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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 nonprotein 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. Response 4.1: A more detailed description of the data analysis is provided in the statistical analysis section, where non-normalized and normalized qPCR data were analysed following the standards in the field. Nonetheless, statements as the one referred have been removed from the text. Response 4.2: As was mentioned before, the focus on the description of

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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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1	Tolerance in <i>Salix atrocinera</i> Highlights		Formatted: Font: Bold, Spanish
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	Salix atrocinerea accumulates and tolerates high As concentrations in its tissues.		Formatted: Normal, Justified, Indent: Left: 0 cm, Space After: 0 pt, Line spacing: Double
	Inside the roots As V rapidly reduces to As III and accumulates.	Ň	Formatted: Spanish (Spain, International Sort)
	• 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.		Formatted: List Paragraph, Justified, Space After: 0 pt, Line spacing: Double, Bulleted + Level: 1 + Aligned at: 0.63 cm + Indent at: 1.27 cm
	 An early regulation of the flavonoid pathway alleviates As toxicity in leaves. 		
	• <i>De novo</i> synthesis and accumulation of thiols occurs in As-exposed plants.		
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Tolerance in Salix atrocinera 64 65 66 67 68 69 70 71 72 73 74 75 76 Introduction Formatted: Indent: Left: 0 cm, First 1 line: 0 cm, Space After: 0 pt 77 Arsenic (As) is a metalloid widely spread in the upper Earth's crust although at very low Formatted: Font color: Text 1 78 concentrations. The overall mean value of the total arsenieAs for different soils is estimated as 6.83 Formatted: Font color: Text 1 79 mg Kgkg⁻¹ soil. Nonetheless, arsenieHowever, As soil concentrations may range from 0.1 to more Formatted: Font color: Text 1 80 than 1,000 mg Kgkg¹ in some locations due to both anthropological and geological factors (Kabata-Formatted: Font color: Text 1 81 Pendias, 2010). ArsenicConcerning its toxicity, As is the only known human carcinogen for which Formatted: Font color: Text 1 82 there is adequate evidence of carcinogenic risk for both exposure routes, inhalation and ingestion Formatted: Font color: Text 1 83 (Smith et al., 2009). Therefore, As has been defined as a group 1 carcinogen and is placed in the Formatted: Font color: Text 1 84 highest health hazard category by the international agency for research on cancer (Naidu et al., 2006). 85 By the use of natural resources, humans release arsenicAs into the air, water and soil (Mandal and Formatted: Font color: Text 1 86 Suzuki, 2002). Sixty percent of the anthropogenic As emissions can be accounted to only two

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

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

90 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.

93 Once inside the cell, arsenicAs toxicity depends on its speciation state. Arsenite (As III) has a 94 high affinity for sulfhydryl groups found in the amino acid cysteine. As such, it inactivates a wide 95 range of enzymes by disrupting protein structure and impairs the metabolism by preventing protein-96 protein interactions (Ehlrich, 1990). This affects many key metabolic processes in the cell such as 97 fatty acid metabolism, glucose uptake and glutathione production (Paul et al., 2007; Ahsan et al., 98 2008; Wang et al., 2015). Arsenate (As V) is a phosphate analogue and can substitute inorganic 99 phosphate affecting ATP synthesis and therefore interrupting the production of energy, carbon 100 metabolism and nucleic acid synthesis (Singh et al., 2011; Spratlen et al., 2017). This can also 101 negatively affect DNA repair and methylation and thus impact on gene expression (Reichard and 102 Puga, 2010). Therefore, removal or reductionlowering of arsenicAs concentrations from highly As-103 polluted soil and water is an environmental priority. Among the most eco-friendly cleanup 104 technologies and opposite to traditional excavation and disposal in landfills, emerges the 105 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. 106 107 (1994) exploit, exploits the ability of certain plants species to accumulate metal(loid)s in their 108 tissues, thus reducing their concentrations or attenuating their mobility in the environment

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Tolerance in Salix atrocinera 109 (PilonSmits, 2005)., and therefore offering a solution to the above-mentioned pollution challenge 110 (Pilon-Smits, 2005; Kidd et al., 2015). 111 It is well known that toxic metal(loid)s induce loss of plant biomass, among other deleterious 112 effects, mainly associated with growth inhibition (Gill et al., 2015). Plants differ in As tolerance, 113 from sensitive plant species like all major crops, to tolerant plants such as certain ecotypes of the 114 grass 115 Holcus lanatus (Quaghebeur and Rengel, 2003), as well as hyperaccumulators like Pteris-116 vittata (Chinese break fern), which can accumulate 2% of its dry weight as arsenicAs (Wang et al., 117 2002). However, hyperaccumulator species are usually limited by a low biomass production, which 118 may pose serious restrictions to this cleaning procedure (Shelmerdine et al., 2009, Fernández et al., 119 2010). Some plant species and soil biota populations, usually autochthonous to polluted soils, are 120 able to colonize and thrive in highly polluted environments, even when high concentrations of metals 121 are found in their cells and tissues. This is the case of Salix atrocinerea (grey willow). AboutSo far, 122 about 450 species of Salix worldwide have been described (Argus, 1995), with some of them reported 123 as efficientsuitable in phytoremediation processes because of their high growth rate and deep-rooting 124 traits (Kuzovkina and Quigley, 2005; Janssen et al., 2015). Whereas Nevertheless, the focus on the 125 use of *Salix* for arsenicAs uptake is still low because it is not a metal(loid) hyperaccumulating 126 species. However, some investigations have highlighted its phytoremediation potential for As (Purdy 127 and Smart, 2008; Puckett et al., 2012; Yanitch et al., 2017). In addition, complementary studies 128 exploring the feasibility of high biomass plants to extract metals from polluted soils such as willow,

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

129 concluded that the high biomass compensates for the moderate metal concentrations found in the

130 aboveground tissues (Hammer et al., 2003; Ruttens et al., 2011).

131 Understanding <u>arsenie As</u> tolerance in plants is <u>potentially</u> useful <u>knowledge</u> to <u>know</u> whether
 132 plants avoid As uptake and, thus, reduce <u>human arsenic the As</u> intake <u>by humans</u> and the As 133 associated health problems (Song et al., 2010), or enhance As uptake and its removal by

134 phytoremediation (Yang et al., 2012). To achieve this, it is necessary to study the As behavior from

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135	the soil to its accumulation in the aboveground plant tissues. Although arsenicAs is toxic and not		Formatted: Font color: Text 1
136	essential for plants it is effectively absorbed through various transporters into the roots, mainly as As		
137	V, the most thermodynamically stable and hence dominant species in aerobic environments		Formatted: Font: Bold, Font color:
138	(Quaghebeur and Rengel, 2003). Specific As V-such, specific As transporters have been identified		
139	that include the high affinity phosphate uptake systems for As V (Shin et al., 2004; Catarecha et al.,	\bigtriangledown	Formatted: Font color: Text 1
140	2007;	\wedge	Formatted: Font color: Text 1
1 / 1	LaDlana et al. 2012) arbits de Ulares the silien termeneters are reconstituted as de Ularbury	M/	Formatted: Font color: Text 1
141	Leblanc et al., 2015), while <u>As III uses the shicon transporters are responsible for As III influx</u> .		Formatted: Font color: Text 1
142	(Xu et al., 2015; Lindsay and Maathuis, 2016). Once inside the plant cells, a small amount may be		Formatted: Font: Not Italic, Font color: Text 1
			Formatted: Font color: Text 1
143	transported to the xylem but the majority is reduced to As III by arsenate reductases (Ellis et al.,	// //	Formatted: Font color: Text 1
1 4 4		$\left(\left(\right) \right) $	Formatted: Font color: Text 1
144	2006; Duan et al., 2007; Zhao et al., 2009). In this form, As can be exported back into soil,		Formatted: Justified, Indent: Left: 0
145	transported via the xylem to stem and leaves, or complexed with thiol-rich molecules like		cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text,
146	metallothioneins (MTs), glutathione (GSH) or, more stably, by phytochelatins (PCs) (Schmöger et		and numbers
147	al 2000: Hartley-Whitaker et al 2001: Dave et al 2013: Batista et al 2014) Then thisthese As-	1	Formatted: Font color: Text 1
14/	al., 2000, Hartiey- wintaker et al., 2001, Dave et al., 2015, Datista et al., 2014). Then this unser As-		Formatted: Font color: Text 1
148	PCs complexes can subsequently be transferred from the cytosol into the vacuole by ABC		Formatted: Font color: Text 1
149	transporters for storage in order to prevent cell damage (Song et al., 2010). This Therefore, this		Formatted: Font color: Text 1
150	suggests that PCsnon-protein thiols (NPTs) compounds play an important role in decreasing As		Formatted: Font color: Text 1
151	toxicity in plants- <u>and preventing its transport from roots to shoots</u> .		Formatted: Font color: Text 1
			Formatted: Font color: Text 1
152	Apart from the works on arsenic with Salix of Purdy and Smart (2008), Puckett et al. (2012),	\leq	Formatted: Font color: Text 1
153	and more recently the extensive transcriptomic study by Yanitch et al. (2017), only limited, but not		
154	integrated (2017) that have provided unequivocal useful information to understand the tolerance of		Formatted: Font color: Text 1
155	Salix to As, still an integrative approach concerning the tolerance mechanisms of Salix to As is		Formatted: Font color: Text 1
156	available and this information has hardly any emphasis onneeded. Besides, special attention needs to		
157	be paid to the speciation state of As-, since this determines its uptake and also its tolerance by the		Formatted: Font color: Text 1
158	plant. In the current study, a S. atrocinerea clone, previously selected for its As accumulation,		Formatted: Font color: Text 1
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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and		
	Tolerance in Salix atrocinera		
159	(unpublished data), was grown hydroponically in the presence of As V. Samples were harvested at		Formatted: Font color: Text 1
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160	different time points to kinetically study arsenicAs accumulation, and its chemical speciation in the		Formatted: Font color: Text 1
1.61		\geq	Formatted: Font color: Text 1
161	different plant tissues, roots and shoots. In addition, the production of NPTs as well as the synthesis of	$ \langle \rangle $	Formatted: Font color: Text 1
162	phytocholating as a machanism implied in As deterrification. In addition, some expression of the main		Formatted: Font color: Text 1
102	phytochelatins as a mechanism implied in As detoxineation. In addition, gene expression of <u>the main</u>		Formatted: Font color: Text 1
163	transcripts related to the entry of arsenic into the roots and its storage into vacuoles, thiol metabolism	(Formatted: Font color: Text 1
164	and As stress related responses was analyzed.involved in the genetic response behind As tolerance		
165	were also measured. Therefore, this study aims to describe the As uptake and transport pathways as		Formatted: Font color: Text 1
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166	well as possible accumulation in S. atrocinerea, together with the changes in the mechanisms	(Formatted: Font color: Text 1
167	involved in arsenicAs tolerance in grey willow at different biological organization levels.		Formatted: Font color: Text 1
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168			Formatted: Font color: Text 1
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169	2 Material and Methods		Formatted: Indent: Left: 0 cm, First line: 0 cm, Space After: 0 pt
170			
170 171	2.1 Plant material and hydrononic culture conditions		
170 171	<u>2.1</u> Plant material and hydroponic culture conditions		
170 171 172	 <u>2.1</u> Plant material and hydroponic culture conditions <u>2.1</u> Solir structure plants were calculated from an in withe willow along providently obtained from 	(Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm
 170 171 172 173 174 	 <u>2.1</u> Plant material and hydroponic culture conditions <u>2.1</u> Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias Spain). Non lignified stemStem cuttings of 15 cm 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1
 170 171 172 173 174 175 	 <u>2.1</u> Plant material and hydroponic culture conditions <u>2.1</u> Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Non lignified stemStem cuttings of 15 cm length were placed on cellulose plugs in a hydroponic system containing 50 mL of 1/10 Woody Plant 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1
170 171 172 173 174 175 176	 2.1 Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Non lignified stemStem cuttings of 15 cm length were placed on cellulose plugs in a hydroponic system containing 50 mL of 1/10 Woody Plant Medium 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1
 170 171 172 173 174 175 176 177 	 <u>2.1</u> Plant material and hydroponic culture conditions <u>2.1</u> Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Non lignified stemStem 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- 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, line ansier: Derythe Deryth editort
 170 171 172 173 174 175 176 177 178 	 2.1 Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Non lignified stemStem 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 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text
 170 171 172 173 174 175 176 177 178 179 	 2.1 Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Non lignified stemStem 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. 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers
170 171 172 173 174 175 176 177 178 179 180	 2.1 Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain), Non lignified stemStem 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, Na₂HAsO₄.7H₂O_{, since in this form the As is} 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers Formatted: Font color: Black
 170 171 172 173 174 175 176 177 178 179 180 181 	 2.1 Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i>, willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain), Non lignified stemStem 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 etal., 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, Na₂HAsO₄.7H₂O since in this form the As is freely soluble. 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers Formatted: Font color: Black
170 171 172 173 174 175 176 177 178 179 180 181 182	 2.1Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Non lignified stemStem 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 eteal., 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, Na₂HAsO₄.7H₂O₋, 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 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers Formatted: Font color: Black
 170 171 172 173 174 175 176 177 178 179 180 181 182 183 	 2.1_Plant material and hydroponic culture conditions 2.1 Salix atrocinerea plants were selected from an <i>in vitro</i> willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain), Non lignified stemStem 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, Na₂HAsO₄.7H₂O, since in this form the As is freely soluble. Plants were cultured under a 12h 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 		Formatted: Normal, Indent: Left: 0 cm, First line: 0 cm Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Font color: Text 1 Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers Formatted: Font color: Black

184 modules, simulating the photosynthetically active radiation (PAR) of sunlight. The PAR level

185 reached $\underline{170 \ \mu mol \ m^{-2} \ s^{-1}}$ at the plant apex level.

186 $\frac{170 \ \mu \text{mol m}^{=2} \text{s}^{=1}}{\text{at the plant apex level.}}$

After 1, 3, 10 and 30 days (d), plants were carefully removed from beakers and rootsexhaustively 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.

193

194 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 sampledry 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 Formatted: Justified, Indent: Left: 0 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers

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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and		
205	Tolerance in Salix atrocinera filtered through a 0.45 μ m PTFE membrane filter. The solutions were neutralized by the addition of		
206	NaOH. The arsenicAs species were separated through a mobile phase of 0.2 M EDTA dissolved in 2	_	Formatted: Font color: Text 1
207	M PBS (Phosphate Buffered Saline; pH 6.0) in a separation column with a 1260 Infinity HPLC		
208	coupled to a 7700 ICPMS (both from Agilent Technologies). Identification of arsenicAs species was		Formatted: Font color: Text 1
209	confirmed by spiking real extracts with a mixture of standard solutions: As III, As V,		
210	monomethylarsenic acid (MMA), and dimethylarsenic acid (DMA).	_	Formatted: Font color: Text 1
211			
212	2.3 Analysis of non-protein thiolic compounds		Formatted: Indent: Left: 0 cm, First line: 0 cm, Line spacing: Double
213	The extraction and analysis of non-protein thiols (NPTs) were carried out infrom 150 mg of	_	Formatted: Font color: Text 1
214	fresh weight leaves and roots of S. atrocinerea following the protocol described by Fernández et al.		Formatted: Font color: Text 1
215	(2012). The high-performance liquid chromatography (HPLC) separation was performed using a		Formatted: Font color: Text 1
216	chromatograph Waters 600 (Waters		Formatted: Font color: Text 1
217	Corporation) with a post-column derivatization with Ellman's reagent (Ellman, 1959). The sample		Formatted: Font color: Text 1
218	(100-µL) was injected into a Kromasil 100 C18 5-µm (250- \times 4.6-mm) column (Scharlau)*		Formatted: Normal (Web), Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double
219	and eluted with solvent A (acetonitrile: H ₂ O, 2: 98 (v/v) to which 0.05% trifluoroacetic acid (TFA)	\mathbb{N}^{\sim}	Formatted: Font color: Text 1
			Formatted: Font color: Text 1
220	was added) and solvent B (acetonitrile: H_2O , 98: 2 (v/v) also with 0.05% TFA). Samples were		Formatted: Font color: Text 1
221	separated using a linear gradient (0-25% in 25- min and 25-50% in 5- min) of solvent B at 1.5- mI		Formatted: Font color: Text 1
221	separated using a linear gradient (0–25% in 25–1111 and 25–56% in 5–1111) of solvent B at 1.5–1112	\leq	Formatted: Font color: Text 1
222	min ⁻¹ -flow for 30-min. The derivatized thiols were detected at 412-nm using a Waters 996	\backslash	Formatted: Font color: Text 1
			Formatted: Font color: Text 1
223	photodiode-array detector and the obtained peaks were identified by comparison with the standards	\mathbb{N}	Formatted: Font color: Text 1
224		///	Formatted: Font color: Text 1
224	of GSH and a mix of PCs. The quantitative changes in the thiol compounds observed were calculated	1/ //	Formatted: Font color: Text 1
225	by the integration of their neak areas at 412, nm of absorbance converted into nmol and quantified as		Formatted: Font color: Text 1
225	by the integration of their peak areas at +12- intr or absorbance converted into intor and qualititied as		Formatted: Font color: Text 1
226	GSH equivalents.	U	Formatted: Font color: Text 1
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228 2.4 Gene expression analysis

229	Isolation of RNA was carried out using the protocol described by Chang et al. (1993) with	-
230	slight modifications. Frozen leaves or roots (100-mg) were homogenized with 550-µL of buffer	X
231	containing 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP),	
232	100- mM TrisHCITris-HCI (pH 8.0), 25- mM EDTA, 2- M NaCl, 0.5- g- L ⁻¹ - spermidine and 2% β-	
233	mercaptoethanol. Then, it was extracted twice by adding 550-µL of chloroform:isoamyl alcohol	
234	(24:1) and centrifuged at 14,000 g-for 20-min at 4-°C. After addition of 10-µL LiCl (10-M), RNA	
235	was precipitated overnight at 4- $^{\circ}C$ and harvested by centrifugation at 14,000 g-for 20-min at 4- $^{\circ}C$.	
236	The pellet obtained was washed with 75% ethanol and resuspended in RNase free water. The	
237	concentration of RNA was determined spectrophotometrically at 260-nm using Nanodrop equipment	STREET, STREET
238	(Isogen Life Science) and the RNA quality was tested using the Experion TM automated	
239	electrophoresis system (Bio-Rad). DNA was removed using a TURBO DNA-free Kit (Ambion) and	
240	the cDNA synthesis was performed using PrimerScript RT reagent Kit (Takara) with equal amounts	\backslash
241	of RNA input (1 µg). Finally, the cDNA was ten-fold diluted using a 1/10 dilution of TE (Tris-	
242	EDTA) buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C,	
243 244 245	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	

246 8.0) and stored at -20 °C.

