein; *NIP1*, aquaporin NIP1.1; *PCS*, phytochelatin synthase; *PHO1*, phosphate transporter PHO1; *SIP1*, aquaporin SIP.1; *WBABCT*, white-brown-complex ABC transporter.



Supplementary Fig. 1. qRT-PCR Ct values of the 6 candidate RGs in control and As conditions. Mean values.



Supplementary Fig. 2. Relative fold change of the gene expression levels in roots of *S. atrocinerea* exposed to As regarding those genes involved in stress response that showed the most markedly regulation along the 30 days. Values represented are the fold change (± S.D.) of mean normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *ACCS*, aminocyclopropane-1-carboxylate synthase; *AIP-1*, arsenite-inducible RNA-associated protein AIP-1-related.



Supplementary Fig. 3. Heat map representations of the gene expression data obtained in samples of roots **(A)** and leaves **(B)** of *S. atrocinerea* exposed to arsenic for 30 days and hierarchical clustering based on the most differentially expressed genes with a fold regulation equal or lower than 2. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see ***Supplementary Table 1***.

Figure 1

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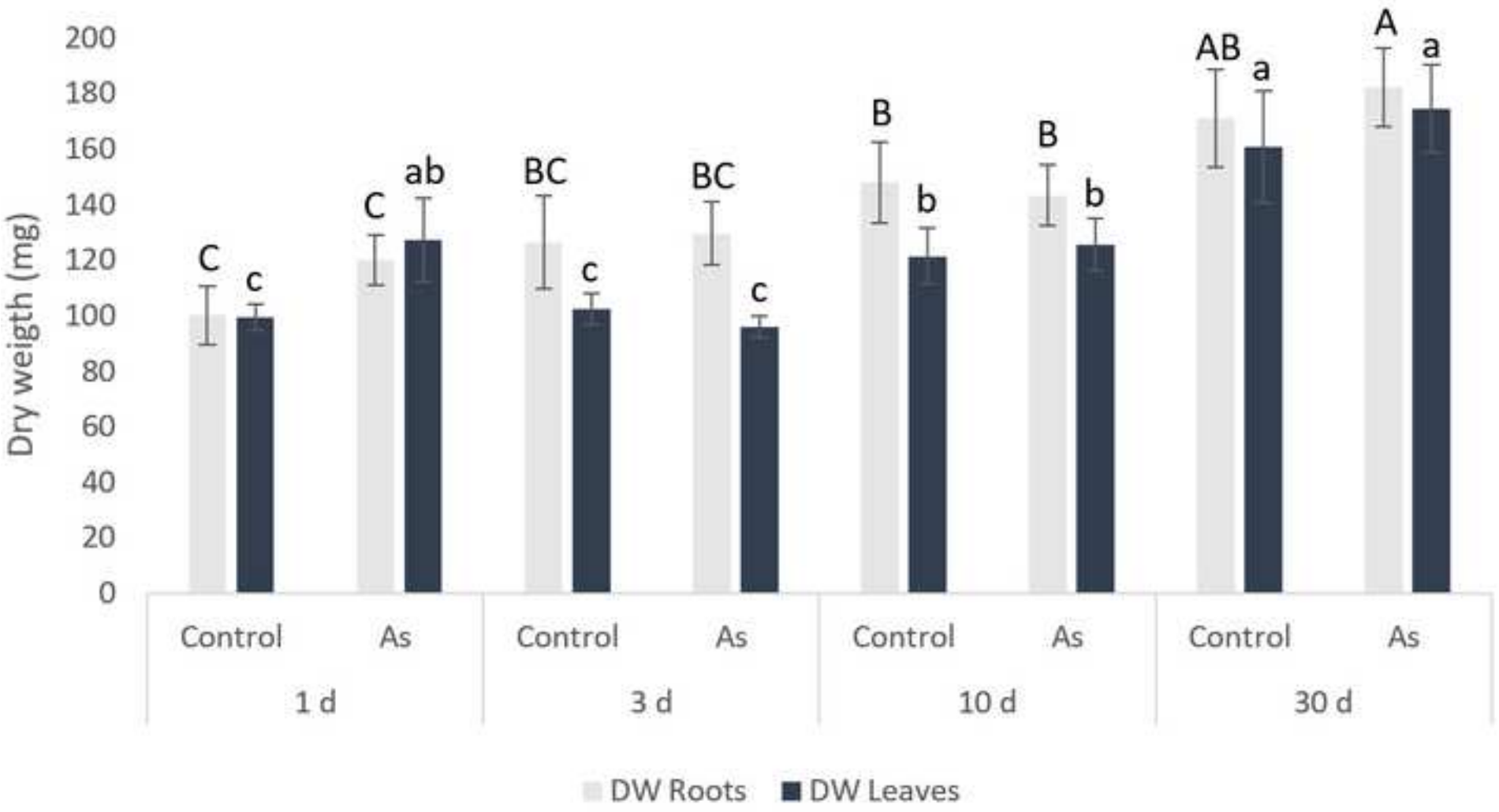