247 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.,

250 2012), according to sequences of genes obtained in the *Phytozome* nucleotide database of the closely

al., 2012). Genes measured were selected based on a transcriptomic study of Yanitch et al. (2017)

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related species-*Salix purpurea* v1.0, for which the whole genome has been sequenced (Goodstein et

	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and	
	Tolerance in Salix atrocinera	
253	addressing different interesting aspects of arsenic metabolism. Only), only for three genes,	
254	highaffinityhigh-affinity, phosphate transporter (HAPO4), arsenite-inducible RNA-associated protein	
255	(AIP-1) and metallothioneins (MT1A), their sequences were obtained from willow reference	
256	sequences annotated at the NCBI (National Center for Biotechnology Information) (O'Leary et al.,	
257	2016). Their specificity was verified in silico using BLAST	
258	(http://www.arabidopsis.org/Blast/index.jsp). The genes measured (Supplementary Table 1) were	
259	selected based on different genetic aspects behind As tolerance (Konlechner et al., 2013; Puckett et	
260	al., 2012; Yanitch et al., 2017). The gPCR efficiency of the primers was determined using a standard	
261	curve consisting of a two-fold dilution series of a pooled sample. Only primers with an efficiency	
262	between 90 and 110% were used for qPCR analysis and their amplification specificity of the primers	
263	was validated by melting curves. PCR amplifications were done in a total volume of 10-µL	
264	containing 2- µL cDNA sample, 5- µL SYBR Green,	\sum
265	0.6- uL of primers (10 uM 300 nM) and 2.4- uL RNase free water. The reaction cycle was as follows:	\mathbb{N}
266	20- s at 95- °C, 40 cycles of 1- s at 95- °C and 20- s at 60- °C. Gene expression was calculated	
267	relatively as $2^{-\Delta Cq}$ in which ΔCq represents each corresponding quantification cycle (Cq) value	\mathbb{N}
268	minus the minimum Cq value observed (Schmittgen, 2008). Gene expression was normalized with a	
269	factor suggested by the Graynorm algorithm (Remans et al., 2014) based on the expression of six	
270	reference genes from Salix selected from literature (Li et al., 2016; Zhang et al., 2017).	
271	In roots AFR2 OUT	
271		
272	Salix selected from literature under As and other abiotic stresses (Li et al., 2016; Zhang et al., 2017).	\
273	The 6 selected candidate reference genes, a-TUB2, Alpha-tubulin 2; ACT7, Actin 7; ARF2, ADP-	
		/
274	ribosylation factor 2; DNAJ, Chaperone protein DnaJ 49; EF1a, Elongation factor 1-alpha and	
275	OTU, OTU like systems protected (Supplementary Table 1) are also orthology of gapes in S	
213	010; 010-like cystelle protease (Supplementary Table 1) are also orthologs of genes in S.	
276	<i>purpurea</i> . The primer sequences, amplicon length, PCR amplification efficiency and correlation	
	<u></u>	
277	coefficient are shown in Supplementary Table 1. To evaluate the stability of the 6 candidate	
278	reference genes (RG) at the transcript level under As exposure, the gene expression levels were	
279	determined by the average Cq values (Supplementary Fig. 1). In order to detect the stabilities of 6	
200	condidete DCs, the best combination of DC for normalization of our transmints of interest was	
200	candidate KOS, the best combination of KO for normalization of our transcripts of interest was	
281	suggested by the Gravnorm algorithm (Remans et al. 2014) In roots AFR2 OTU and EF1a were the	
201		
282	three most stable reference genes in all the sample sets according to the <i>GrayNorm</i> algorithm and the	/
283	combination of the three was used for normalization- (Supplementary Fig. 1A). In leaves a different	
284	combination of genes than that obtained in roots showed the most stable pattern in all the sample sets,	_

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285 and therefore a combination of α -TUB2, OUT and ACT7 was used for OTU and ACT7 was used for

286 normalization (Supplementary Fig. 1B). In both roots a leaves the suggestion selected by GrayNorm

287 corresponded to the genes less affected by As exposure.

288 normalization.

289 HeatA principal component analysis and heat maps were constructed to compare expression-

- 290 levels between different genes and samples at different time points.
- 291

311

292 2.5 Statistical analysis

293 To evaluate the effects of arsenicAs toxicity in S. atrocinerea over the different time points on 294 the measured variables, depending on the number of variables to compare a one-way or a two-way 295 Analysis of variance (ANOVA) was performed. Log transformation was applied to approximate 296 normality when it was necessary (e.g. to determine statistical significance of gene expression data, 297 datasets were first log-transformed). Data normality was tested using the Shapiro-Wilk test, while 298 homoscedasticity was verified via Bartlett' s and Levene's tests. If data did not meet the normality 299 assumption, a non-parametric Kruskall-Wallis test was used, followed by the Wilcoxon rank sum test. When the F ratio was significant ($p \le 0.05$), Tukey's least significant difference test (HSD, $p \le 0.05$) 300 301 was employed to compare between individual means. A principal component of different data groups 302 (e.g. different treatments). In the gene analysis (PCA)the previous was performed using the log-303 transformed data of gene expression on both the normalized and the non-normalized data, although 304 only the first are presented both were taking into account to establish the significance of the different 305 treatments and exposure periods results. Results are expressed as the mean \pm standard deviation of at least three independent replicates. DataAll data were analyzed using R (version 306

307 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1,

308 http://www.mixOmics.org) and agricolae (version 1.2e4, http://tarwi.lamolina.edu.pe/--fmendiburu).

- 309 http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized
- 310 Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level $p \le 0.05$,
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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and	
I	Tolerance in Salix atrocinera	
312	3 Results	Formatted: Indent: Left: 0 cm, First line: 0 cm, Space After: 0 pt, Line spacing: Double
313	3.1 Plant growth and nutrient analysis	Formatted: Font color: Text 1
314	After 30 d of exposure, no external symptoms of phytotoxicity (data not shown) nor growth	Formatted: Normal (Web), Justified, Indent: Left: 0 cm, Line spacing: Double
515	reduction, measured as dry weight, were observed between plants grown on control of As-containing	
316	medium (Fig. 1).	Formatted: Font: Bold
317	With regard to nutrient concentrations, total P concentration significantly decreased in roots	
318	from 10 d onwards in As-exposed plants as compared to controls, whereas in leaves the P	
319	concentration was lower in As-exposed plants as compared to controls at 3 and 10 d. However, P	
320	concentration in leaves was similar in both treatments after 30 d of exposure (Table 21).	Formatted: Font: Bold
321	Accumulation of Ca increased in AsexposedAs-exposed roots along the exposure time when	
322	compared to control conditions. However, in leaves a Ca decrease was observed in As-exposed	
323	plants, except at 3 d (Table 21). Although the B concentration was slightly higher in roots of As-	Formatted: Font: Bold
324	exposed plants, this increase was only significant at 3 d. In leaves-there was, an increase in B	
325	concentration at 3 d <u>was observed</u> and this increase was maintained in As-exposed plants till the end	
326	of the experiment. For Zn concentrations, an opposite accumulation trend was observed between	
327	roots and leaves of As exposed plants at 1 d withthere was an increase in roots and a decrease in	
328	leaves as compared to control conditions- at 1 d of As exposure. However, no differences were	
329	observed in Zn concentrations for this elements at other time periods. Fe concentrations were higher	
330	in roots of As-exposed plants throughout the experiment, whereas the opposite trend was observed in	
331	leaves (Table 2).<u>1).</u>	Formatted: Font: Bold

332	With regard to the pH of the culture medium, we generally observed a decrease during the first
333	3 days of exposure and the experiment. However, an increase from 10 d onwards was observed under
555	5 days of exposure and <u>the experiment. However</u> all increase noin 10 a <u>onwards was observed</u> ander
334	As exposure as compared to control medium (Fig. 2). 2A).

335

336 **3.2** Arsenic accumulation and speciation

We observed that roots of S. atrocinerea accumulated As concentrations ranging from 180 mg-337 As Kgkg⁻¹ dry weight at 1 d to more than 2,400 mg As Kgkg⁻¹ dry weight after 30 d of exposure 338 339 (Table <u>32</u>). In leaves, arsenicAs accumulation was much lower, although after 30 d of exposure, it 340 reached an As concentration higher than that present in the culture medium (Table 32). Although 341 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. 22B). Total arsenicAs 342 343 concentration in the medium decreased 14 % due to plant uptake and no spontaneous arsenie As speciation was detected in the medium when *S. atrocinerea* was not present (data not shown). 344

In plant tissues, the arsenieAs was detected as As III or As V, but no arsenieAs methylated species were observed (**Table 32**). In roots, As V was more abundant (91%) during the first 3 d of exposure but after 10 d, As III was the predominant arsenieAs form (**Fig. 22C**). 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).

350

351 3.3 Analysis of non-protein thiolic compounds

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352	In roots of control plants, only GSH was observed and present at a 2-fold higher concentration
353	than that observed in the As-exposed roots (Table 4). Nevertheless, already after 1 d of exposure <u>3</u>).
354	However, in roots and leaves of S. atrocinerea plants exposed to As, changes in the concentrations of
355	non-protein thiols (NPTs) in roots and leaves were <u>already observed</u> in Asexposed plants after 1 d
356	and this trend was maintained over time (Table 4). Interestingly3). Besides, in the roots of
357	AsexposedAs-exposed plants the total concentration of NPTs increased over time (up to 4.5-fold
358	higher after 30 d compared to 1 d) and it was always higher than that observed in leaves. This NPTs
359	increase in roots under As exposure was mainly due to an increment of <i>de novo</i> synthesized
360	compounds such as PC ₂ , Cys-PC ₂ , PC ₃ , desGly-PC ₃ , Cys-PC ₃ and also two unidentified thiolic
361	compounds that were named TC_1 and TC_2 (Table 4).<u>3</u> .

362 In leaves of control plants, the thiolic compounds GSH, desGly-PC₄, and TC₃ were detected. 363 (Table 43), whereas under arsenie As exposure we observed *de novo* synthesis of desGly-PC₂ at 364 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 365 366 than in control plants (**Table 43**). This increase in GSH, together with *de novo* synthesis of desGly-367 PC₂, 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 368 369 As did not significantly differ from that observed in leaves of plants grown under control conditions

370 (**Table** 4). <u>3).</u>

371

372 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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375	reduction to As III, thiol metabolism and vacuolar transports. In this way changes were observed in		
376	transcripts coding for the phosphate transporter (PHO1, the), aquaporins (NIP1, SIP1 and SILICON,		Formatted: Font color: Text 1
377	the), boron transporter (BORON, the), As V reductase CDC25-like tyrosine phosphatase (arsenate		Formatted: Font color: Text 1
378	reductase; CDC25-1), the proteins related to thiol metabolism such as glutathione synthase (GS) and		Formatted: Font color: Text 1
379	metallothioneins (MT1A), the vacuolar transporters like CAX2 2, white brown complex ABC		Formatted: Font color: Text 1
380	transporter (WBABCT and ABCG and the) (Fig. 3). This differential regulation was also	())	Formatted: Font color: Text 1
381	accompanied by changes in transcripts for As stress-related proteins like cellulose synthase (CSA),	$\langle \langle \langle \rangle \rangle$	Formatted: Font color: Text 1
382	arsenite inducible protein (AIP-1) and aminocyclopropane-1-carboxylate synthase (ACCS).		Formatted: Font color: Text 1
383) () In leaves, regulation of the transcripts measured was not so noticeable as in roots and		Formatted: Font color: Text 1
505	(). In reaves, regulation of the transcripts measured was not so noticeable as in roots and		Formatted: Font color: Text 1
384	differences in gene expression between leaves from control plants and leaves from Asexposed As-	$\langle \rangle \rangle$	Formatted: Font color: Text 1
		$\langle \rangle$	Formatted: Font color: Text 1
385	exposed plant were due to the expression of NIP1, CDC25-1 and CDC25-2 transcripts and genes of	$\langle \rangle$	Formatted: Font color: Text 1
• • •			Formatted: Font color: Text 1
386	the flavonoid pathway coding for chalcone synthases (CHS1, CSH2 and CSH3), flavanone	$\langle \rangle$	Formatted: Font color: Text 1
207			Formatted: Font color: Text 1
381	singuroxylase (F3H), Havonola 5 hydroxylase (FLH), dingdronavonol 4 reductase (2HFLK) and		Formatted: Font color: Text 1
388	anthocyanidin reductase (ANR); and also the alterations in mainly to the overexpression of ACCS		Formatted: Font color: Text 1
389 390	(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		
391	leaves (Supplementary Fig. 3B).		
392	To establish the kinetic gene expression observed for MT1A,		Formatted: Font color: Black
393	BORON and PHOL.		
394			
395	3.4.1 Principal Component Analysis		
396	A of related target genes, a principal components component analysis (PCA) was performed		Formatted: Font color: Text 1
397	using the normalized gene expression levels data, allowing us to identify the correlations among the		Formatted: Font color: Text 1
398	measured genes simultaneously.data obtained in leaves and roots of <i>S. atrocinerea</i> plants collected at		Formatted: Font color: Text 1
399	<u>1, 3, 10 and 30 d.</u> According to <u>PCA</u> component 1, roots of plants exposed to arsenicAs for 1 d		Formatted: Font color: Text 1

400 showed the highest gene expression for GS, Formatted: Font color: Text 1

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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and			
	Tolerance in Salix atrocinera			
401	NIP1, SIP1, CSA, AIP-1, vacuolar transporter (CAX2-2,), glutathione reductase (GR) and	_	F	ormatted: Font color: Text 1
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402	ACCS, whereas the expression of BORON, PHO1, MT1A, CDC25-2, WBABCT, vacuolar transporter	$\langle \rangle$	F	ormatted: Font color: Text 1
403 404	(ABCG) was higher at later time points. On the other hand, <u>PCA</u> component 2 in roots (24% of the total variation) indicated that the increased expression of ABCG clustered samples at 3 d, whereas		F CI D Li	ormatted: Justified, Indent: Left: 0 n, First line: 1 cm, Line spacing: ouble, Don't adjust space between atin and Asian text, Don't adjust space
101	total valuation, maleated that the meleased expression of the ed, endered samples at 5 d, whereas	11 1	F	ormatted: Font: 8 nt. Not Italic Font
405	the decrease in expression for phytochelatin synthase (PCS) and transcripts for a high-affinity		C	olor: Black, Not Superscript/ Subscript
106	phosphate transporter (HAPOA) at 3 d separated this group from the rest (Fig. 3) (A)	- //	Ľ	
400	phosphate transporter (1171 04) at 5 d separated this group from the rest (Fig. 5). 4A).		Ľ	ormatted: Font color: Text 1
		\mathbb{N}	F	ormatted: Font color: Text 1
407	In leaves, according to <u>PCA</u> component 1, the differential gene expression collected from plants		F	ormatted: Font color: Text 1
408	growing under arsenie <u>As</u> exposure at 1 and 3 d, had a more similar pattern than that observed at 10	<u>, </u>	F	ormatted: Font color: Text 1
409	and		F	ormatted: Font: Bold, Font color: ext 1
410	30 d. Main differences were attributed to the up-regulation of genes involved in the flavonoid		F	ormatted: Font color: Text 1
		())	F	ormatted: Font color: Text 1
411	pathway, CHS3, CHS2, ANR, F3H, FLH, 2HFLR, BORON and CHS1 expression at 1 and 3 d and of	$\langle \rangle \rangle$	F	ormatted: Font color: Text 1
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412	CDC25-2 at 10 and 30 d. Component 2 (20% of the total variation), however, indicated a separation	$\left(\right)$	F	ormatted: Font color: Text 1
413	between the initial (1 d) and the last time point (30 d) from the intermediate points (3 and 10 d) as a		F CI Li	ormatted: Justified, Indent: Left: 0 m, First line: 1 cm, Space After: 0 pt, ne spacing: Double, Don't adjust
414	consequence of lower ACCS and higher ABCG2 expression in these intermediate points (Fig. 3).		sj D ai	bace between Latin and Asian text, on't adjust space between Asian text ad numbers
415	<u>4B),</u>	\sim	F	ormatted: Font color: Text 1
416		\nearrow	F	ormatted: Font: Bold, Font color: ext 1
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417	3.4.2 Differential gene expression in roots			
418	Concerning As transporters, transcripts encoding for PHO1 were 2 fold up regulated at 1 d and			
419	kept steady throughout the experiment in roots of As exposed willows (Fig. 4). Likewise, transcripts			
420	encoding for HAPO4 were 1.5 fold up regulated after 10 and 30 d of arsenic exposure (Fig. 4) in			
421	comparison to control plants. Unanges involving transcripts coding for arsenite transport related			
422	proteins were also detected. As such, <i>WPT</i> transcripts were 12, 5.5, 5 and 4 total up regulated in the			
423	presence of As at 1, 5, 10 and 50 d, respectively (Table 5). For transcripts encoding 10f 51P1 a 5, 2 and 1.8 fold up regulation was observed at 1, 2 and 10 d, respectively (Table 5). On the sector			
424	there uses a 2 fold down regulation at 2 d in SILICON transportate (Fig. 4). Interpretingly,			
423 426	transcripts were			
-T20	tunsenpts were			
427 428	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).			