Figure 2

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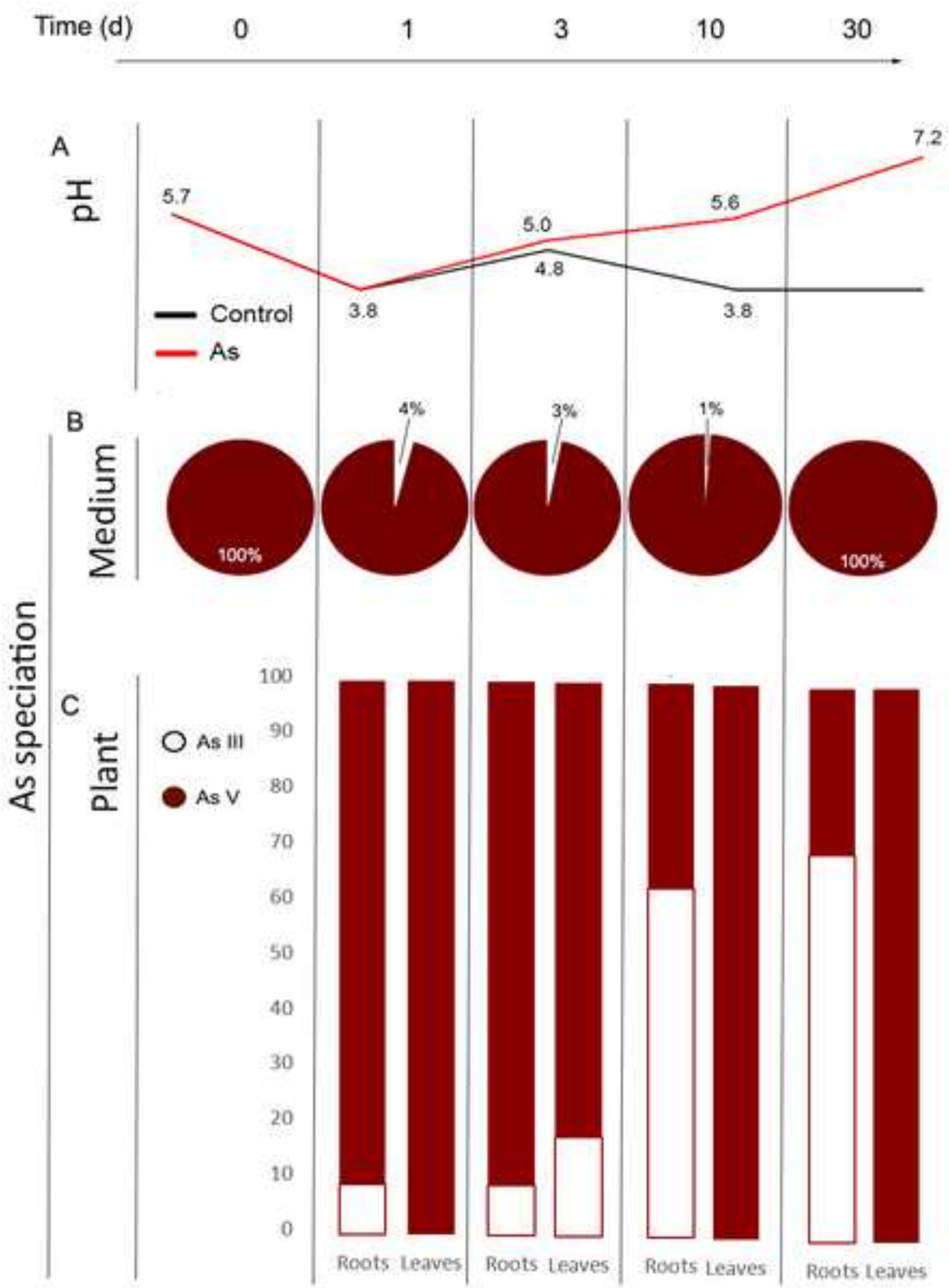