429	Regarding genes involved in arsenic reduction, two transcripts of CDC25-like tyrosine
430	phosphatase, an arsenate reductase, <i>i.e. CD25 1</i> and <i>CD25 2</i> , were measured in roots of As exposed
431	plants. The transcript levels of CD25 1 showed an up regulation of 2 fold at 1 and 3 d, 2.5 fold at 10
432	d and 3 fold at 30 d after As exposure (Table 5), whereas no differential expression was observed in
433	the transcript levels for CD25-2 (Fig. 4).
434	Concerning genes related to the thiol metabolism, expression levels of GS transcripts were 17,
435	5, 5 and 3 fold up regulated after 1, 3, 10 and 30 d As exposure, respectively (Fig. 4). Transcripts for
436	GR, an enzyme involved in the turnover of GSH, were only found to be 2-fold overexpressed at 1 d
437	after arsenic exposure, whereas no changes were detected in glutathione transferase (GST) transcript
438	levels. In As exposed roots, there was a 1.5 fold up regulation in PCS transcripts at 1 d after
439	exposure and MT1A was 2 fold down regulated under arsenic exposure at 1, 3 and 10 d. For
440	transcripts related to the vacuolar accumulation of metals and PC metal complexes, a 1.5 and 2 fold
441	up regulation of CAX2 1 and CAX2 2 was respectively observed under arsenic exposure at 1 d (Fig.
442	4). At 3 d there was a 1.8 fold increase in ABCG transcripts (Fig. 4). In addition, a 2, 4 and 2 fold
443	increase in WBABCT transcripts was detected at 3, 10 and 2014, respectively (Table 5).
444	Studying the arsenic stress related genes, an up regulation of 40, 8, 16 and 11 fold was
445	observed after As exposure for transcript levels of AIP 4 at 1, 3, 10 and 30 d, respectively (Table 5).
446	One transcript encoding an ACCS, producing the ethylene precursor ACC, was up regulated
447	throughout the experiment in the As exposed plants, but transcript quantities decreased from 30 fold
448	at 1 and 3 d to 9 and 8 fold at 10 and 30 d, respectively (Table 5). There were no changes in
449	transcripts associated to the ethylene receptor protein ER under As exposure (Fig. 4). For cellulose
450	synthesis, there was a 2fold down regulation in transcript levels of CSA at 10 and 30 d after As
451	exposure (Fig. 4).
452	

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453 **3.4.3 Differential gene expression in leaves**

454 Changes observed in transcripts encoding for As related transporters in leaves were minor and 455 showed a different trend than that seen in roots. Transcripts associated to PHO1 were 1.3, 1.4, 1.5-456 fold down regulated in leaves of As exposed willows at 1, 3 and 10 d, respectively (Fig. 4). However, 457 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 458 459 in the presence of As and SIP.1 expression was 1.5 fold up regulated but only at 3 d (Fig. 4). In 460 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 1.5-461 fold upregulated at 30 d of As exposure (Fig. 4). 462

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

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Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in Salix atrocinera 466 With regard to gene expression of the thiol metabolism related genes, there were no changes in 467 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 468 the pattern in the roots, MT1A transcripts were up regulated under arsenic exposure throughout the 469 experiment, with a 2, 1.7, 1.5, 1.9 fold increase at 1, 3, 10 and 30 d, respectively (Fig. 4). Vacuolar 470 471 transport dynamics in leaves of As-exposed plants were represented by a 1.4, 1.7 and 1.5 fold 472 upregulation of ABCG at 1, 3 and 10 d, respectively; and by a 1.6-fold up-regulation in transcript 473 levels of CAX2 1 at 1 d. No differences were observed in transcript levels of CAX2 2 or wBABCT (Fig. Formatted: Font: 8 pt, Font color: Black 474 4). 475 For the As stress related genes, a 2.3 fold up regulation in transcript levels of AIP 1 after 30 d 476 As exposure was observed (Fig. 4). Accs transcripts were 3, 2 and 4 fold higher up regulated after As Formatted: Font: 8 pt, Font color: Black exposure at 1, 10 and 30 d, respectively (Table 5). In leaves, transcript levels of ER were 1.5 fold 477 478 upregulated in S. atrocinerea plants after 30 d under arsenic exposure (Fig. 4). Concerning the 479 cellulose biosynthesis, there was a 2 fold down regulation in transcript levels of CSA after 30 d of As 480 exposure (Fig. 4). 481 Transcript levels for genes encoding enzymes regulating the phenylpropanoid and flavonoid athways were differentially expressed in leaves in response to As treatment (Fig. 4). For the initial 482 starting point of the biosynthetic pathway, the levels of three transcripts encoding a chalcone synthase 483 484 (CHS) were measured (CHS1, CHS2 and cuse): CHS1 was 1.3 and 1.5-fold down-regulated at 10 and Formatted: Font: 8 pt, Font color: Black 485 30 d, respectively; whereas cuse transcripts were 1.6 and 1.4 fold up regulated at 1 and 3 d, Formatted: Font: 8 pt, Font color: 486 respectively. Also CHS3 transcripts were 1.6 and 1.3 fold up regulated at 1 and 3 d, respectively; but Black 2.5 fold down regulated at 10 and 30 d. For the remainder of the pathway, transcript levels of F3H 487 488 were 1.5 fold up regulated at 1 d and 1.5 fold down regulated at 10 and 30 d. A similar trend was 489 observed for FLH and 2HFLR transcripts. No changes in the flavonol synthase transcripts (FLS) were 490 detected under As exposure. With regard to transcript levels of key enzymes regulating the Formatted: Justified, Indent: First 491 production of anthocyanins, it was observed that anthocyanidin synthase transcripts (ANS) were 2line: 1 cm, Space After: 0 pt, Line 492 fold downregulated at 10 d and transcript levels of the gene encoding for ANR were 1.5-fold spacing: Double, Don't adjust space between Latin and Asian text, Don't 493 downregulated at 10 and 30 d in As exposed leaves of S. atrocinerea. adjust space between Asjan text and numbers 494 Formatted: Font color: Text 1 Formatted: English (United Kingdom) Formatted: Indent: Left: 0 cm. First 495 4 Discussion line: 0 cm, Space After: 0 pt Formatted: English (United Kingdom) 496 The total pollutant concentration of a certain element in the soil is not a representation of the Formatted: Font color: Text 1 497 amount of metal that is available (exchangeable) for the plantsplant uptake, neither a good indicator Formatted: Font color: Text 1 498 to establish plant toxicity limits. Therefore, for a successful when phytoremediation processprocesses Formatted: Font color: Text 1 499 will rely on the use of certain plant species that tolerate and accumulate high concentrations of 500 metal(loid)s, it is very important that the studies conducted in the laboratory under controlled Formatted: Font color: Text 1 501 conditions, on which the basic physiological knowledge is set, are based on well reflected pollutant Formatted: Font color: Text 1 502 concentrations. Many hydroponic studies have used higher As concentrations than those found in soil Formatted: Font color: Text 1 503 solution, and their environmental relevance has been questioned (Fitz and Wenzel, 2002). According Formatted: Font color: Text 1 504 to this, some authors propose that hydroponic cultures should include As doses in the range of 0 - 10Formatted: Font color: Text 1 505 µM to allow the extrapolation of the results to As-polluted soils (Moreno-Jimenez et al., 2010). Formatted: Font color: Text 1

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

Tolerance in Salix atrocinera

506	Nonetheless, the fact that S. atrocinerea plants used in this study already grow on the Nitrastura		Formatted: Font color: Text 1
507	brownfield with an As exchangeable fraction of 18 mg Kgkg ⁻¹ (data not shown), suggests that the As		Formatted: Font color: Text 1
508	dose could be increased for this hydroponic assay. Furthermore, this As concentration matches that		
509	recommended in previous hydroponic studies with willow (Purdy and Smart, 2008) and it has already		
510	been used in analyzing differential As gene expression under hydroponic conditions (Puckett et al.,	l	
511	2012). Although some authors have reported that willows have the capability to translocate As from		Formatted: Font color: Text 1
512	roots to above ground tissues (Thustos et al., 2007 , Puckett et al., 2012 ; Sylvain et al., 2010), in		
513	our case,) the As accumulation in leaves diddoes not reach those quantities accumulated by		Formatted: Font color: Text 1
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514	present in hyperaccumulating species like Pteris vittata (Caille et al., 2004)), as it was our		Formatted: Justified, Indent: Left: 0
515	case, but the phytoremediation potential is compensated with a higher biomass as it has also been		cm, First line: 1 cm, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers
516	proposed by Jiang(Meers et al. (2015) for other species., 2007, Witters, 2009), Furthermore, after 30	$\langle \rangle$	Formatted: Font color: Text 1
			Formatted: Font color: Text 1
517	d of As exposure, S. atrocinerea did not show any phytotoxic symptoms and was capable of	$\langle \rangle$	Formatted: Font color: Text 1
510			Formatted: Font color: Text 1
518	accumulating a higher As concentration than that present in the culture medium, showing therefore a		Formatted: Font color: Text 1
519	bioaccumulation factor higher than 1. In its natural habitat. S. atrogingreg, which is also able to thrive		Formatted: Font color: Text 1
517	bioaccumulation factor ingher than 1. In its natural natitat, 5. <i>anotherea</i> , when is also able to unive		
520	in a brownfield environment underan added value for the presence of other metal(loid)s at toxic	_	Formatted: Font color: Text 1
521	concentrations (e.g. Zn, Pb). This multi metal(loids) tolerance, together with a great biomass,		
522	highlights the potential of S. atrocinerea in phytoremediation of polluted environments. As.		Formatted: Font color: Text 1
			Formatted: Font color: Text 1
523	It is well knownhas been reported that exposure to toxic metalloids, such as As, can disturb the		Formatted: Font color: Text 1
524	nutrient profile of the plant and hence lead to toxicity (Lou et al., 2010) and also that arsenate uptake		
525	and tolerance to the induced toxicity is intimately linked to phosphate nutrition. Some authors		
526	propose that an increased phosphate accumulation will lead to a reduced arsenate uptake (Meharg and		
527	Macnair, 1994). Therefore, a higher cytoplasmic phosphate accumulation may enable phosphate to		
528	compete more effectively with arsenate for ATP synthesis, decreasing arsenate toxicity within the		
529	plant cells. Nonetheless, different studies have seen a reduction of phosphate uptake in plants		
530	exposed to arsenate, which indicates that arsenate uptake occurs via the phosphate transporters and		
531	can also replace prosphate groups in diomolecules (wang et al., 2002; Patra et al., 2004). Our results		
532 533	showed that <i>b. dirocinered</i> does not rely on F accumulation to prevent As toxicity and that AS uptake		
534	accumulation observed in the roots and its reduced translocation to the leaves is a response to As		
535	accumulation in the roots of <u>S</u> atrocinered. Ca is an essential plant macronutrient.) and also that As		
536	V uptake and tolerance to its induced toxicity is intimately linked to phosphate nutrition. In the soil.		
537	As is mainly present in its As V form (Cordos et al., 2006) and once it is in contact with the roots, As		

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

Tolerance in Salix atrocinera 538 V can enter via phosphate transporters (Maciaszczyk-Dziubinska et al., 2012). Therefore, changes in 539 transcripts encoding for As V-related transporter proteins could be expected and, in our case, the As 540 V added to the hydroponic solution caused a differential regulation of transcripts for phosphate 541 transporters. The up-regulation of *PHO1* in roots of *S. atrocinerea* from the onset of the As exposure 542 (Fig. 3) and that observed at 10 and 30 d of transcripts encoding for a high-affinity phosphate 543 transporter protein (HAPO4) (Supplementary Fig. 3A), relate to the first lower and then similar P 544 concentrations in roots of As-exposed S. atrocinerea as compared to non-exposed plants (Table 1). It 545 has been suggested that reduced uptake of As V is a well-known mechanism of As V resistance 546 employed by many plant species, which is achieved through a reduction of the phosphate/arsenate 547 uptake system in resistant plants (Meharg and Hartley-Whitaker, 2002). Moreover, it is thought that 548 this reduction decreases As V influx to a level at which the plant can detoxify As, presumably by 549 constitutive mechanisms (Catarecha et al., 2007). However, according to our results of As 550 accumulation and a lower concentration of P under As exposure as compared to the control condition, 551 it can be suggested that the transcript upregulation of phosphate-related transcripts in roots is based 552 on preventing As V competition and avoiding P deprivation. Therefore, since As does accumulate at 553 high concentrations in roots of willow, a more effective detoxification mechanism than inhibition of 554 phosphate transporters as seen in other plants would be necessary in S. atrocinerea. After 30 d, As 555 concentration in leaves of S. atrocinerea reached levels higher than toxicity levels established for 556 non-tolerant plants (1-20 mg As kg⁻¹ dry weight; White and Brown, 2010). Under these conditions, a 557 differential regulation of As V-related transporters in leaves of S. atrocinerea was observed. The 558 decrease in PHO1 transcripts at 1, 3 and 10 d (Supplementary Fig. 3B), is a similar response to that 559 of As resistant species, where avoiding As uptake in leaves by reducing phosphate uptake constitutes 560 a tolerance mechanism (Meharg and Hartley-Whitaker, 2002). However, at 30 d the down-regulation 561 ceased and there were no differences in transcript levels of PHO1 compared to those observed in 562 leaves of control plants and it matched with a similar P concentration in leaves of both treatments 563 (Table 1). According to the Ca concentrations observed in plants of S. atrocinerea (Table 1), it can 564 be suggested that Ca accumulation in the roots and its reduced translocation to the leaves is a 565 response to As accumulation. Ca is an essential plant macronutrient and it plays an important role in 566 cell wall and membrane stabilization and regulates nutrient uptake as well as different stress 567 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. 568 More recently, Ji et al. (2017) have identified a Ca dependent protein kinase (CPK31) responsible for 569 570 As III tolerance in Arabidopsis. CPK31 is an interacting protein of NIP1.1, an aquaporin involved in 571 As III uptake. Similarly to the nip1.1 mutants, the loss of function mutants of CPK31 improved the 572 tolerance against As III but not As V, and accumulated less As III in roots than that of the wild type 573 plants. This might indicate that this Ca dependent CPK31 protein might be a target to regulate 574 NIP1.1 for As III tolerance. 575 In multiple studies 2015), including an increase of the antioxidant defense under As exposure-

576 and reducing As uptake (Rahman et al., 2015). In multiple studies, it has been shown that 577 micronutrient accumulation is affected by As exposure, but it can also have an impact on As uptake

578 and hence As toxicity (Srivastava et al., 2017).2017). It has been proposed that B channels might Formatted: Font color: Text 1

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579	have a role in As transport into the cell (Yanitch et al., 2017), which is also reflected in our results	
580	with increased BORON transcript levels in roots (Fig. 3) and leaves (Supplementary Fig. 3B).	
581	Furthermore, boric acid transporter NIP5.1 from Arabidopsis is also permeable to As III (Mitani-	
582	Ueno et al., 2011), and our data showed that B accumulation in plant tissues changes along the time	
583	of exposure to As (Table 1), with BORON transcripts 5-fold down-regulated at 1 d (Fig. 3), when As	
584	III concentration in the medium was the highest (Fig. 2B). In leaves, BORON transcripts are induced	
585	at 1 and 3 d in response to As (Supplementary Fig. 3B), coinciding at 3 d with the highest B	
586	concentration (Table 1). Whereas Zn is described as an indispensible micronutrient, which mitigates	
587	As toxicity by modulating ROS and the antioxidant function in plants (Das et al. 2016) or improveby	
588	improving the thiol metabolism (Srivastava and Srivastava, 2017), no major changes were detected in	
589	Zn concentration, appart apart from the decrease increase at 1 d, highlights the tolerance of S.	
590	atrocinerea to in As, exposed plants. With regard to Fe, our data showed that Fe translocation to	
591	leaves was more affected by As than any of the other elements, with an increased Fe concentration in	
592	roots exposed to As, whereas in leaves a Fe decrease was observedit decreased (Table 21). Shaibur et	
593	al. (2008) described that one of the symptoms of As toxicity is the formation of Fe plaques in roots	
594	and, as also seen in our case, Fe:P ratios in the roots of the As-exposed plants were higher than those	
595	observed in the control roots. This suggests that, in the liquid culture medium, As may have been	
596	adsorbed with Fe on the surface of the roots, forming Fe-As plaques. Thus, the iron plaque formed on	
597	the root surface will act as a natural As barrier and reduce As uptake by the plant and its	
598	translocation. <u>to shoots.</u>	
599	Besides the impact of other elements in the medium on As uptake, the speciation of As also	
600	plays an important role in the accumulation of As and tolerance by the plant (Moreno-Jimenez et al.,	