Figure 3

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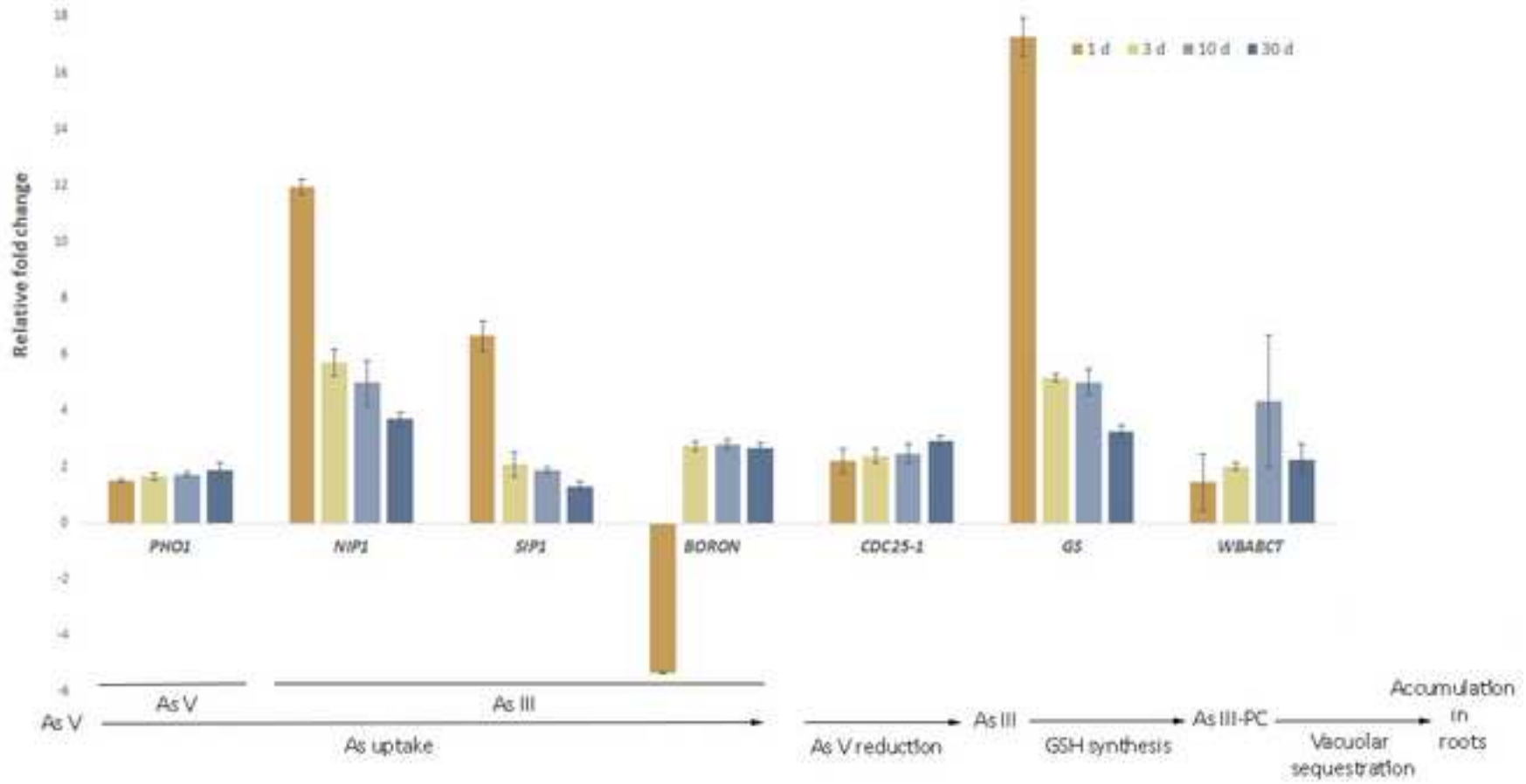

Figure 4

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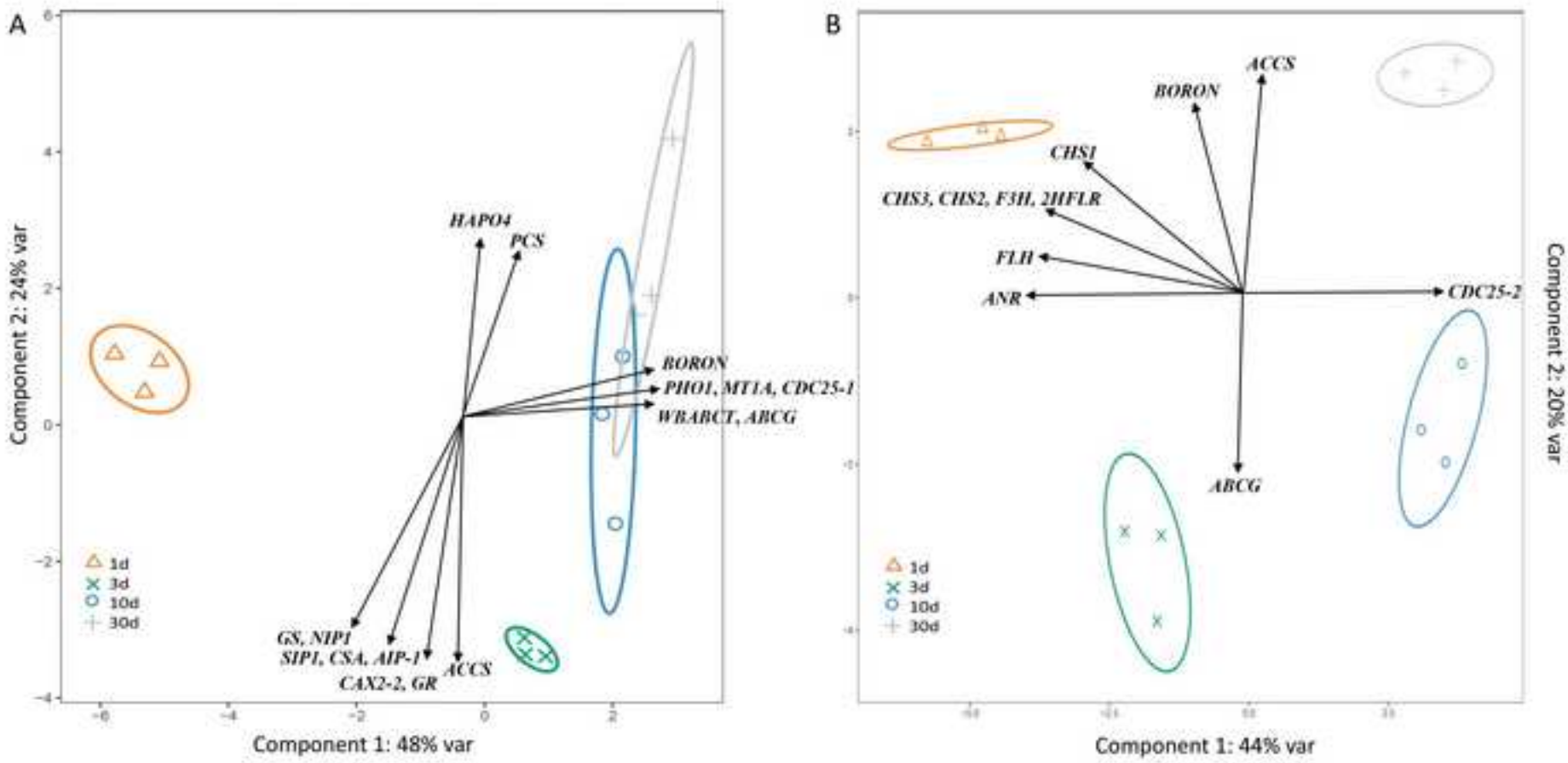