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	Changes in Non-Frotein Thione Compounds and Gene Transcripts Contributing to Arsenic accumulation and		
501	Tolerance in Salix atrocinera		
601	2010). The arsenicAs was added to the culture medium as As V and after 24 h a 4% reduction to As		Formatted: Font color: Text 1
602	III was observed (Fig. 22B). This chemical reduction can be attributed to metabolic activities of S.		Formatted: Font: Bold, Font co Text 1
603	atrocinerea since no speciation was detected when the plant cuttings were not present in the culture	$\langle \rangle$	Formatted: Font color: Text 1
			Formatted: Font color: Text 1
604	medium. It is known that For this observation, two possible explanations can be given. On one side,	_	Formatted: Font color: Text 1
605	plants might induce changes in the pH and in the redox potential of the culture medium, affecting the		Formatted: Font color: Text 1
			Formatted: Font color: Text 1
606	As as it was observed in this study (Fig. 2A), and those changes might affect the speciation-		Formatted: Font color: Text 1
607	Furthermore, it is also known that arsenite efflux from the plant to the medium can be linked to the		Formatted: Font color: Text 1
608	proton gradient across the plasma membranes or dependent of the plant metabolism (e.g. arsenite		Formatted: Font color: Text 1
609	efflux by yet unidentified transporters) (Xu et al., As. For an example, it 2007; Park et al., 2016). It		Formatted: Font color: Text 1
610	has been proposed that protons released from organic acids (R-COOH) and excreted by plant roots		Formatted: Font color: Text 1
611	may contribute to the reduction of As V to As III, while increasing the pH as the process consumes		
612	H ⁺ (Park et al., 2016). Interestingly, only arsenate As V was detected after 30 d matching and it	_	Formatted: Font color: Text 1
613	matched with the highest pH value, in the medium; whereas the highest arsenite As III concentration		Formatted: Font color: Text 1
614	in the medium was detected at 24 hl d when the pH was the lowest (Fig. 2). Possibly 24.)	$\overline{\ }$	Formatted: Font color: Text 1
014	in the medium was detected at 24 minute pri was the lowest (Fig. 2). Possiol, 24.		Formatted: Font color: Text 1
615	Therefore, another possible explanation for the presence of As III in the medium is a direct efflux of		Formatted: Font color: Text 1
616	As III from the plant to the medium that can be linked to the proton gradient across the plasma	\backslash	Formatted: Font: Bold, Font co
617	membranes or dependent of the plant metabolism (e.g. direct As III from plant cells to the medium)		Formatted: Font color: Text 1
618	(Xu et al., 2007; Park et al., 2016). Taking transcriptional regulations into account, since willow		Formatted: Font color: Text 1
619	plants were able to induce the occurrence of As III in the medium, differences in transcript levels of		
620	genes encoding for As III transport were expected in roots of As-exposed plants.		
621	In our case, we observed a noticeable up-regulation of the transcripts encoding the aquaporin+		Formatted: Justified, Indent: L cm, First line: 1 cm, Space Afte
622	NIP1.1, reported for As III uptake into the roots (Ma et al., 2008), and in transcripts for SIP1 at 1 d of		Line spacing: Double, Don't adj space between Latin and Asian Don't adjust space between Asia
623	As exposure (Fig. 3). This up-regulation diminishes over time, probably as a consequence of a very		and numbers

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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and Tolerance in <i>Salix atrocinera</i>	
624	active As V reduction to As III during the first days of exposure, with a lot of free As III initially in	
625	the cytoplasm. This transcript up-regulation for As III transporters in roots, suggests that As V	
626	reduction has occurred even before its entry into the roots, which is supported by the presence of 4%	
627	of As III in the medium at 1 d of exposure (Fig. 2B), and which coincides with the highest up-	
628	regulation of NIP1 and SIP1. In leaves, where As was mainly present as As V, no changes in	
629	transcript levels for the aquaporin transcripts were observed (Supplementary Fig. 3B).	Formatted: Font color: Text 1
630	Once inside the cell, since As V has no affinity for the "-SH" groups in the PCs, the first step in	
631	As detoxification is As reduction (Finnegan and Chen, 2012). The main mechanism for As V	
632	reduction is the presence of As V reductases where GSH acts as electron donor (Dhankher et al.,	
633	2002). Arsenate reductases are believed to have evolved from the CDC-25 (cell division cycle) dual-	
634	specificity tyrosine phosphatases (Duan et al., 2007). Based on homology with the yeast As V	
635	reductase, ACR2P, Bleeker et al. (2006) identified a CDC25-like plant candidate and showed that it	Formatted: Font color: Text 1
636	had arsenate reductase activity like it was also observed in other assays (Dhankher et al., 2006).	
637	arseniteIn our study. As V reduction to As III was observed in roots right after As uptake with an	
638	increasing As III concentration in root tissues from 9% to 70% by the end of the study (Fig. 2C).	
639	This coincides with the CDC25-1 up-regulation in roots of plants exposed to As (Fig. 3), whereas no	
640	changes were observed for CDC25-2 (Supplementary Fig. 3A).	
641 642 643 644 645 646 647 648 649	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 <i>GS</i> transcripts along the As-exposure time to PC production as a detoxification mechanism. This was reflected by the increased NPT concentrations in <i>S. atrocinerea</i> roots after As uptake (Table 3). Our results showed that although there was a clear up-regulation of <i>GS</i> 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	

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

Tolerance in Salix atrocinera

observations of As speciation in the medium; where it seems that As III efflux by roots occurs right
after arsenateAs V uptake and that this efflux diminishes once the arseniteAs 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.

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,

657 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 666 667 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 668 669 transporter proteins could be expected. In our case, As V was added to the hydroponic solution and a 670 differential regulation of transcripts for PHO1 was observed. An up regulation of PHO1 in roots of S. 671 atrocinerea (Fig. 4) might indicate that As V enters via these transporters from the onset of the As 672 exposure. It has been suggested that reduced uptake of arsenate is a well known mechanism of 673 arsenate resistance employed by many plant species, which is achieved through a reduction of the 674 phosphate/arsenate uptake system in resistant plants (Meharg and Hartley Whitaker, 2002). 675 Moreover, it is thought that this reduction decreases arsenate influx to a level at which the plant can 676 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. 677 678 thaliana, pht1.1.3, which harbours a semidominant allele encoding for the high affinity phosphate 679 transporter (PHT1.1). This allowed the *pht1.1-3* mutant to decrease arsenate uptake in the short term 680 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 681 682 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 683 reinforced. Moreover, As accumulation did not cause phytotoxic symptoms in the plants. The fact 684 that S. atrocinerea does not show an As excluding behavior, is an added value for phytoremediation. 685 686 Thus, since As is accumulated in plant tissues of willow, a more effective detoxification mechanism 687 than inhibition of phosphate transporters would be necessary in this plant as suggested by Yanitch et al. (2017). 688

In addition, although PCs were synthesized in *S. atrocinerea* in response to As exposure and their
 concentration increased over time in roots (**Table 3**), Ansenie 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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693 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 694 695 arsenic resistant species (Meharg and HartleyWhitaker, 2002), where avoiding As uptake is a 696 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 697 absorb P to maintain the P homeostasis in the plants. This is reflected in our data, since after 1 d the P 698 concentration in leaves of As exposed plants is lower than that under control conditions, whereas at 699 700 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 701 702 that higher PHO1 transcript levels in roots can counteract arsenate competition and avoid P 703 deprivation. 704 Since willow plants were able to induce the occurrence of arsenite in the medium, differences in 705 transcript levels of genes encoding for arsenite transport were also expected in roots of As exposed 706 plants. Especially an up regulation in transcripts encoding the aquaporin NIP1.1 that has been 707 reported previously as responsible for arsenite uptake into the roots (Ma et al., 2008) was observed 708 (Table 5). This suggests that arsenate reduction has occurred even before its entry into the roots, 709 which is supported by the presence of 4% of As III in the medium at 1 d of exposure (Fig. 2), and 710 which coincides with the highest up regulation of NIP1.1. Another aquaporin, SIP.1 was also up-711 regulated up to 10 d. In leaves, where As was mainly present as arsenate, no changes in transcript 712 levels for the aquaporin NIP1.1 was observed (Fig. 4). These results differ from those of Yanitch et 713 al. (2017) as was also seen for SILICON and the SIP.1 regulation pattern. Despite arsenite uptake, As 714 metabolism is different in different species and growth conditions and a different gene expression 715 pattern can be expected (Ma et al., 2008; Li et al., 2009). 716 Interestingly, in a study of Yanitch et al. (2017) it has also been proposed that B channels might-Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust 717 have a role in As transport into the cell, which is also reflected in our results with increased BORON space between Latin and Asian text, Don't adjust space between Asian text and numbers 718 transcript levels in roots and leaves (Table 5 and Fig. 4). This is interesting since it has been shown 719 that boric acid transporter NIP5.1 from Arabidopsis is also permeable to arsenite (Mitani Ueno et al., 720 2011) and our data showed that B accumulation in roots is increased under arsenic treatment. It is 721 interesting that BORON transcripts in leaves are induced at 1 and 3 d in response to arsenic treatment 722 (Fig. 4) coinciding at 3 d with the highest B concentration (Table 2). Formatted: Font color: Text 1 723 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 724 reduction is the presence of arsenate reductases where GSH acts as electron donor (Dhankher et al., 725 726 2002). Arsenate reductases are believed to have evolved from protein tyrosine phosphatases
	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and					
	Tolerance in Salix atrocinera					
727	(PTPases), which include the CDC 25 (cell division cycle) dual specificity tyrosine phosphatases					
728	(Duan et al.,					
729	2007). Based on homology with the yeast As(V) reductase, ACR2P, Bleeker et al. (2006) identified a					
730	CDC25-like plant candidate and showed that it had arsenate reductase activity like it was also					
731	observed in other assays (Dhankher et al., 2006). In both studies, they focused on maximizing As					
732	uptake and increasing translocation from roots to shoots with a phytoremediation purpose. In our					
733	study, arsenate reduction to arsenite was observed in roots right after As uptake with an increasing					
734	arsenite concentration in root tissues from 9% to 70% by the end of the study (Fig. 2). This coincides					
735	with the CDC25 1 up regulation in roots of plants exposed to As (Table 5), whereas no changes were					
736	observed for CDC25 2 (Fig. 4). In the study by Dhankher et al. (2006). As accumulation in shoots of					
737	Arabidopsis was enhanced, through enhanced As V translocation in knockdown ACR2 lines.					
738	Therefore. CDC25 1 offers a target to increase As translocation in S. atrocinerea, where by					
739	decreasing As reduction and its further complexation to PCs and sequestration into the vacuole, it can					
740	be transported and accumulated in aboveground tissues.					
741	Another form to reduce accumulated arsenate in the plant is through a non enzymatic reduction					
742	where GSH is implied. Since this process is relatively slow, we can attribute the large up regulation					
743	observed in GS transcripts to PCs production as a detoxification mechanism. This was shown by the					
744	increased total thiol concentrations in S. atrocinerea roots after As uptake (Table 4). Our results					
745	showed that although there was a clear up regulation of GS (Table 5), GSH concentrations in roots of					
746	As exposed plants remained constant over time and lower than those of the control plants, as also					
747	reported by Hasanuzzaman et al. (2017). In addition, although phytochelatins were synthesized as a					
748	detoxification mechanism under As exposure and their concentration increased over time in roots,	_				
749	there was only a slight increase, in transcripts coding for PCS at 1 d, similar to the behavior observed	_				
750	in transcripts of <i>GR</i> (<i>Supplementary Fig.</i> 4 <u>3</u> <i>A</i>). This suggests that the induction of <i>PCS</i> expression is	\leq				
751	unlikely to play a significant role in regulating PC biosynthesis (Cobbett 2000). It is knownThis					
752	agrees with Rea el al. (2004), who reported that PCS enzymes are expressed constitutively at	<				
753	relatively high levels and are generally unaffected by exposure of cell cultures or plants to heavy					
754	metal(loid)s-(Rea et al., 2004). As reported <u>described</u> in other plant species, phytochelatinPC-based					
755	sequestration is considered to be essential for arsenicAs tolerance, where hypertolerant ecotypes					

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756	present higher PCs concentration PC concentrations under arsenie As exposure compared to		Formatted
757	nontolerantnon-tolerant ecotypes and in both cases the plants exhibited arsenic hypersensitivity when		Formatted Formatted
758	PC synthesis was blocked by buthionine sulfoximine (BSO) (Meharg and Hartley-Whitaker, 2002;		Formatted
759	Schat et al., 2002; Fernández et al., 2013). The big7-fold increase in NPTs_NPT concentration		Formatted
760	observed in our case in roots of willow after 1 d of As exposure, could be it is then related to a fast		Formatted
761	arsenate As V reduction to arsenite in the plant As III and to the need to synthetize longerchain longer-	\sim	Formatted Formatted
762	chain PCs to chelate arsenite. the increasing concentrations of As III, and therefore maintaining		Formatted
763	cellular stability. As it has been reported, by Sharma et al. (2016), longer chain PCs contribute to a		Formatted
764	more effective cellular detoxification due to a higher metal-binding capacity and formation of more		Formatted
765	stable Ascomplexes As-complexes that will prevent the interaction with sulphydrylsulfhydryl groups		Formatted Formatted
766	of other proteins and hence affect the metabolism (Sharma et al., 2016).		Text 1 Formatted
767	We also observed that in the roots, the organ where more As was accumulated, a greater PCs.		Formatted Formatted
768	synthesis was present than in leaves where As accumulation is lower. Another interesting observation		Formatted cm, First lin Double
769	of our study is the presence of many unknown thiol products. This is in accordance with the results of		
770	Li et al. (2004) in Arabidopsis, where arsenicAs exposure resulted in the expression of many		
771	unknown thiol products, whereas cadmium induced greaterhigher increases in traditional PCs (PC2,		Formatted
772	PC ₃ , PC ₄).		Formatted Formatted
773	Most of the arsenicAs speciation experiments described in literature propose As III as being the		cm, Line spa
774	predominant As form in leaves (Zhang et al., 2009; Kertulis et al., 2005; Zhang et al., 2009; Yan et	\angle	Formatted Formatted
775	al., 2012; Park et al., 2016), however). However, in our case As V was the main As species observed		Formatted
776	in leaves throughout the experiment (Fig. 2). A possible explanation for the lack of arsenite observed		Text 1
777	in leaves could be that, despite <u>2C</u>). Despite As exposure caused de novo synthesis of desGly-PC ₂ and		color: Text
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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and		
	Tolerance in Salix atrocinera		
778	anthe increase of desGly-PC4 in leaves of As-exposed plants, a possible explanation for the lack of As	/	Formatted: Font color: Text 1
		$\overline{\langle}$	Formatted: Font color: Text 1
//9	III isobserved in leaves, with the exception at 3 d, could be attributed to the relatively low	$\langle \rangle$	Formatted: Font color: Text 1
780	concentration of As in leaves as compared to roots (Table 2), which might require a less effective		Formatted: Font color: Text 1
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		Formatted: Font color: Text 1
783	than in roots, and represent the main NPT in leaves (Table 3). Since As III – GSH complexes are less		Formatted: Font color: Text 1
784	stable that As III – PCs, a dissociation of these complexes could take place with the consequent re-	<	Formatted: Font: Bold, Font co Text 1
785	oxidation of As III to As V (Bluemlein et al., 2009; Zhao et al., 2009). This-In relation to this, the As		Formatted: Font color: Text 1
786	V presence in leaves of <i>S. atrocinerea</i> might explain the need for constant the up-regulation of the	_	Formatted: Font color: Text 1
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787	CDC25-like tyrosine phosphatases pathway (Fig. 4). Taking into account that speciationobserved at		
788	10 and distribution of arsenic in the 30 d, when As increased in shoots (Supplementary Fig. 3B), and		Formatted: Font color: Text 1
789	exceeded plant ean provide important information and help to understand the mechanisms for arsenie		Formatted: Font color: Text 1
790	accumulation, translocation, toxic limits (White and transformation as noted by Zhang et al. (2002),		Formatted: Font color: Text 1
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		Formatted: Font color: Text 1
			Formatted: Font color: Black
795	contrast to PCs that rely on enzymatic synthesis, MTs, which are also important metal chelators in		Formatted: Font color: Black
796	plant cells, MTs are encoded by genes and thus areare direct products of mRNA translation (Anjum		Formatted: Font color: Black
797	et al., 2015). Examples of MTs-examples induction under metal exposure in <i>Salix</i> have been	_	Formatted: Font color: Black
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798	described by Konlechner et al. (2013). However, the metal(2013), and it is known that metals like Zn		Formatted: Font color: Black
700	hindeen To hind to MTo with the highest offinity and also To and other assertial watch (Dividence of		Formatted: Font color: Black
000	onnasor re onna to wris with the nightst and the life of the second other essential metals (Blindauer et	\leq	Formatted: Font: Times New F Font color: Black
800	al. 2010). Inerefore, in this study according to the differential transcription pattern of <i>MTTA</i> between	//	Formattad: Font color: Black

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801	roots and leaves, due to the Fe accumulation in roots and its reduction in leaves, it might be that		Formatted: Font color: Black
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802	MT1A induction in leaves (Supplementary Fig. 3B) corresponds to the need of supplying enough Fe		Formatted: Font color: Black
803	in leaves and that this up-regulation is not be involved in direct As chelation. AnywayHowever, its		Formatted: Font color: Black
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804	induced expression in leaves forms part of the response to the As-induced stress.	\backslash	Formatted: Font color: Black
		$\langle \rangle$	Formatted: Font color: Black
805	Once $\frac{\text{arsenate} As V}{\text{arsenite} As III}$ and complexed to NPTs to limit its toxicity,	$\langle \rangle \rangle$	Formatted: Font color: Black
806	these complexes could heare taken up by ABC transporters and stored in the vacuale. It is known that	$\langle \rangle \rangle$	Formatted: Font color: Text 1
800	these complexes could be and taken up by ADC transporters and stored in the vacuole. It is known that		Formatted: Justified, Indent: Left: 0
807	As III PCs complexes have a low stability and their storage into the acidic environment of vacuoles		cm, First line: 1 cm, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space
808	can improve their stability and counteract a redox based destabilization of the complex (Schmöger et		Formatted: Font color: Text 1
000			Formatted: Font color: Text 1
809	al., 2000), ABC transporters constitute one of the largest protein families, present in organisms		Formatted: Font color: Text 1
810	ranging from bacteria to humans, and have been identified as transporters involved in detovification		Formatted: Font color: Text 1
010	ranging from bacteria to numans, and have been identified as transporters involved in detoxification		
811	processes by transporting metal(loid)-PC complexes (Kang et al., 2011). It is known that As III-PCs		
812	complexes have a low stability and their storage into the acidic environment of vacuoles can limit its		
813	dissociation and As release back into the cytosol (Schmöger et al., 2000). Song et al. (2010) already		Formatted: Font color: Text 1
814	made emphasis inemphasized that engineering of vacuolar PC transporters in plants may be of		Formatted: Font color: Text 1
815	potential use in phytoremediation-as they observed that Arabidopsis overexpressing ABCC1 and		
816	ABCC2 mutants resulted in arsenic hypersensitivity. Later on, it was also proven by Park et al. (2012)		
817	that overexpression of these transporters is also involved in greater vacuolar sequestration of		
818	cadmium and mercury. According to this, in our study, the up-regulation inobserved of WBABCT		Formatted: Font color: Text 1
819	transcripts encoding for the white brown complex ABC transporter (WBABCT) (Table 5)		Formatted: Font color: Text 1
820	highlight(Fig. 3) highlights its role in metal(loid)-PC complexes transportation and constitutes an		Formatted: Font color: Text 1
821	interesting target gene to increase accumulation for phytoremediation purposes. Since Interestingly, in		Formatted: Font color: Text 1
877	leaves since only As V was present throughout the exposure time and at low concentration compared		Formatted: Font color: Text 1
022	interves, since one is a was present throughout the exposure time and at low concentration compared	\leq	Formatted: Font color: Text 1