Supplementary Figure 1

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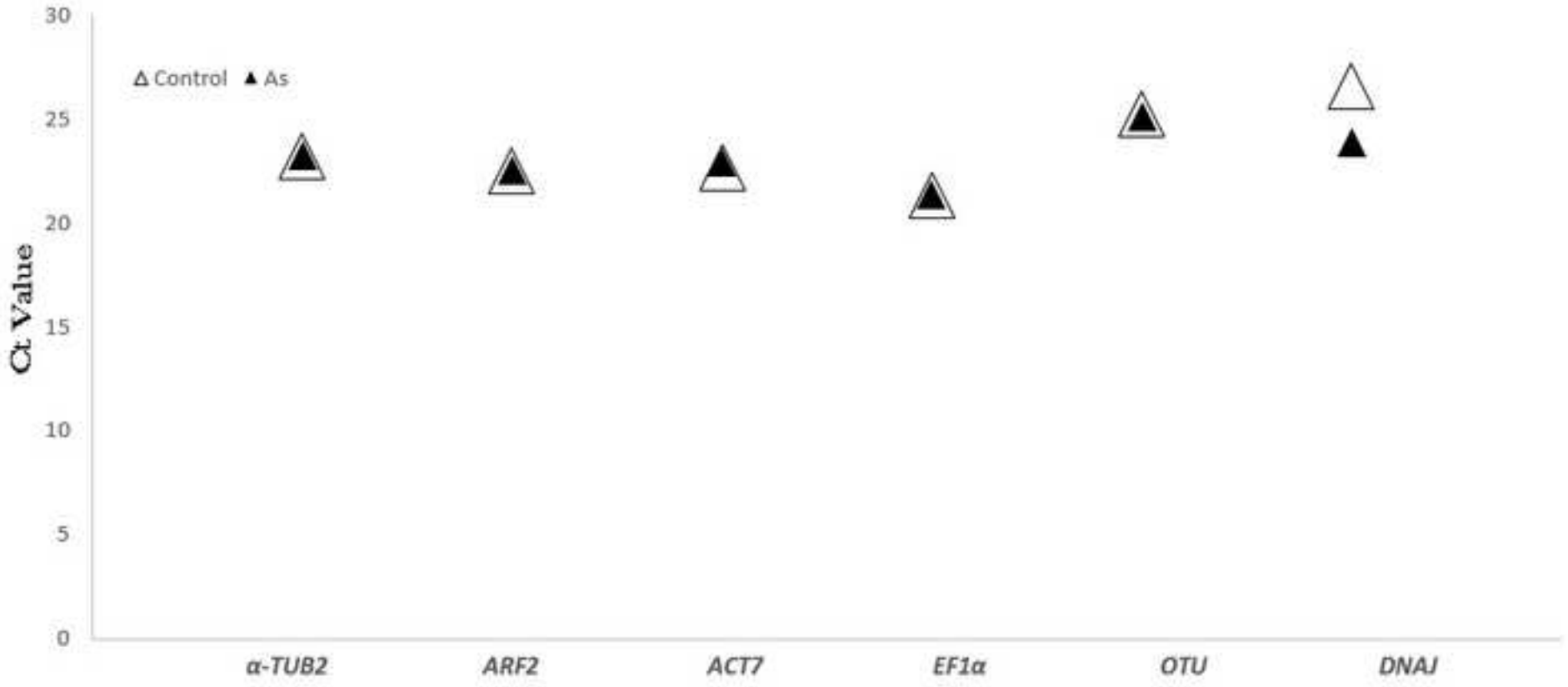