823 to As accumulated in roots, no differential up-regulation of the vacuolar transporter WBACT 824 transcripts was observed- as compared to control (Supplementary Fig. 3B). 825 Taking into account that speciation and distribution of As in the plant can provide important 826 information and help to understand the mechanisms for As accumulation, translocation, and 827 transformation as noted by Zhang et al. (2002), our results suggest that the As tolerance mechanism 828 of S. atrocinerea relies on As V reduction in roots but not in leaves. Therefore, limited As V 829 translocation by an effective As V reduction to As III and its complexation to NPT compounds and 830 further sequestration into the root vacuoles, as supported by the gene expression, seems to be the 831 reason for the tolerance of S. atrocinerea to As. 832 Under arsenate or arseniteAs accumulation, stress is induced in plant cells. The Although in 833 leaves As detoxification processes are not really activated as seen in roots, S. atrocinerea plants 834 respond to As in both roots and leaves by altering gene expression related to general stress. This 835 response includes alterations at the gene level of transcripts related to the cell wall synthesis, as the 836 down-regulation of genes involved in cellulose biosynthesis could indicate a limitation in cell 837 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 838 transcriptome analysis under arsenate stress showed changes in gene expression including 40 cell 839 840 wall related genes among 637 transcripts (Huang et al., 2012). Down regulated genes included the 841 cellulose synthase like A (CSA) that is also downregulated in our study in roots and leaves of S. 842 atrocinerea exposed to As (Fig. 4). Although no growth inhibition is observed in the current 843 experimental time frame on hydroponics, long term experiments should be foreseen in the future. 844 Within a short experimental time frame, it is well described that ethylene is involved in cross 845 communication between plant organs during stress, such as Cd stress (Schellingen et al., 2014). In 846 our experiment, (Supplementary Fig. 3A,B); ethylene biosynthesis, with ACCS was notably up-847 regulated in roots (Table 5), highlighting the presence of ACC or ethylene as an As stress response as 848 was also previously seen by Yanitch et al. (2017) and that, according to transcript data as also seen in 849 our data, ACC might act as a signaling molecule independent from ethylene synthesis (Yanitch et al., 850 2017). 851 It has been described that one of the most prominent features of the eukaryotic cellular response to 852 arsenic is the inductionand to a lesser extent in leaves, probably explained a low As translocation 853 (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 854 expression of heat shock genes and therefore, molecular chaperones are rapidly synthesized and 855 856 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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858	proteinproteins, like AIP-1-related, AIP-1, is, a highly conserved gene selectively activated by		Formatted: Font color: Text 1
859	arsenite As III in many cell types. Inactivation of the Caenorhabditis elegans homolog aip 1		Formatted: Font color: Text 1
860	compromises survival of worms exposed to arsenite, but not to other stressors (Sok et al., 2001). In).		Formatted: Font color: Text 1
861	that in our experiment, transcripts for AIP-1 showed in roots at 1 d the highest up-regulation observed		Formatted: Font color: Text 1
862	for any of the measured genes, and these results were only observed in roots, where arsenite	$\langle \rangle$	Formatted: Font color: Text 1
863	accumulation increased drastically throughout the experiment (Table 5; Fig. 2). (Supplementary Fig. 2)	$\langle \rangle$	Formatted: Font color: Text 1
804 865	2), and it decreased overtime. Since this protein is As III-induced, and As III concentrations increase		Formatted: Font color: Text 1
866	its toxicity. Another As-related response of willow to arcenic contamination is the biosynthesis of		Eormatted: Font color: Text 1
867	phenylpropanoids that may culminate with the increased production of tanning (Yanitch et al. 2017)	\langle	Formatted: Font color: Text 1
868	In our study, we observed an early up-regulation of chalcone synthase (<i>CHS1</i>, <i>CHS2</i>) and in other		Formatted: Font color: Text 1
869	selected genes (<i>CHS1</i> , <i>CHS2</i> , 2HFLR and F3H) in the flavonoid pathway at 1 and 3-d		Formatted: Font color: Text 1
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870	(Fig. 4 B and Supplementary Fig. 3B), whereas at 10 and 30 d no major changes in the		Formatted: Font color: Text 1
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871	expression pattern or a down-regulation were noticed- <u>later on as compared to control conditions</u> .	\mathbb{N}	Formatted: Font color: Text 1
070			Formatted: Font: Bold, Font color: Text 1
872	Therefore, by the information provided by the transcript levels, we suggest that S. atrocinerea relies		Formatted: Font color: Text 1
873	on the phenylpropanoid pathway to cope with As toxicity during the early times of exposure, but		Formatted: Font color: Text 1
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874 875	further investigation at metabolic level is essential.		Formatted: Justified, Indent: Left: 0 cm, First line: 1 cm, Space After: 0 pt, Line spacing: Double, Don't adjust space between Latin and Asian text, Don't adjust space between Asian text and numbers
0/0	5 Conclusions	$\langle \rangle$	Formatted: Font color: Text 1
		$\langle \rangle$	Formatted: Font color: Text 1
877	The selected S. atrocinerea clone naturally growing in an As-contaminated brownfield showed	\backslash	Formatted: Indent: Left: 0 cm, Space After: 0 pt, Line spacing: single
878	great tolerance when grown in the presence of a high concentration of arsenicAs and accumulated		Formatted: Indent: Left: 0 cm, First line: 0 cm, Space After: 0 pt, Line spacing: Double
879	more than 2,400 mg As Kgkg ⁻¹ dry weight in its roots without showing phytotoxicity symptoms. Our		Formatted: Font color: Black
880	findings reveal that tolerance to arsenic in S. atrocinerea is associated withunder the following		Formatted: Font color: Black
881	mechanisms: (I) increased arsenatepresence of As V in hydroponic conditions, willow plants show a		
882	transcriptional regulation of genes involved in nutrient transporters, As V reduction in roots, resulting		
883	in high arsenite concentrations in this organ, (II) increased GS expression in roots (III), de novo		
884	thiolic compound, glutathione synthesis and accumulation in both roots and leaves leading to		

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

 885
 vacuolar As sequestration, and (IV) mediated arsenic defense of As into the vacuoles, together with

 886
 genes involved in stress responses by ACC signaling, which coincides with a rapid As III presence

 887
 and accumulation in root tissues, altered nutrient profile and de novo synthesis and chaperone

 888
 induction.-increase of NPT compounds, all of which contribute to the tolerance to the metalloid by S.

 889
 atrocinerea.

890 The high As accumulation together with a high biomass yield makes this willow species a 891 potential tool for its use in As phytoremediation. Overall, a better understanding of the physiological 892 mechanisms of tolerance to arsenic toxicity in S. atrocinerea was achieved through this study by 893 experimental verification of the significance of particular transcripts complemented by 894 comprehensive an integrative analysis of nutrient profile. As accumulation and speciation, as well as 895 non protein thiolicNPT compounds synthesis. More research is neededHowever, according to our observations, further elarify the exact molecular and biochemical responses that confer tolerance to 896 897 overlooked that 898 actual protein levels 899 ptein stability and activity. Therefore, targeted metabolomics and proteomics studies will help to 900 a plethora of physiological effects that can be applied to improve the capabilities for As 901 phytoremediation in the field. Further research should also focus on what happens in real field 902 conditions polluted soils where, apart from As, there are, usually, other metal(loid)s at high 903 concentrations in As polluted soils that can affect As remediation. the plant detoxification responses.

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905 6 Conflict of Interest

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Tolerance in Salix atrocinera

925	Ahsan, N., Lee, D.G., Alam, I., Kim, P.J., Lee, J.J., Ahn, YO., Kwak, S.S., Lee, I.J., Bahk, J.D.,		
926	Kang, K.Y., Renaut, J., Komatsu, S., Lee, B.H. (2008) Comparative proteomic study of		
927	arsenicinducedarsenic-induced differentially expressed proteins in rice roots reveals		
928	glutathione plays a central role during As stress. PROTEOMICSProteomics. 8, 3561-		
929	3576.		
930	Anjum, N. A., M. Hasanuzzaman, M. A. Hossain, P. Thangavel, A. Roychoudhury, S. S. Gill, M. A.		
931	M. Rodrigo, V. Adam, M. Fujita, R. Kizek, A. C. Duarte, E. Pereira and I. Ahmad		
932	(2015). "Jacks of metal/metalloid chelation trade in plants—an overview." Frontiers in		
933	Plant Science 6(192)		
934		/	Formatted: Font: (Default) Times New Roman, 12 pt
935	Argus, G.W. (1995) Salicaceae Willow Family: Part Two: Salix L. Willow. Journal of the		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt, Line spacing: single
936	Arizona-Nevada Academy of Science 29, 39-62.	/	Formatted: Font: (Default) Times
937	Batista, B.L., Nigar, M., Mestrot, A., Rocha, B.A., Barbosa Junior, F., Price, A.H., Raab, A.,		New Roman, 12 pt
938	Feldmann, J. (2014) Identification and quantification of phytochelatins in roots of rice to	,	Formatted: Font: (Default) Times
939	long-term exposure: evidence of individual role on arsenic accumulation and		New Roman, 12 pt
940	translocation. J Exp Bot 65, 14671479. <u>1467-1479.</u>	/ /	Formatted: Font: (Default) Times
941	Bleeker, P.M., Hakvoort, H.W., Bliek, M., Souer, E., Schat, H. (2006) Enhanced arsenate reduction /	_ /	Remented Fast (Default) Times
942	by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in		New Roman, 12 pt
943	arsenate-tolerant Holcus lanatus. Plant J 45, 917-929.		Formatted: EndNote Bibliography.
944	Blindauer, C. A. and O. I. Leszczyszyn (2010). "Metallothioneins: unparalleled diversity in structures /		Justified, Indent: Left: 0 cm, Hanging:
945	and functions for metal ion homeostasis and more." Nat Prod Rep 27(5): 720-741.		2 cm
946	Gong, J. M., D. A. Lee and J. I. Schroeder (2003). "Long-distance root-to-shoot transport of		Formatted: Font: (Default) Times New Roman, 12 pt
947	phytochelatins and cadmium in Arabidopsis." Proc Natl Acad Sci U S A 100(17): 10118-10123	[/]	Formatted: Font: (Default) Times
0.49	Diversion K. Dech. A. Feldmann, I. (2000). Stability of ensenie nentides in plant sytuests, offlingoff,	11	New Roman, 12 pt
948	Bidemieni, K., Raao, A., Feldmann, J. (2009) Stability of arsenic peptides in plant extracts: online online to the stability of arsenic peptides in plant extracts.	/ /	Formatted: Font: (Default) Times
949	the versus on-line parallel elemental and molecular mass spectrometric detection for	/ /	International Sort)
950	Geille N. Surgerich S. Zhao, E.L. McCreth, S.D. (2004) America homemory support from the Density	/ /	Formatted: EndNote Bibliography
931	Came, N., Swanwick, S., Zhao, F.J., McGrath, S.P. (2004) Arsenic hyperaccumulation by Piens		Justified, Indent: Left: 0 cm, Hanging:
932	fortilization Environmental Dellution 122, 112, 120		2 cm
933	Tertifisation. Environmental Fondulon 152, 115-120.		Formatted: Font: (Default) Times New Roman, 12 pt
954	Catarecha, P., Segura, M.D., Franco-Zorrilla, J.M., García-Ponce, B., Lanza, M., Solano, R.,		Formatted: Font: (Default) Times
055	Des Ares I. Learne A. (2007). A Materit of the Archidencie Dhearthete Transporter DUT1.1 Displayer	11	New Roman, 12 pt
955	Paz-Ares, J., Leyva, A. (2007) A Mutant of the Arabidopsis Phosphate Transporter PHT1;1 Displays	-	Formatted: Font: (Default) Times
930	Change C. Durginger L. Common L. (1002) A simple and officient method for isolating DNA from nine	/	New Roman, 12 pt
937	Chang, S., Puryear, J., Carney, J. (1995) A simple and efficient method for isolaung KINA from pine		Formatted: EndNote Bibliography,
938	trees. Plant Molecular Blology Reporter 11, 115-116.		2 cm, Space After: 0 pt, Line spacing:
959	Cobbett, C. S. (2000). Phytochelatins and Their Roles in Heavy Metal Detoxification. Plant		single
960	Physiology 123(3): 825-832.	/	Formatted: Font: (Default) Times New Roman, 12 pt
0.01			Formatted: EndNote Bibliography,
961	Commission,-European2000Ambient Air Pollution by AS, CD and NI Compounds (Position		Justified, Indent: Left: 0 cm, Hanging: 2 cm, Line spacing: single
962	Paper—Final),-Luxembourg: Office for Official Publications of the European Communities.	_	Formatted: Font: (Default) Times
			ivew Koman, 12 pt

963	Cordos, E. A., T. Frentiua, M. Pontaa, I. Marginean, B. Abrahamb and C. Roman (2006).		
0.01			Formatted: Font: (Default) Times New Roman, 12 pt
964	Distribution study of inorganic arsenic (III) and (V) species in soil and their mobility in the area of		Formatted: EndNote Bibliography,
965	Bala-Mare, Romania. Chemical Speciation & Bioavailability 18(1): 11-25		Justified, Indent: Left: 0 cm, Hanging:
966	Baia Mare, Romania. Chemical Speciation & Bioavailability 18(1): 11-25	\backslash	single
967	Das, I., Sanyal, S. K., Koushik G., and Das, D. K (2016. 'Arsenic). Arsenic mitigation in soil-plant		Formatted: Font: (Default) Times New Roman, 12 pt
968 969	system through zinc application in West Bengal soils, Bioremediation journal, 20:	\sum	Formatted: Font: (Default) Times New Roman, 12 pt
970	Dave R. Singh P.K. Tripathi P. Shri M. Divit G. Dwivedi S. Chakrabarty D. Trivedi P.K.	$\langle \rangle \rangle$	Formatted: EndNote Bibliography.
971	Sharma V.K. Dhankher, O.P. Cornas F.I. Barroso, I.B. Tripathi, R.D. (2013) Arsenite	\mathbb{N}	Justified, Indent: Left: 0 cm, Hanging:
972	tolerance is related to proportional thiolic metabolite synthesis in rice (Oryza sativa L)	$\ \rangle$	2 cm
073	Arch Environ Contam Toxicol 64, 235, 242	/ /// /	Formatted: Font: (Default) Times
074	Dhankhar O D V Li D D Docon L Shi D Salt L E Sanacoff N A Sachti and D D Maaghar	//// /	New Roman, 12 pc
974	(2002) Engineering tolerance and hypersecumulation of arconic in plants by combining	1///	Formatted: Font: (Default) Times
975	(2002). Engineering toterance and hyperaccumulation of arsenic in plants by combining		Formatted: Fort: (Default) Times
970	20(11): 11401145 1140 1145		New Roman, 12 pt
911	$20(11)$. $\frac{11401145}{1140-1145}$	$\langle \rangle$	Formatted: Font: (Default) Times
978	Dhankher, O.P., Li, Y., P Rosen, B., Shi, J., Salt, D., Senecoff, J., A Sashti, N., Meagher, R.		New Roman, 12 pt, Not Italic
979	(2002) Engineering Tolerance and Hyperaccumulation of Arsenic in Plants by Combining Arsenate		Formatted: Font: (Default) Times New Roman, 12 pt
980	Reductase and v. Glutamylevsteine Synthetase Expressions.		Formatted: Font: (Default) Times
			New Roman, 12 pt
981	Dhankher, O.P., Rosen, B.P., McKinney, E.C., Meagher, R.B. (2006) Hyperaccumulation of arsenic		Formatted: Font: (Default) Times
982	in the shoots of Arabidopsis silenced for arsenate reductase (ACR2). Proceedings of the	\sim	New Roman, 12 pt
983	National Academy of Sciences of the United States of America 103, 5413-5418.	$\langle \rangle$	Formatted: Font: (Default) Times
984	Duan, G.L., Zhou, Y., Tong, Y.P., Mukhopadhyay, R., Rosen, B.P., Zhu, Y.G. (2007) A CDC25		
985	homologue from rice functions as an arsenate reductase. New Phytol 174, 311-321.		Formatted: EndNote Bibliography,
986	Ehlrich, H.L. (1990) Geomicrobiology. Marcel Dekker New York.	$\langle \rangle$	2 cm
987	Ellis, D.R., Gumaelius, L., Indriolo, E., Pickering, I.J., Banks, J.A., Salt, D.E. (2006) A novel		Formatted: Font: (Default) Times
988	arsenate reductase from the arsenic hyperaccumulating fern Pteris vittata. Plant Physiol	$\langle \ \rangle$	New Roman, 12 pt
989	141, 15441554. <u>1544-1554.</u>	\backslash	Formatted: Font: (Default) Times New Roman, 12 pt
990	Ellman, G.L. (1959) Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82,		Formatted: Font: (Default) Times New Roman, 12 pt
991	70-77.		Formatted: Font: (Default) Times
		\leq	New Roman, 12 pt, English (United
992	Fernández Fernández, R., I. Carballo, H. Nava, R. Sánchez-Tamés, A. Bertrand and A.	$\langle \rangle$	Formatted: EndNote Bibliography,
993	González (2011). Looking for Native Hyperaccumulator Species Useful in Phytoremediation:	$\langle \rangle$	Justified, Indent: Left: 0 cm, Hanging:
994	297330.	$\langle \rangle \rangle$	single
005	Formandar D. A. Dartsand D. Dais M. D. Maynott, J. J. Martine and A. Camadar (2012). Co., (1)		Formatted: English (United States)
993 006	remanuez, K., A. Bertrand, K. Keis, M. P. Mourato, L. L. Martins and A. Gonzalez (2013). Growth		Formatted: English (United States)
990 007	and physiological responses to cadmium stress of two populations of Dittrictua Viscosa		Formatted: English (Onfault) Times
997	(L.) Greuter. J Hazaru Mater 244-245: 555-502.		New Roman, 12 pt

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	Changes in Non-Protein Thiolic Compounds and Gene Transcripts Contributing to Arsenic accumulation and		
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998	Fernández, R., Bertrand, A., García, J.I., Tamés, R.S., González, A. (2012) Lead accumulation and		Formatted: Font: (Default) Times
999	synthesis of non-protein thiolic peptides in selected clones of Melilotus alba and		New Roman, 12 pt
1000	Melilotus officinalis. Environmental and Experimental Botany 78, 18-24.	<	Formatted: Font: (Default) Times New Roman, 12 pt, English (United
1001	Fernández, R., Carballo, I., Nava, H., Sánchez-Tamés, R., Bertrand, A., González, A. (2010)		Formatted: English (United States)
1002	Looking for notive hyperseumulator species useful in phyteremediation. In: Handbook of		Formatted: English (United States)
1002	Phytoremediation. Golubev, I.A (ed), Nova Science Publishers, Inc.New York, pp.297-		Formatted: Font: (Default) Times New Roman, 12 pt
1004 1005	330. Finnegan, P. M. and W. Chen (2012). Arsenic Toxicity: The Effects on Plant Metabolism.		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt, Line spacing: single
1006 1007	Frontiers in Physiology 3: 182.		Formatted: Font: (Default) Times New Roman, 12 pt
1008 1009	fundamentals and potential application to phytoremediation. Journal of Biotechnology 99, 259-278.	\backslash	Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt, Line spacing:
1010 1011 1012	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.		Formatted: Font: (Default) Times New Roman, 12 pt
1013 1014	Gallego, J.R., Esquinas, N., Rodríguez-Valdés, E., Menéndez-Aguado, J.M., Sierra, C. (2015)		Formatted: Font: (Default) Times New Roman, 12 pt
1015 1016	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-		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
1017 1018	induced physio-chemical and ultrastructural changes in four cultivars of Brassica napus L. Chemosphere 120, 154-164.		Formatted: Font: (Default) Times New Roman, 12 pt
1019 1020 1021	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		
1022	Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W.,		Formatted: Font: (Default) Times New Roman, 12 pt
1023 1024	Hellsten, U., Putnam, N., Rokhsar, D.S. (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res 40, D1178-D1186.		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
1025 1026	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.		Formatted: Font: (Default) Times New Roman, 12 pt
1027	Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W.T., Schat, H., Meharg, A.A.	/	Formatted: Font: (Default) Times New Roman, 12 pt
1028 1029	(2001) Phytochelatins Are Involved in Differential Arsenate Tolerance in Holcus lanatus. Plant Physiol 126, 299-306.		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
1030	Hasanuzzaman, M., K. Nahar, T. I. Anee and M. Fujita (2017). Glutathione in plants:	/	Formatted: Font: (Default) Times New Roman, 12 pt
1031 1032	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.		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
1033	Huang, J.H., Fecher, P., Ilgen, G., Hu, K.N., Yang, J. (2012) Speciation of arsenite and arsenate in		Formatted: Font: (Default) Times New Roman, 12 pt
1034	Food Chemistry 130, 453-459.		Formatted: Font: (Default) Times New Roman, 12 pt
			·

1036 1037	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:		Formatted: Font: (Default) Times New Roman, 12 pt
1038	Exploiting Plant-Associated Bacteria to Improve Biomass Production and Metal Uptake.		Formatted: Font: (Default) Times New Roman, 12 pt
1039	Ji, R., L. Zhou, J. Liu, Y. Wang, L. Yang, Q. Zheng, C. Zhang, B. Zhang, H. Ge, Y. Yang, F. Zhao,		Formatted: Font: (Default) Times New Roman, 12 pt
1041 1042	S. Luan, and W. Lan. (2017. 'Calcium). <u>Calcium</u> -dependent protein kinase CPK31 interacts with arsenic transporter AtNIP1;1 and regulates arsenite uptake in Arabidopsis		Formatted: Font: (Default) Times New Roman, 12 pt
1043 1044	thaliana'thaliana, <i>PLoS One</i> , 12: e0173681. Jiang Y Lei M Duan L Longhurst P (2015) Integrating phytoremediation with biomass		Formatted: Font: (Default) Times New Roman, 12 pt
1045	valorisation and critical element recovery: A UK contaminated land perspective. Biomass		Formatted: Font: (Default) Times New Roman, 12 pt
1040	Kabata-Pendias, (2010), Trace Elements in Soils and Plants, Fourth Edition. CRC Press, pp. 407-505.		Formatted: Font: (Default) Times New Roman, 12 pt
1048	Kang, J., Park, J., Choi, H., Burla, B., Kretzschmar, T., Lee, Y., Martinoia, E. (2011) Plant		Formatted: Font: (Default) Times New Roman, 12 pt
1049	ABC Transporters. The Arabidopsis Book / American Society of Plant Biologists 9, e0153.	/	Formatted: EndNote Bibliography,
1050	Kertulis, G.M., Ma, L.Q., MacDonald, G.E., Chen, R., Winefordner, J.D., Cai, Y. (2005)		2 cm, Space After: 0 pt, Line spacing: single
1051 1052	Arsenic speciation and transport in Pteris vittata L. and the effects on phosphorus in the xylem sap.	\langle	Formatted: Font: (Default) Times New Roman, 12 pt
1052	Kidd, P., Mench, M., Álvarez-López, V., Bert, V., Dimitriou, I., Friesl-Hanl, W., Herzig, R.,		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
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1054 1055	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 Tracet		Formatted: English (United States) Formatted: Font: (Default) Times New Roman, 12 pt
1054 1055 1056 1057	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- <u>ElementContaminatedElement-Contaminated</u> Soils. International Journal of Phytoremediation 17, 1005-1037.		Formatted: English (United States) Formatted: Font: (Default) Times New Roman, 12 pt Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt
1054 1055 1056 1057 1058 1059	 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 Tracet <u>ElementContaminatedElement-Contaminated</u> 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 		Formatted: English (United States) Formatted: Font: (Default) Times New Roman, 12 pt Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt Formatted: Font: (Default) Times New Roman, 12 pt
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	Tolerance in Salix atrocinera		
1074	Li, RY., Ago, Y., Liu, WJ., Mitani, N., Feldmann, J., McGrath, S.P., Ma, J.F., Zhao, FJ. (2009)		Formatted: Font: Times New Roman,
1075 1076	<u>The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. Plant Physiol</u> 150, 2071-2080.		12 pt
1077	Li, Y., O. P. Dhankher, L. Carreira, D. Lee, A. Chen, J. I. Schroeder, R. S. Balish and R. B. Meagher		Formatted: Justified, Indent: Left: 0
1078	(2004). "Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced		cm, Hanging: 2 cm, Line spacing: single
1079	arsenic tolerance and cadmium hypersensitivity." Plant Cell Physiol 45(12): 1787-1797.		
1080	Li, J., Jia, H., Han, X., Zhang, J., Sun, P., Lu, M., Hu, J. (2016) Selection of Reliable Reference		
1081	Genes for Gene Expression Analysis under Abiotic Stresses in the Desert Biomass Willow, Salix		
1082	psammophila. Front Plant Sci 7, 1505.		
1083	Li D. V. Ago, V. Lin, W. I. Mitani, N. Faldmann, I. McCrath, S.D. Ma, I.E. Zhao, E. L.		
1083	(2000) The Diver A queporin Leil Mediates Untake of Methylated Arsonic Species, Diant Drysiel 150.		
1084	$\frac{2009}{110}$ rice Aquapoint Estrated acts optake of weatyrated Atsente Species. Frank Physiol 190, 2071-2080		
1005	2011 2000.		
1086	Lindsay, E.R., Maathuis, F.J.M. (2016) Arabidopsis thaliana NIP7;1 is involved in tissue arsenic.		Formatted: Font: (Default) Times
1087	distribution and tolerance in response to arsenate. FEBS Letters 590, 779-786.	$\overline{\ }$	New Roman, 12 pt
1088	Lloyd, G.a.M., B.H. (1981) Woody Plant Medium (WPM)-A Mineral Nutrient Formulation for		Formatted: EndNote Bibliography,
1089	Microculture of Woody Plant Species. HortScience 16, 453.		2 cm
1090	Lou, L.Q., Ye, Z.H., Lin, A.J., Wong, M.H. (2010) Interaction of arsenic and phosphate on their		Formatted: Font: (Default) Times
1091	uptake and accumulation in Chinese brake fern. Int J Phytoremediation 12, 487-502.	$\overline{\ }$	New Roman, 12 pt
1092	Ma, J.F., Yamaji, N., Mitani, N., Xu, XY., Su, YH., McGrath, S.P., Zhao, FJ. (2008)		Formatted: Font: (Default) Times
1095	Proceedings of the National Academy of Sciences 105, 0021, 0025	\sim	Formatted: Font: (Default) Times
1094	Proceedings of the <u>Ivational Academy of Sciences 103</u> , 9931-9933.		New Roman, 12 pt
1095	National Academy of Sciences 105, 9931-9935.		
1000			
1096	Maciaszczyk-Dziubinska, E., Wawrzycka, D., Wysocki, R. (2012) Arsenic and Antimony		
1097	Transporters in Eukaryotes. International Journal of Molecular Sciences 13, 3527-3548	/	Formatted: Font: (Default) Times
1098	Mandal, B.K., Suzuki, K.T. (2002) Arsenic round the world: a review. Talanta 58, 201-235.	~	Formatted: EndNote Bibliography
1000			Justified, Indent: Left: 0 cm, Hanging:
1099	Matschullat, J. (2000) Arsenic in the geosphere — a review. Science of The Total Environment	$\langle \rangle$	2 cm, Space After: 0 pt, Line spacing: single
1100	249 297 312		Formatted: Font: (Default) Times
1100	Meers E Vandecasteele B Ruttens A Vangronsveld I Tack EMG (2007) Potential of five	\checkmark	New Roman, 12 pt
1102	willow species (Salix spp.) for phytoextraction of heavy metals. Environmental and	$\langle \rangle$	Formatted: Font: (Default) Times
1103	Experimental Botany 60, 57-68.	$\langle \rangle$	New Roman, 12 pt
1104	Meharg, A.A., Hartley-Whitaker, J. (2002) Arsenic uptake and metabolism in arsenic resistant and		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging:
1105	nonresistant plant species. New Phytologist 154, 29-43.		2 cm, Space After: 0 pt, Line spacing:
1106	Mehara A. Macnair M. R. (1002) Suppression of the High Affinity Phosphate Untake	$\langle \rangle$	single
1100	monung, m.m., maonan, m.m. (1992) Suppression of the ringh mining radisplate optake		Formatted: Font: (Default) Times New Roman, 12 pt
1107	System: A Mechanism of Arsenate Tolerance in Holcus lanatus L. Journal of Experimental Botany	/	Formatted: EndNote Bibliography
1108	4 3, 519-524.		Justified, Indent: Left: 0 cm, Hanging:
			2 cm

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.
- 1115Moreno-Jimenez, E., Esteban, E., Fresno, T., de Egea, C.L., Penalosa, J.M. (2010) Hydroponics as a1116valid 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.
- 1120Naidu, R., Smith, E., Owens, G., Bhattacharya, P., Nadebaum, P. (2006) Managing arsenic in the1121environment: from soil to human health. CSIRO Publishing, Collingwood.
- 1122Nie, L., Shah, S., Rashid, A., I. Burd, G., Dixon, D.G., Glick, B. (2002) Phytoremediation of arsenate1123contaminated soil by transgenic canola and the plant growth-promoting bacterium1124Enterobacter 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.

1128Park, J., Song, W.Y., Ko, D., Eom, Y., Hansen, T.H., Schiller, M., Lee, T.G., Martinoia, E.,1129Lee, Y. (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to1120Lei, Y. (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to

- 1130 cadmium and mercury. Plant J 69, 278 288.
- 1131Park, J.H., Han, Y.S., Seong, H.J., Ahn, J.S., Nam, I.H. (2016) Arsenic uptake and speciation in1132Arabidopsis 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.

- 1136 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.
- 1140 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.

1145 Purdy, J.J., Smart, L.B. (2008) Hydroponic Screening of Shrub Willow (Salix Spp.) for Arsenic

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1146	Tolerance and Uptake. International Journal of Phytoremediation 10, 515-528.		Formatted: Font: (Default) Times New Roman, 12 pt
1147	Quaghebeur, M., Rengel, Z. (2003) The Distribution of Arsenate and Arsenite in Shoots and		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm. Line spacing: single
1140 1149	Plant Physiol. 132(3):1600-1609.	$\left< - \right>$	Formatted: Font: (Default) Times
1150 1151 1152	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		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
1153 1154	Phytologist 168, 551558. 551-558. Rahman, A., M. G. Mostofa, M. M. Alam, K. Nahar, M. Hasanuzzaman and M. Fujita (2015).	\backslash	Formatted: Font: (Default) Times New Roman, 12 pt
1155 1156	Calcium Mitigates Arsenic Toxicity in Rice Seedlings by Reducing Arsenic Uptake and Modulating the Antioxidant Defense and Glyoxalase Systems and Stress Markers	\backslash	Formatted: Font: (Default) Times New Roman, 12 pt
1157	BioMed research international 2015: 340812-340812.		Formatted: Font: (Default) Times New Roman, 12 pt
1158	plants. Current Opinion in Biotechnology 5, 285-290.		Formatted: Font: (Default) Times New Roman, 12 pt
1161 1162 1163 1164	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		
1165	Long-Lost Cousin, Phytochelatin Synthase. Plant Physiology 136(1): 2463-2474.		Formatted: Font: (Default) Times
1166 1167 1168 1169	 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 PCB: reporting and minimizing. 		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt, Line spacing: single
1170	the uncertainty in data accuracy. Plant Cell 26, 3829-3837.	\backslash	Formatted: Font: (Default) Times
1171	Rousseau, MC., Straif, K., Siemiatycki, J. (2005) IARC Carcinogen Update. Environmental		Formatted: Font: (Default) Times
1172	Health Perspectives 113, A580 A581.		
1173	Ruttens, A., Boulet, J., Weyens, N., Smeets, K., Adriaensen, K., Meers, E., Van Slycken, S.,		
1174 1175	Tack, F., Meiresonne, L., Thewys, T., Witters, N., Carleer, R., Dupae, J., Vangronsveld, J. (2011) Short		
1176 1177	Rotation Coppice Culture of Willows and Poplars as Energy Crops on Metal Contaminated Agricultural Soils. International Journal of Phytoremediation 13, 194-207.		Formatted: Font: (Default) Times New Roman, 12 pt
1178 1179	Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., Bleeker, P.M. (2002) The role of		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging:
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1181 1182 1183	Schellingen K., Van Der Straeten D., Vandenbussehe 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.		
1184 1185	<u>Schmittgen, T. D. and K. J. Livak (2008). Analyzing real-time PCK data by the comparative C(T)</u> method." Nat Protoc 3(6): 1101-1108.		
1186	Schmöger, M.E.V., Oven, M., Grill, E. (2000) Detoxification of Arsenic by Phytochelatins in		
1187	Plants. Plant Physiol 122, 793-802.	\langle	Formatted: Font: (Default) Times New Roman, 12 pt
1188	Shaibur, M. R., N. Kitajima, R. Sugawara, T. Kondo, S. M. Imamul Huq and S. Kawai (2008).		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt, Line spacing: single
1189	Physiological and Mineralogical Properties of Arsenic-Induced Chlorosis in Barley Seedlings Grown		
1190	Hydroponically. Journal of Plant Nutrition $31(2)$: $333-353$.	\mathbf{i}	New Roman, 12 pt
1191	Sharma, R., R. Bhardwaj, N. Handa, V. Gautam, S. K. Kohli, S. Bali, P. Kaur, A. K. Thukral, S.		Formatted: EndNote Bibliography
1192	Arora, P. Onri and A. P. Vig (2016). Chapter 10 - Responses of Phytochelatins and	$\langle \rangle$	Justified, Indent: Left: 0 cm, Hanging:
1193	Metallotnioneins in Alleviation of Heavy Metal-Stress in Plants: An-Overview. Plant	$\langle \rangle$	2 cm
1194	Metal Interaction. P. Anmad, Elsevier: 205-285.	\mathbb{N}	Formatted: Font: (Default) Times New Roman, 12 pt
1175	Shehirefune, F.A., Black, C.K., McGlaui, S.F., Toung, S.D. (2007) Wodening	$\langle \rangle \rangle$	Formatted: Font: (Default) Times New Roman, 12 pt
1196 1197	phytoremediation by the hyperaccumulating fern, Pteris vittata, of soils historically contaminated with arsenic. Environmental Pollution 157, 1589-1596.		Formatted: Font: (Default) Times New Roman, 12 pt
1198	Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: Pht1;1	\mathbb{V} ,	Formatted: Font: (Default) Times
1200	environments Plant I 39, 629-642	, //	Formattad: Font: (Dofault) Times
1200	Singh, A.P., Goel, R.K., Kaur, T. (2011) Mechanisms Pertaining to Arsenic Toxicity. Toxicology	$\langle \rangle$	New Roman, 12 pt
1202	International 18, 87-93.	$\langle \rangle$	Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging:
1205	Sintedrey, P. L. (2007). Managing Alsenic in the Environment: from Son to Fundan realth- Edited by R. Naidu, F. Smith, G. Owens, P. Rhattacharya & P. Nadebaum, European Journal of Soil	$\langle \rangle$	Formatted: Font: (Default) Times
1204	Science 58(2): 519-520.		New Roman, 12 pt
1206	Smith A H A Ercumen X Yuan and C M Steinmaus (2000) "Increased lung cancer risks area	1	Formatted: Font: (Default) Times New Roman, 12 pt
1200	similar whether arsenic is ingested or inhaled." Journal of exposure science &		Formatted: Font: (Default) Times New Roman, 12 pt
1208 1209 1210	environmental epidemiology 19(4): 343-348. Sok, J., Calfon, M., Lu, J., Lichtlen, P., Clark, S.G., Ron, D. (2001) Arsenite-inducible RNAassociatedRNA-associated protein (AIRAP) protects cells from arsenite toxicity. Cell		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
1211	Stress Chaperones 6, 6-15.	$\langle \rangle$	Formatted: Font: (Default) Times New Roman, 12 pt
1212	Song, W.Y., Park, J., Mendoza-Cozatl, D.G., Suter-Grotemeyer, M., Shim, D., Hortensteiner,		Formatted: Font: (Default) Times New Roman, 12 pt
1213	S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E. (2010)	/	Formatted: Font: (Default) Times New Roman, 12 pt
1214 1215	Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proc- Natl Acad Sci U S A 107, 21187-21192.		Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm

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1216	Spratlen, M.J., Gamble, M.V., Grau-Perez, M., Kuo, CC., Best, L.G., Yracheta, J., Francesconi, K.,		Formatted: Font: (Default) Times
1217	Goessler, W., Mossavar-Rahmani, Y., Hall, M., Umans, J.G., Fretts, A., Navas-Acien, A.	l	New Roman, 12 pt
1218	(2017) Arsenic metabolism and one-carbon metabolism at low-moderate arsenic	(
1219	exposure: Evidence from the Strong Heart Study. Food and Chemical Toxicology 105,	_	Formatted: Font: (Default) Times
1220	<u>387-397.</u>	l	
1221	Evidence from the Strong Heart Study. Food and Chemical Toxicology 105, 387-397.		
1222	Srivastava, S. and M. Shrivastava (2017). Zinc supplementation imparts tolerance to arsenite stress in		Formatted: Font: (Default) Times
1223	Hydrilla verticillata (L.f.) Royle. Int J Phytoremediation 19(4): 353-359.		New Roman, 12 pt
1224	Srivesteve S. A. K. Srivesteve G. Seblok T. H. Deshpende and P. Supresenne (2015)		Formatted: EndNote Bibliography,
1224	Transcriptomics profiling of Indian mustard (Brassica juncea) under arsenate stress identifies key		2 cm
1226	candidate genes and regulatory pathways. Front Plant Sci 6: 646.		
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1227	Stanhill, A., Haynes, C.M., Zhang, Y., Min, G., Steele, M.C., Kalinina, J., Martinez, E., Pickart,		Formatted: Font: (Default) Times
1228	C.M., Kong, XP., Ron, D. (2006) An Arsenite-Inducible 19S Regulatory Particle-		New Roman, 12 pt
1229	Associated Protein Adapts Proteasomes to Proteotoxicity. Molecular Cell 23, 875-885.		Justified, Indent: Left: 0 cm, Hanging:
1230	Sylvain, B., Mikael, MH., Florie, M., Emmanuel, J., Marilyne, S., Sylvain, B., Domenico, M.		2 cm
1231	(2016) Phytostabilization of As, Sb and Pb by two willow species (S. viminalis and S.		Formatted: Font: (Default) Times
1232	Thetoš P. Száková Li. Vysloužilová M. Pavlíková D. Weger I. Javorská H. (2007) Variation		New Roman, 12 pt
1233	in the untake of Arsenic Cadmium Lead and Zinc by different species of willows Salix		New Roman, 12 pt
1235	spp. grown in contaminated soils. Central European Journal of Biology 2, 254-275.		•
1026	Id E. Ahmed D. Ahmed N. Dese Messed K. Hussein M. Echern Melik M. Oswann		
1230	A (2018) Devergendiation of argonic contaminated soils by Eucolyptus completions. Terminalia		
1237	ariuna and Saliv tetrasporma		
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1239	Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. (2012)		Formatted: Font: (Default) Times
1240	Primer3new capabilities and interfaces. Nucleic Acids Res 40, e115.		New Roman, 12 pt
1241	Wang, J., Zhao, F.J., Meharg, A.A., Raab, A., Feldmann, J., McGrath, S.P. (2002) Mechanisms of		Formatted: EndNote Bibliography,
1242	arsenic hyperaccumulation in Pteris vittata. Uptake kinetics, interactions with phosphate,	$\langle $	2 cm
1243	and arsenic speciation. Plant Physiol 130, 1552-1561.		Formatted: Font: (Default) Times
1244	Wang, X., Mu, X., Zhang, J., Huang, Q., Alamdar, A., Tian, M., Liu, L., Shen, H. (2015) Serum		New Roman, 12 pt
1245	rets: a step forward in understanding abranic arsonic toxicity. Matellomics 7, 544, 552		Formatted: Font: (Default) Times
1240 1247	White P L and P H Brown (2010) Plant nutrition for sustainable development and global health		Formatted: Font: (Default) Times
1247	Annals of botany 105(7): 1073-1080		New Roman, 12 pt
1249	Witters, N., Slvcken, S., Ruttens, A., Adriaensen, K., Meers, E., Meiresonne, L., Tack, F.M.G.,		
1250	Thewys, T., Laes, E., Vangronsveld, J. (2009) Short-rotation coppice of willow for		
1251	phytoremediation of a metal-contaminated agricultural area: a sustainability assessment.		
1252	BioEnergy Res 2.		
1253	Xu W Dai W Yan H Li S Shen H Chen Y Xu H Sun Y He 7 Ma M (2015)		
1233	230, 40, 200, 40, 100, 100, 100, 100, 0000, 100, 0000, 100, 200, 100, 1	(Formatted: Font: (Default) Times
1254	Arabidopsis NIP3:1 Plays an Important Role in Arsenic Uptake and Root-to-Shoot Translocation		New Roman, 12 pt
1255	under Arsenite Stress Conditions. Molecular Plant 8, 722-733.	\searrow	Formatted: EndNote Bibliography,
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1256 1257 1258 1259 1260 1261 1262 1263 1264	 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) 	Formatted: Font: (Default) Times New Roman, 12 pt Formatted: Font: (Default) Times New Roman, 12 pt Formatted: Font: (Default) Times New Roman, 12 pt Formatted: English (United States)
1265 1266 1267 1268 1269 1270 1271 1272 1273 1274 1275	 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', <i>New Phytol</i>, 181: 777 94. 	Formatted: Font: (Default) Times New Roman, 12 pt Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm, Space After: 0 pt, Line spacing: single Formatted: Font: (Default) Times New Roman, 12 pt Formatted: Font: (Default) Times New Roman, 12 pt Formatted: Font: (Default) Times New Roman, 12 pt
1276 1277 1278 1279 1280 1281	 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 	Formatted: Font: (Default) Times New Roman, 12 pt Formatted: EndNote Bibliography, Justified, Indent: Left: 0 cm, Hanging: 2 cm
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Tolerance in Salix atrocinera

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

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Gene	Gene description	S. purpurea ortholog locus or	Primer sequence	Product size	Effi	Formatted
Gene	Sene description	NCBI annotation	F/R (5/3/_)	(bp) _▲	ency	Formatted
Reference	e genes Reference genes (1: used to normalize gene expression data in roots, 2:	_		4		Deleted Cells
used to no	rmalize gene expression data in leaves		000100000			Deleted Cells
OTU		SapurV1A.0615s020	CTCTTCGAA			Deleted Cells
OTU ^{1,2}	OTU-like cysteine protease family protein	0.1	ATCCCCATCTTT	114	91.2	Deleted Cells
			CGCAGTCG		M \ X	Deleted Cells
ACT7		SapurV1A.0231s032	TGTATGCCA	•		Deleted Cells
ACT7 ²	Actin 7	0.1	GTCACGACCAG	140	90.6	Deleted Celle
			CAAGATCCA			Deleteu Celis
α-		SamurV1A 0508e003	TTTGTCCAC			Formatted
TUB2	Alpha-tubulin 2	0.1	CCCTCGTCATCA	133	97.6	Formatted Table
TUB2		•	CCACCTTC			Formatted
		C	GCTCCCGGTTCT			
DNAJ	Chaperone protein DnaJ 49	Sapurv 1A.0212s011	AAATTAACCCC	117		Formatted
			TCTCTGCGTAGT			Formatted
			ACCAGATTTCC			Formatted
EF1a	Elongation factor 1-alpha	SapurV1A.0023s030	GAGCCCAAG	150	90.1	
EFIa		0.1	GTGCAAACC		IN/MIN	Formatted
			TGGGGCTGTCTT		I WANN	Formatted
ARF2	ADP-ribosylation factor 2.	SapurV1A.0014s016	TCACCAAG	131.	96.9	Formatted
ARF2		0.1	<u>GGTCACAATCT</u>			Formattad
Arsenate	transport		CACCOAOCI			Formatteu
			GAACGACGAGC			Formatted
HAPO4	High-affinity phosphate transporter 4	HO228362.1	ACCTGGTT	108	2.4	Formatted
			ACGGGTTCTATT			Formatted
NIA			CAGCCACTTATC			Formatted
<u>NA-</u> DPHO	Sodium-dependent phosphate transporter	SapurV1A.0139s026	CCCAGCAA	134		
T		<u>0.1</u>	TCAAGGCGAAT			Formatted
			AGAGGCTGCGA			Formatted
PHO1	Phosphate transporter PHO1-like protein	SapurV1A.0063s055	TGTTGAACA	115		Formatted
<u></u>		<u>0.1</u>	GTCTGAAGCAA	<u></u>		Formattod
A reanita t	renenart		GOCGAOICA			
Arsenite			TCATTCGCGGA			Formatted
<u>BORO</u>	Poren transporter	SapurV1A.0014s020	ACAACTGGAG	142		Formatted
<u>N</u>	Boton transporter	<u>0.1</u>	ACTGTCGGCTCT	145		Formatted
			CAAGGTTGTGA			Formatted
NIP1	Aquaporin NIP1.1	SapurV1A.0029s017	CTCTTCCAGGA	106	89.9	Formatted
		<u>0.1</u>	TGAAATGGG			Formatted
		Somur V1 & 1059-006	GCCAGTTCAGT			Formatted
<u>SIP1</u>	Aquaporin SIP.1	<u>5apur v 1A.10588006</u> <u>0.1</u>	TGCAGCAGAGG	<u>147</u>		Formatted
			GTTTCGAG			Francieu
SILICO		SapurV1A 1225s008	GGTAGCAGTCT CAGCAGGTG			Formatted
N	<u>Silicon 1</u>	<u>0.1</u>	TGAAAGGTTCC	<u>94</u>	5 8	Formatted
			CAGCAACTGT			Formatted

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	Arsenate	reductases		A	CA	TCACAATCT
	CDC25-1	Tyrosine phosphatase	SapurV1A.0142s0310.1	ACGGCATCT	TAGGTCTGGTT	<u>97</u>
	CDC25-2	Turosine phosphatase	SapurV1A 0243c0430 1	TCAACTTTCA	CCACAGAAGACCT	147
	Thiol met	tabolism chelating response.	<u>Supur (111.02+530+50.1</u>	CACTAGTTG/	ACGAGCCAGGA	
acabata	-				ACGAAATGAGG	
sporter PHO1-	GR	Glutathione reductase		SapurV1A.0056s077	GCTGTGGGTT	126
protein	SapurV	1A.0063s0550.1 AGAGGCTGCGATGTTGAA	CA 11	5	CTGTGCGA	
				SapurV1A.1124s008	GCTGTCAAGTG CCCATCCAT	
	GS	Glutathione synthetase		0.1	CAGACTCCATA	91
					AGCCAGCGA	
DPHOT <mark>Flavonoi</mark>	d Synthesis	Director de al active accuration and		SapurV1A.0160s021	TTGCTGTAAGG	ant phosphate
	<u>61</u>			0.1	TGAGATGAAGG	157
					AACCAGCACA	
	GST	Glutathion S-transferase		SapurV1A.0016s107	GGAGATGA	120
	001			0.1	CCTCCCCACATT TTCCCTGG.	120
					CTTCGGTGCTGA	
	MTIA	Metallothionein-like protein		S. matsudana EF157299.1	CTGCTTTGTTGG	97
					GACCATGC	COTTOTTO
	Arsenic n	aetabolism-<u>Vacuolar transporters</u>			GA	CCATGC
					AGGCTTGGATT	
	<u>ABCG</u>	ABC transporter G		SapurV1A.0258s022 0.1	TGGCTGGTGGA	<u>94</u>
					TTGTTGTCA	
	CDC25				ACGGCATCTTTA	
	-1				GGTCTGGTT	
	Tyrosin e	Vacuolar cation/proton exchanger 2		SapurV1A.0142s031	TACGGCTCGGG	97-94
	phosph	<u></u>		0 <u>1071s0020</u> 1	TCTTGCAATCGT	
	atase <u>CAX2-1</u>				CGTCCACA	
					CAGCCAAGG	
	CDC25				TCAACTTTCACC	•
	Tyrosin			SapurV1A.0243s043	ACAGAAGACCT TTGTTGGTGCTT	
	e phosph	Vacuolar cation/proton exchanger 2		00338s0120.1	GGATGTGC	<u>147-142</u>
	atase				GCAGGACAGCA GGAAAGAG	•
	WBAB					-
	<u>CT</u> Arse			CACTAGTIGACG	GCAAGAGGTGG TAGGACTGT	
	transn	White-brown-complex ABC transporter		<u>SapurV1A.0084s002</u>	ACACCCATCCG	<u>96</u>
					ACAAAACCA	
	ort			0.1		
	ort related			<u>0.1</u>		
	ort related			0.1		
2 <mark>04 ∺</mark>	ert related	osphate transporter 4		0.1		
2 04 Hi	ort related	rosphate transporter 4		0.1		
104 Hi	ort related	iosphate transporter 4		0.1		
204 Hi	eff related	tosphate transporter 4		0.1		
1 04 Hi	ert related	iosphate transporter 4	CATTCCGTGGC	0.1	00 07.0%	0.007.1
2 0 4 Hi <u>L (Ch</u>	ert related gh affinity ph	105phate transporter 4 Se CAX2-1 SapurV1A:0820s0070	CATTCCGTGGC	CCTAGTGAC¥acuo xchanger 2	<u>20 26.9%</u> 49	<u>0.9974</u>
2 04 Hi <u>1 Ch</u>	eref related	wosphate transporter 4 Se <u>CAX2-1</u> Sapur V1A-0820s0070	CATTCCGTGGC	CCTAGTGAC Vacuo	<u>20 26.9%</u> 49	<u>0.9974</u>
₽ 04 Hi	ert related	10000000000000000000000000000000000000	CATTCCGTGGC	CCTAGTGAC Vacuo	<u>20 26.9%</u> 49	<u>0.9974</u>
204 Hi <u>7 Ch</u>	ert related	iosphate transporter 4 se <u>CAX2-1</u> SapurV1A.0820s0070	CATTCCGTGGC	CCTAGTGAC Vacuo	<u>90 96.9%</u> 49	<u>0.9974</u>
204 Hi	ere related	iosphate transporter 4 Se <u>CAX2-1</u> SapurV1A.0820s0070	CATTCCGTGGC	CCTAGTGAC Vacuo	<u>90 96.9%</u> 49	<u>0.9974</u> *
PO4 Hi	igh affinity ph	iosphate transporter 4 Se <u>CAX2-1</u> SapurV1A.0820s0070	CATTCCGTGGC	CCTAGTGAC Vacuo xchanger 2	<u>90 96.9%</u> 49	<u>0.9974</u> *
PO4 Hi	igh affinity pk	105phate transporter 4 Se CAX2-1 SapurV1A.0820s0070	CATTCCGTGGC	CCTAGTGAC¥acuo xchanger 2	<u>90 96.9%</u> 49	<u>0.9974</u>
₽04 Hi <u>1/ (Ch</u>	igh affinity pl	105phate transporter 4 See	CATTCCGTGGC	CCTAGTGAC Vacus	<u>90 96.9%</u> 49	<u>0.9974</u>
PO4 Hi	igh affinity pl	iosphate transporter 4 EAX2-1 SapurV1A.0820s0070	CATTCCGTGGC	CCTAGTGAC¥acus	<u>90 96.9%</u> 49	<u>0.9974</u>
₽04 Hi 1/	igh affinity pl	iosphate transporter 4 <u>EAX2-1</u> <u>SapurV1A.0820s0076</u>	CATTCCGTGGC	CCTAGTGAC¥acu6 xchanger 2	<u>90 96.9%</u> 49	<u>0.9974</u>