Supplementary Figure 2

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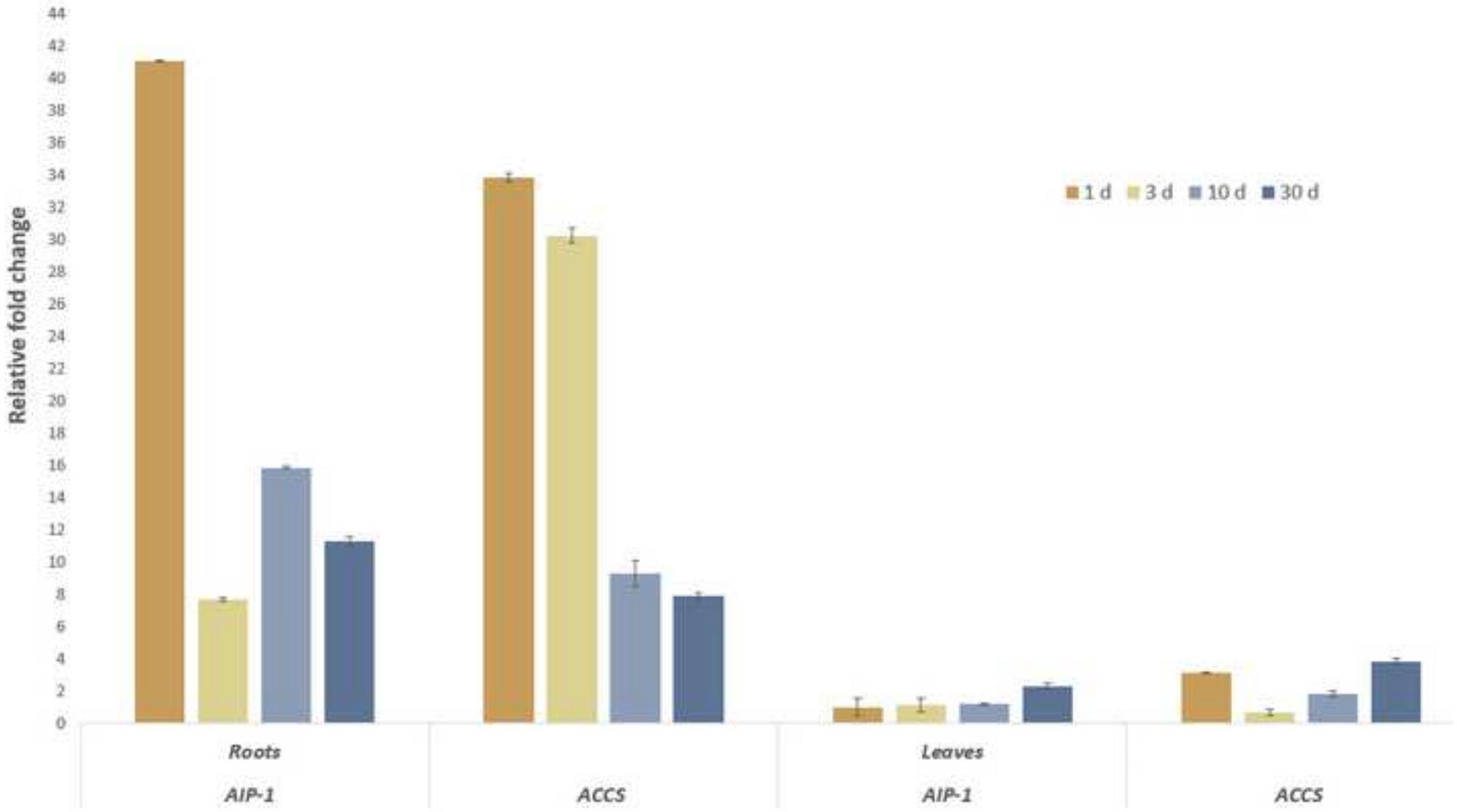

Supplementary Figure 3

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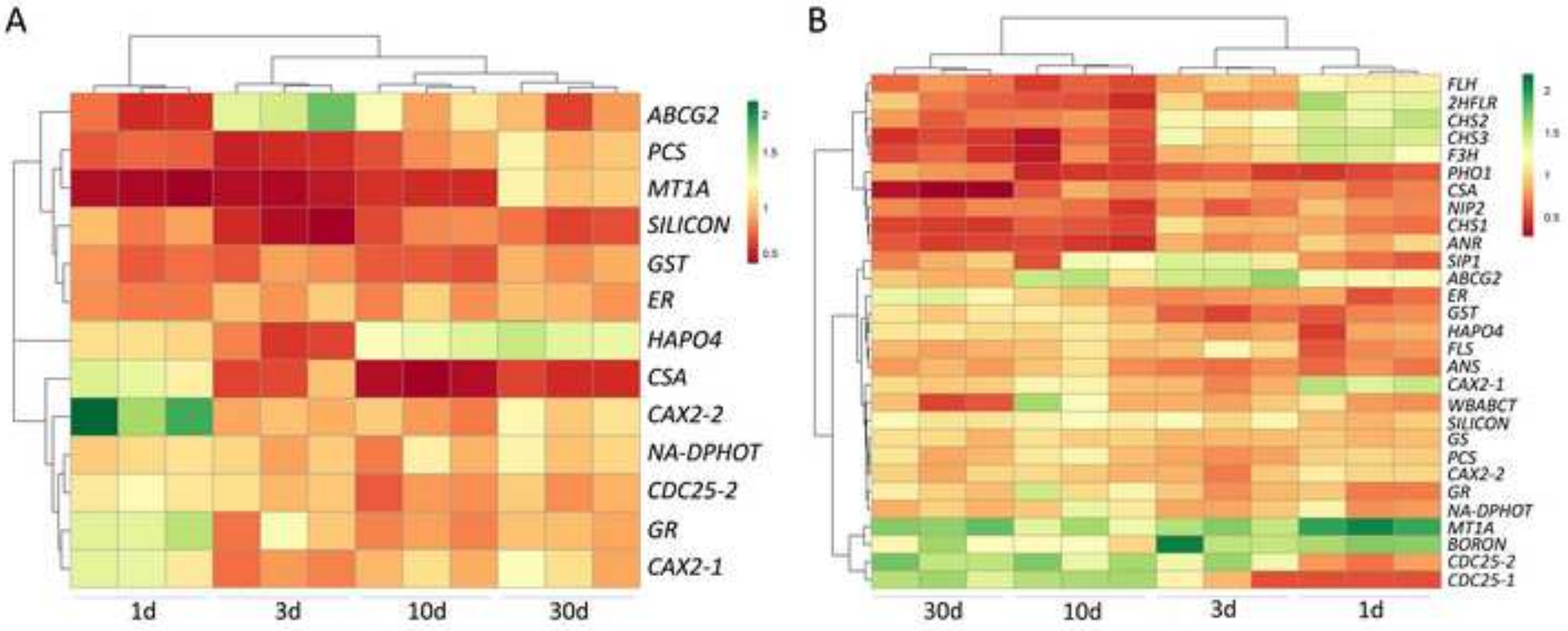

Table 1
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Table 1. Nutrients (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters within each column and tissue indicate significant differences among treatments and time points on HSD test at $p < 0.05$.

Organ	Time (d)	Treatment	Nutrient				
			P	Ca	B	Zn	Fe
Roots	1	Control	5757.89 ± 430.12 a	4322.53 ± 207.32 b	19.62 ± 1.12 a	512.35 ± 53.12 b	1955.93 ± 98.36 b
		As	5720.65 ± 379.34 a	4929.41 ± 242.98 a	21.98 ± 1.23 a	625.22 ± 29.75 a	2364.78 ± 115.93 a
	3	Control	5791.72 ± 456.56 a	3271.75 ± 245.56 b	15.83 ± 0.89 c	439.97 ± 23.45 bc	1219.43 ± 62.34 d
		As	5507.21 ± 412.42 a	4556.62 ± 342.45 a	19.46 ± 0.93 a	469.75 ± 30.45 bc	1354.59 ± 49.45 c
	10	Control	3772.06 ± 235.67 b	3041.54 ± 289.87 c	15.53 ± 0.92 b	481.45 ± 32.34 bc	1087.50 ± 83.12 e
		As	2883.34 ± 176.34 c	4257.52 ± 458.96 b	17.53 ± 1.09 ab	432.36 ± 23.56 c	1287.73 ± 69.32 c
	30	Control	2729.45 ± 278.45 d	3093.95 ± 334.56 c	13.26 ± 1.01 d	330.76 ± 22.34 d	943.27 ± 34.23 f
		As	1633.24 ± 99.83 e	4154.46 ± 354.98 a	14.38 ± 0.89 d	332.44 ± 15.69 d	1082.25± 50.54 e
Leaves	1	Control	3403.13 ± 179.33 a	6227.33 ± 434.93 a	64.22 ± 6.73 ab	633.62 ± 40.93 a	234.31 ± 12.45 b
		As	3112.77 ± 143.54 ab	5290.13 ± 302.34 b	49.23 ± 5.34 bc	544.83 ± 30.87 b	226.23 ± 10.15 b
	3	Control	3591.96 ± 123.43 a	5886.58 ± 478.23 b	49.31 ± 3.53 bc	646.95 ± 51.23 a	382.15 ± 21.54 a
		As	2942.52 ± 174.23 b	6909.65 ± 398.12 a	68.55 ± 4.52 a	532.08 ± 79.56 ab	180.67 ± 7.28 c
	10	Control	2358.36 ± 132.34 c	6378.05 ± 403.23 ab	47.97 ± 2.23 c	503.68 ± 23.13 b	250.83 ± 10.23 b
		As	2046.62 ± 124.54 d	4603.75 ± 345.21 c	57.26 ± 3.21 b	516.58 ± 31.22 b	143.65 ± 18.23 d
	30	Control	2035.46 ± 121.23 d	7001.72 ± 421.23 a	43.32 ± 2.34 d	429.35 ± 28.78 c	239.40 ± 12.12 b
		As	2106.37 ± 134.24 cd	5492.63 ± 324.12 b	60.67 ± 3.11 ab	400.92 ± 33.21 c	152.47 ± 13.52 d

Table 2
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Table 2. Arsenic accumulation (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to arsenic for 30 days. Different letters within each column and plant tissue indicate significant differences among time points on HSD test at $p < 0.05$. nd: not detected.

Organ	Time point (d)	Arsenic		
		III	V	Total
Roots	1	16.14 ± 2.34 d	164.88 ± 149.33 d	182.43 ± 20.10 d
	3	33.45 ± 4.78 c	318.86 ± 21.95 c	353.65 ± 23.98 c
	10	929 ± 80.21 b	542.35 ± 41.29 b	1471.92 ± 123.87 b
	30	1688 ± 148.43 a	734.90 ± 65.20 a	2448 ± 178.32 a
Leaves	1	nd	2.78 ± 0.24 d	2.78 ± 0.24 d
	3	1.30 ± 0.08 e	5.76 ± 0.45 c	7.23 ± 0.39 c
	10	nd	18.75 ± 1.14 b	18.75 ± 1.14 b
	30	nd	25.45 ± 2.57 a	25.45 ± 2.57 a

Table 3
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Table 3. Non-protein thiolic peptides (nmol GSH g^{-1} FW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters within each row and plant tissue indicate significant differences among treatments and time points on HSD test at $p < 0.05$. nd: not detected.

		1 d		3 d		10 d		30 d	
Organ	Thiol	Control	As	Control	As	Control	As	Control	As
Roots	Cys	nd	5.85 ± 1.73 a	nd	19.38 ± 0.18 b	nd	7.99 ± 2.15 a	Nd	4.06 ± 0.15 c
	GSH	13.73 ± 0.93 a	7.90 ± 0.66 b	11.68 ± 1.59 a	6.91 ± 0.55 bc	15.15 ± 1.83 a	6.15 ± 0.74 c	12.80 ± 1.40 a	6.05 ± 0.52 c
	TC_1	nd	14.69 ± 1.11 a	nd	10.62 ± 0.74 b	nd	6.72 ± 1.25 c	Nd	nd
	PC_2	nd	13.63 ± 1.85 b	nd	16.72 ± 1.78 ab	nd	19.10 ± 1.03 a	Nd	18.42 ± 1.75 a
	Cys-PC_2	nd	10.37 ± 0.76 d	nd	13.46 ± 1.51 c	nd	23.13 ± 0.02 b	Nd	34.35 ± 1.48 a
	TC_2	nd	6.78 ± 0.14 d	nd	10.17 ± 0.32 c	nd	17.12 ± 0.45 b	Nd	21.79 ± 0.29 a
	PC_3	nd	20.32 ± 1.40 d	nd	33.64 ± 1.09 c	nd	47.01 ± 9.54 b	Nd	65.38 ± 1.06 a
	desGly-PC_3	nd	8.88 ± 0.53 d	nd	34.35 ± 2.27 c	nd	73.86 ± 4.27 b	Nd	150.19 ± 12.24 a
	Cys-PC_3	nd	10.91 ± 0.71 d	nd	61.14 ± 1.74 c	nd	74.34 ± 3.73 b	Nd	169.27 ± 11.71 a
	Total ΣNPTs	13.73 ± 0.93 e	99.34 ± 2.71 d	11.68 ± 1.59 e	174.85 ± 18.70 c	15.15 ± 1.83 e	267.57 ± 12.93 b	12.80 ± 1.40 e	469.52 ± 21.32 a
Leaves	GSH	43.09 ± 2.53 b	49.35 ± 1.80 a	40.30 ± 3.45 bc	49.87 ± 3.33 a	44.70 ± 2.78 ab	37.74 ± 1.88 c	45.63 ± 2.45 ab	41.41 ± 1.86 b
	desGly-PC_2	nd	2.22 ± 0.31 d	nd	5.18 ± 1.03 c	nd	7.65 ± 0.53 b	Nd	9.39 ± 0.88 a
	desGly-PC_4	2.50 ± 0.11 d	2.52 ± 0.10 d	2.46 ± 0.14 d	3.06 ± 0.36 c	2.74 ±0.12 cd	2.87 ± 0.14 c	4.09 ± 0.52 b	6.46 ± 0.48 a
	TC_3	4.48 ± 0.38 b	5.45 ± 0.23 a	4.04 ± 0.49 b	6.25 ± 0.64 a	4.04 ± 0.34 b	5.08 ± 0.70 ab	3.03 ± 0.18 c	3.95 ± 0.41 b
	Total ΣNPTs	55.40 ± 1.89 bc	67.60 ± 1.45 a	50.54 ± 3.45 c	69.58 ± 5.70 a	53.56 ± 4.05 bc	58.16 ± 2.50 b	54.70 ± 4.11 bc	62.51 ± 5.94 ab

Supplementary Table 1

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Supplementary Table 1. Primer sequences used for the real time RT-PCR analyses.

Gene	Gene description	*S. purpurea* ortholog locus or NCBI annotation	Primer sequence F/R (5′-3′)	Product size (bp)	Efficiency	R^2
Reference genes (1: used to normalize gene expression data in roots, 2: used to normalize gene expression data in leaves)						
OTU[1,2]	OTU-like cysteine protease family protein	SapurV1A.0615s0200.1	GGCAGTGGTTCCTCTTCGAA ATCCCCATCTTTCGCAGTCG	114	91.2%	0.9951
ACT7[2]	Actin 7	SapurV1A.0231s0320.1	CTGTCCTTTCCCTGTATGCCA GTCACGACCAGCAAGATCCA	140	90.6%	0.9939
α-TUB2[2]	Alpha-tubulin 2	SapurV1A.0598s0030.1	CCAAGCGAGCATTTGTCCAC CCCTCGTCATCACCACCTTC	133	97.6%	0.9917
DNAJ	Chaperone protein DnaJ 49	SapurV1A.0212s0110.1	GCTCCCGGTTCTTCTTATTTTCC AAATTAACCCCTCTCTGCGTAGT	117	81.3 %	0.9871
EF1α[1]	Elongation factor 1-alpha	SapurV1A.0023s0300.1	ACCAGATTTCCGAGCCCAAG TTGGCCCAAAAGTGCAAACC	150	90.1%	0.9932
ARF2[1]	ADP-ribosylation factor 2	SapurV1A.0014s0160.1	TGGGGCTGTCTTTCACCAAG GGTCACAATCTCACCGAGCT	131	96.9%	0.9992
Arsenate transport						
HAPO4	High-affinity phosphate transporter 4	HQ228362.1	GAACGACGAGCACCTGGTT ACGGGTTCTATTCGCCTTGA	108	86.1%	0.9951
NA-DPHOT	Sodium-dependent phosphate transporter	SapurV1A.0139s0260.1	CAGCCACTTATCCCCAGCAA TCAAGGCGAATAGAACCCGT	134	94.5%	0.9873
PHO1	Phosphate transporter PHO1-like protein	SapurV1A.0063s0550.1	AGAGGCTGCGATGTTGAACA GTCTGAAGCAAGGCGAGTCA	115	81.3%	0.9938
Arsenite transport						
BORON	Boron transporter	SapurV1A.0014s0200.1	TCATTCGGGGAACAACTGGAG ACTGTCGGCTCTGCAACTC	143	93.3%	0.9805
NIP1	Aquaporin NIP1.1	SapurV1A.0029s0170.1	CAAGGTTGTGACTCTTCCAGGA GACAGCAGGGTTGAAATGGG	106	89.9%	0.9912
SIP1	Aquaporin SIP.1	SapurV1A.1058s0060.1	GCCAGTTCAGTACAAGCACATG TGCAGCAGAGGGTTTCGAG	147	103.9%	0.9911
SILICON	Silicon 1	SapurV1A.1225s0080.1	GGTAGCAGTCTCAGCAGGTG TGAAAGGTTCCCAGCAACTGT	94	85.2%	0.9977
Arsenate reductases						
CDC25-1	Tyrosine phosphatase	SapurV1A.0142s0310.1	ACGGCATCTTTAGGTCTGGTT TACGGCTCGGGACATAGACA	97	92.9%	0.9851
CDC25-2	Tyrosine phosphatase	SapurV1A.0243s0430.1	TCAACTTTCACCACAGAAGACCT CACTAGTTGACGAGCCAGGA	147	89.9%	0.9960
Thiol chelating response						
GR	Glutathione reductase	SapurV1A.0056s0770.1	ACGAAATGAGGGCTGTGGTT CCTCTCCATGATCTGTGCGA	126	93.9 %	0.9624
GS	Glutathione synthetase	SapurV1A.1124s0080.1	GCTGTCAAGTGCCCATCCAT CAGACTCCATAAGCCAGCGA	91	116.4%	0.9889
PCS	Phytochelatin synthase	SapurV1A.0160s0210.1	GTGGAAGGGTATTGCTGTAAGGA TGAGATGAAGGAACCAGCACA	137	98.53%	0.9922
GST	Glutathion S-transferase	SapurV1A.0016s1070.1	CGGTTCTTGGCTGGAGATGA CCTCCCCACATTTTCCCTGG	120	90.0%	0.9935
MT1A	Metallothionein	*S. matsudana* EF157299.1	CTTCGGTGCTGAGAATGGCT CTGCTTTGTTGGGACCATGC	97	90.5%	0.9998
Vacuolar transporters						
ABCG	ABC transporter G	SapurV1A.0258s0220.1	AGGCTTGGATTCTACAACTGCT TGGCTGGTGGATTGTTGTCA	94	84.3%	0.9778
CAX2-1	Vacuolar cation/proton exchanger 2	SapurV1A.1071s0020.1	TCTTGCAATCGTCGTCCACA ACCTAAACGCTCAGCCAAGG	94	92.9%	0.9954
CAX2-2	Vacuolar cation/proton exchanger 2	SapurV1A.0338s0120.1	TTGTTGGTGCTTGGATGTGC GCAGGACAGCAGGAAAGAG	142	103.4%	0.9826
WBABCT	White-brown-complex ABC transporter	SapurV1A.0084s0020.1	GCAAGAGGTGGTAGGACTGT ACACCCATCCGACAAAACCA	96	97.3%	0.9979

Table 1. Continued

Flavonoid Synthesis						
CHS1	Chalcone synthase	SapurV1A.0820s0070.1	CATTCCGTGGCCCTAGTGAC CGGAGCCTACAATGAGAGCA	90	96.9%	0.9974
CHS2	Chalcone synthase 2	SapurV1A.0056s0660.1	AACTGCGAGCCACTAGACAC AAAAGCACACCCCACTCCAA	145	91.5%	0.9992
CHS3	Chalcone synthase 3	SapurV1A.0820s0080.1	GCGGCCCAGACTATTCTACC AGCCTCGGTCAGACTCTTCT	135	87.7%	0.9999
F3H	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	TCTTGTCGGAGGCTATGGGA TCGGTATGGCGTTTGAGTCC	136	96.7%	0.999
FLH	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT	114	88.6%	0.9949
2HFLR	Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCATCGA	96	92.7%	0.9661
FLS	Flavonol synthase	SapurV1A.1087s0040.1	TCCCAACCCAGATTGTGTCG CAAATAGGCCCCACTGCGAA	94	90.5%	0.9976
ANR	Anthocyanidin reductase	SapurV1A.0028s0410.1	TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT	129	93.8%	0.9930
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG	127	95.8%	0.9823
Stress related						
ACCS	1-aminocyclopropane-1-carboxylate synthase	SapurV1A.2160s0020.1	GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA	115	102.3%	0.9982
AIP-1	Arsenite-inducible RNA-associated protein AIP-1-related	SapurV1A.0229s0030.1	CTTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG	140	93.2%	0.9940
ER	Ethylene receptor	SapurV1A.0052s0240.1	TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA	90	120.0%	0.9870
CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA TCCAGCAACAACTCCAACGA	125	90.5%	0.9919