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Tolerance in Salix atrocinera

			CGGAGCCTACAATGAGAGCA			
CHS2CAX2-2	<u>Chalcone synthase 2</u> Vacuolar cation/proton exchanger 2 .	SapurV1A.0056s0660.1	AACTGCGAGCCACTAGACAC	.145	91.5%	0.9992
			AAAAGCACACCCCACTCCAA			
<u>CHS3</u> NIP1	Chalcone synthase 3	SapurV1A.0820s0080.1 Aquaporin NIP1.1	GCGGCCCAGACTATTCTACC	135	87.7%	0.9999
			AGCCTCGGTCAGACTCTTCT	A		
<u>F3H</u>	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	TCTTGTCGGAGGCTATGGGA TCGGTATGGCGTTTGAGTCC	<u>136</u>	<u>96.7%</u>	<u>0.999</u>
<u>FLH</u>	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT	<u>114</u>	<u>88.6%</u>	<u>0.9949</u>
<u>2HFLR</u>	Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC	<u>96</u>	<u>92.7%</u>	<u>0.9661</u>
FLS	Flavonol synthase	SapurV1A.1087s0040.1	<u>ACTCGCCAAATCCTCATCGA</u> <u>TCCCAACCCAGATTGTGTCG</u>	94	90.5%	0.9976
ANR	Anthoevanidin reductase	SapurV1A 0028c0/10.1	CAAATAGGCCCCACIGCGAA TTCCCAGCAGCGTAAACCTG	129	03.8%	0.0030
Ann	Anthocyantum reductase	<u>54pur v 1A.002030410.1</u>	<u>GGGCTCTGCAAACATCCTCT</u>	127	23.870	0.7750
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	<u>TGTTATGCACCTTGTCAACCATG</u> <u>TCCTGAAGCCTGATCGTTCG</u>	<u>127</u>	<u>95.8%</u>	<u>0.9823</u>
Stress related						
ACCSSIP1	1-aminocyclopropane-1-carboxylate	SapurV1A.2160s0020.1	<u>GCAGCACCAACTTTTGTCTCA</u>	115	102.3%	0.9982
WBABCT	White-brown-complex ABC transporter		GGGGTTGTTCGTAGGGTGAA			4
AIP-1ABCG	Arsenite-inducible RNA-associated transporter G-family, protein AIP-1-related		CTTGCCAGTTGAAGGTGTGC			
			ACAATCTTTTCCGTTCCTCAAGG			
SILICON ER	Ethylene receptor	SapurV1A.0052s0240.1 Silicon 1	TACCATACACCTGCCCACTG	90	120.0%	0.9870
BORON	Boron transporter		GTAGTAGAGGTACACGAACAGCA			4
<u>CSA</u>	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA TCCAGCAACAACTCCAACGA	<u>125</u>	<u>90.5%</u>	<u>0.9919</u>

GTCTGAAGCAAGGCGAGTCA

S	CAGCCACTTATCCCCAGCAA	124	04.5%	0.0873	
Sapur v 1A.013950200.1	TCAAGGCGAATAGAACCCGT	134	94.3%	0.9073	
	GAACGACGAGCACCTGGTT				
HQ228362.1	ACGGGTTCTATTCGCCTTGA	108	86.1%	0.9951	
5 NIA 1071 0020 1	TCTTGCAATCGTCGTCCACA	04	22 201	0.0054	
Sapurv 1A.10/150020.1	ACCTAAACGCTCAGCCAAGG	94	92.9%	0.9954	
SomueV14 0228c0120 1	TTGTTGGTGCTTGGATGTGC	142	102.40	0.9826	
3apur v 1A.033650120.1	GCAGGACAGCAGGAAAGAG	142	103.4%		
S	CAAGGTTGTGACTCTTCCAGGA	100	80.00/	0.0012	
Sapur v 1A.002930170.1	GACAGCAGGGTTGAAATGGG	100	89.9%	0.9912	
S	GCCAGTTCAGTACAAGCACATG	147	102.004	0.0014	
Sapur v 1A.105850060.1	TGCAGCAGAGGGTTTCGAG		103.9%	0.9911	
S	GCAAGAGGTGGTAGGACTGT	06	07.20	0.0070	
5apui v 1A.008450020.1	ACACCCATCCGACAAAACCA	70	77.3%	0.9979	
S	AGGCTTGGATTCTACAACTGCT	94	84.20/	0.0778	
Sapur v 1A.025880220.1	-58s0220.1 TGGCTGGTGGATTGTTGTCA		84.3%	0.9778	

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		SapurV1A.1225s0080.1 SapurV1A.0014s0200.1	GGTAGCAGTCTCAGCAGGTG TGAAAGGTTCCCAGCAACTGT TCATTCGGGGAACAACTGGAG ACTGTCGGCTCTGCAACTC	9. 1	4 4 3	85.2% 93.3%	0.9977 0.9805
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1005 -1	006—Table 1. Continued-				Format After: 0	ted: Indent: pt, Line spa	Left: 0 cm, Space cing: Double
Flavonoid Syn	thesis				Format (Spain, 1	ted: Font: B International	old, Spanish Sort)
CHS1	Chalcone synthase	SapurV1A.0820s0070.1	CATTCCGTGGCCCTAGTGAC CGGAGCCTACAATGAGAGCA	9	θ	96.9%	0.9974
			AACTGCGAGCCACTAGACAC				
CHS2	Chalcone synthase 2	SapurV1A.0056s0660.1	AAAAGCACACCCCACTCCAA	+	4 5	91.5%	0.9992
CHS3	Chalcone synthase 3	SapurV1A.0820s0080.1	GCGGCCCAGACTATTCTACC AGCCTCGGTCAGACTCTTCT	1	35	87.7%	0.9999
F3H	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	TCTTGTCGGAGGCTATGGGA	4	36	96.7%	0.999

Tolerance in Salix atrocinera

FLH	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGCTTCTGTTGCTTCTCA	114	88.6%	0.9949
			TGCAAACACAAGGTCCTGGT			
OHELD	Dibudroflavional 4 reductore	SamurV1A 0198-0260 1	GCCACCATTCACGATCTTGC	06	02.7%	0.0661
ZHITLK	Diriyuronavonor 4 reductase	3apul v 174.018880300.1	ACTEGECAAATECTEATEGA	90	92.170	0.9001
			TCCCAACCCAGATTGTGTCG			
FLS	Flavonol synthase	SapurV1A.1087s0040.1	CAAATAGGCCCCACTGCGAA	94	90.5%	0.9976
			TTCCCAGCAGCGTAAACCTG			
ANR	Anthocyanidin reductase	SapurV1A.0028s0410.1	GGGCTCTGCAAACATCCTCT	129	93.8%	0.9930
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG	127	95.8%	0.9823
			TCCTGAAGCCTGATCGTTCG			
As-stress relat	ted					
			GCAGCACCAACTTTTGTCTCA		100.000	
ACCS	1-aminocyclopropane-1-carboxylate synthase	SapurV1A.2160s0020.1	GGGGTTGTTCGTAGGGTGAA	++>	102.3%	0.9982
	Arsenite-inducible RNA-associated protein		CTTGCCAGTTGAAGGTGTGC			
AIP-1	AIP-1-related	SapurV1A.0229s0030.1	ACAATCTTTTCCGTTCCTCAAGG	140	93.2%	0.9940
ER	Ethylene receptor	SapurV1A.0052s0240.1	TACCATACACCTGCCCACTG	90	120.0%	0.9870
	× 1		GTAGTAGAGGTACACGAACAGCA			
Cell wall syntl	hesis					
CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA	125	90.5%	0.9919
			TECAGEAACAACTECAACGA			

1007

Tolerance in Salix atrocinera

1009 Table **21**. Nutrients (mg Kgkg⁻¹ DW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic

1010 conditions for 30 days. Different letters within each column and tissue indicate significant differences

1011 among treatments and time points on HSD test at p < 0.05.

				Nutrient (mg Kg	;- ⁺ - DW) ▲	 			
	_					 			
0	A			P	Ca	 		Zn	Fe
r		Time_(d	Treatm			 			
g			ent						V
a n			_			 			
<u> </u>	_					 			•
						 			W
Roots	.	l	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 ±	625.22 ± 29.	2364.78 ± 115.93 a
							1.23 a		
		2	01	5701 70 +	2071 75 + 245 56 h		1502.	420.07 + 22	1210 42 : 52 24 4
^	A	3	Control	<u>5/91.72 ±</u> 456.56 a	32/1./5 ± 245.50 0	 	15.85 ± 0.89 c	439.97 ± 23.	1219.43 ± 62.54 u
		•	As	5507.21 ±	4556.62 ± 342.45 a	 	19.46 ±	469.75 ± 30.	1354.59 ± 49.45 c ▲
				412.42 a		 	0.93 a		
							•		
	.	10	Control	3772.06 ± 235	3041.54 ± 289.87 c	 	15.53 ±	481.45 ± 32.	1087.50 ± 83.12 e
							0.92 b		
			4.0	2002 24 + 176	4257 52 ± 458 96 h		17 52 +	422.26 +	1207 72 + 60 32 6
^		-	As	2883.34 ± 170	4257.52 ± 458.90 0	 	17.55 ± 1.09 ab	432.30 ± 23.56 c	1281.13 ± 07.32 C
		30	Control	2729.45 ± 278	3093.95 ± 334.56 c		13.26 ±	330.76 ± 22.	943.27 ± 34.23 f
	-					 	1.01 d		
							•		
			As	1633.24 ± 99.1	4154.46 ± 354.98 a	 	14.38 ±	332.44 ±	1082.25± 50.54 e
							0.89 d	15.69 d	
							•		

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Changes in Non-1 Poten	i Thione Compounds and v	sene mansempts	Contributing to I	AI sellite accumulation and

Tolerance in Salix atrocinera

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	•						
	1			6227.33 ± 434.93 a			234.31 ± 12.45
		Control	2402.12		(100	(22 C2	
	^	Control	3403.13 ± 179.33 a		64.22 ± 6.73 ab	633.62 ± 40.93 a	
	A	As	3112.77 ± 143.54 ab	5290.13 ± 302.34 b	49.23 ±	544.83 ± 30.87 b	226.23 ± 10.15
			145.54 ab		5.54 00	50.07 0	
	3	Control-	3591.96 ±	5886.58 ± 478.23 b	49.31 ±	646.95 ±	382.15 ± 21.54
			123.43 a		3.53 bc	51.23 a	
		Δ.5	2942 52 +	6909 65 + 398 12 1	68 55 +	532.08 +	180.67 + 7.28
	^	715	174.23 b	6767.65 2 576.12 a	4.52 a	79.56 ab	100.07 ± 1.20
			<u> </u>				
•	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
					<u> </u>	<u> </u>	
	_	As	2046.62 ±	4603.75 ± 345.21 c	57.26 ±	516.58 ±	143.65 ± 18.23
			124.54 d		3.21 b	31.22 b	
	30	Control-	2035.46 ±	7001.72 ± 421.23 a	43.32 ±	429.35 ±	239.40 ± 12.12
			121.23 d		2.34 d	28.78 c	
		A.c.	A	5402 62 ± 224 12 b	60.67 +	400.02 +	152 47 + 12 52
	A	<u>~15</u>		<u>1472.03 ± 324.12 0</u>	<u>3.11 ab</u>	<u>33.21 c</u>	102.47 ± 10.02
			As				
			2106.37 ± 134.24 cd				
			5492.63 ±				
			(0.77.)				
			00.0/±				
			60.67 ± 3.11 ab				
			60.07 ± 3.11 ab 				
			$\frac{60.67 \pm}{3.11 \text{ ab}}$				
			00.07 ± 3.11 ab 400.92 ± 33.21 e				
			00.07± 3.11 ab 400.92± 33.21 e				
			400.92 ± 33.21 e				
			$\frac{400.92 \pm}{33.21 \text{ e}}$				

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Tolerance in Salix atrocinera

1014 -	$Γable \frac{32}{2}$. Arsenic accumulation (mg Kgkg ⁻¹ DW) in roots and leaves of <i>S. atrocinerea</i> exposed to	
	arsenic	

1015 for 30 days. Different letters within each column and plant tissue indicate significant differences-

among $\frac{1016}{1000}$ time points on HSD test at p < 0.05. nd: not detected.

1017

Time point (d) Amonia (ma Va DWA) 0-----1018 Or Arse <u>III</u> 16.14 ± 2.34 d <u>Total</u> 182.43 ± 20.10 d 164.88 ± 149.33 d Roots 33.45 ± 4.78 c $318.86 \pm 21.95 \ c$ 353.65 ± 23.98 c 10 929 ± 80.21 b 542.35 ± 41.29 b 1471.92 ± 123.87 b

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r -	Tolerance in <i>Salix a</i>	trocinera					
1019		30 ———	16	88 ± 148.43 a	734.90 ± 65.20 a	— 2448 ± 178.32 a	
						A	
1020				_			
1020	Leaves	. 1	nd.		2.78 ± 0.24 d	2.78 ± 0.24 d.	
		<u> </u>			2.76 ± 0.24 u	2.78 ± 0.24 u	
1021	<u>ــــــــــــــــــــــــــــــــــــ</u>	•	3 +	1.30	● 5.76 ±	● 7.23 ±	
		10	± 0).08 e	0.45 c 18.75 ± 1.14 b	0.39 c 18.75 ± 1.14 b	
1022		30 ———	- nd-		25.45 ± 2.57 a	25.45 ± 2.57 a	
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Tolerance in *Salix atrocinera*

Table 4. Arsenic accumulation (mg Kg3. Non-protein thiolic peptides (nmol GSH g⁻¹ DWFW) in roots and

leaves of S. atrocinerea exposed

1031 to control and arsenic

1032 <u>conditions</u> for 30 days. Different letters within each <u>columnrow</u> and plant tissue indicate significant.

differences among $\frac{1033}{\text{treatments and}}$ 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

	10	То 1 <mark>34</mark> —	olerance in <i>Sa</i>	lix atrociner	а							
<u>Org</u>	<u>an</u>	<u>Thiol</u>	<u>1 d</u>			<u>3 d</u>			<u>10 d</u>		<u>30 d</u>	
Org	m.	Thiol						-		3		
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Roo	ts	Суз	nd	5.85 ± 1.73 a		nd	19.38 ± 0.18 b		nd	7.99 ± 2.15 a	Nd	4.06 ± 0.15 🚭
•		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 🚭
<u>ــــ</u>		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-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-PC ₃	nd	8.88 ± 0.53 d	n	nd	34.35 ± 2.27 c	÷	nd	73.86 ± 4.27 b	Nd	150.19 ± 12-
				A	eł A		_	d		_		
•		Cys-PC ₃	nd	10.91 ± 0.71 d	e e	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 .1
Leav	res	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-PC ₂	nd	2.22 ± 0.31 d	n	nd	5.18 ± 1.03 c	Ħ	nd	7.65 ± 0.53 b	Nd	9.39 ± 0.88 #

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Tolerance in Salix atrocinera

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_	1		2.52 ± 0.10 d		3.06 ± 0.36 c		2.87 ± 0.14 c		
	desGly-PC4	2.50 ± 0.11 d		2.46 ± 0.14 d		2.74 ±0.12 cd		4.09 ± 0.52 b	6.46 ± 0.48
	l		.		_		_		
•	l		5.45 ± 0.23 a		6.25 ± 0.64 a		5.08 ± 0.70 ab		-
	TC ₃	4.48 ± 0.38 b		4.04 ± 0.49 b		4.04 ± 0.34 b		3.03 ± 0.18 c	3.95 ± 0.41 🖢
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			67.60 ± 1.45 a		69.58 ± 5.70 a		58.16 ± 2.50 b		-
	Total ΣNPTs	55.40 ± 1.89 bc		50.54 ± 3.45 c		53.56 ± 4.05 bc		54.70 ± 4.11 bc	62.51 ± 5.94
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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) \pm 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 (a = upregulation; b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time point are indicated by color (b = 0.05 As induced changes in expression relative to the non-exposed plants at each time p

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downregulation). Different letters within each column and plant tissue indicate significant differences among time points on HSD test at p < 0.05.








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

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Fig 3.-Biplots of the principal component analysis















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

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Graphical Abstract



Highlights

- *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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14 Highlights

- 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.
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21 Abstract

22 Despite arsenic (As) being very toxic with deleterious effects on metabolism, it can be tolerated and 23 accumulated by some plants. General genetic mechanisms responsible for As tolerance in plants, 24 including *Salix* species, have been described in transcriptomic analysis, but further experimental 25 verification of the significance of particular transcripts is needed. In this study, a Salix atrocinerea 26 clone, able to thrive in an As-contaminated brownfield, was grown hydroponically in controlled 27 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, 28 29 As accumulation, phytochelatin synthesis, non-protein thiol production and expression of target 30 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 31 32 weight in leaves. Roots reduce As V to As III rapidly, with As III being the predominant form of As 33 accumulated in root tissues, whereas in the leaves it is As V. After 1 d of As exposure, roots and 34 leaves show de novo synthesis and an increase in non-protein thiols as compared to the control. 35 Integrating these data on As accumulation in the plant and its speciation, non-protein thiol production 36 and the kinetic gene expression of related target genes, a fundamental role is highlighted for these 37 processes in As accumulation and tolerance in S. atrocinerea. As such, this study offers new insights 38 in the plant tolerance mechanisms to As, which provides important knowledge for future application 39 of high-biomass willow plants in phytoremediation of As-polluted soils.

40

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

41



60 1 Introduction

61 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} 62 soil. However, As soil concentrations may range from 0.1 to more than 1,000 mg kg⁻¹ in some 63 64 locations due to both anthropological and geological factors (Kabata-Pendias, 2010). Concerning its 65 toxicity, As is the only known human carcinogen for which there is adequate evidence of 66 carcinogenic risk for both exposure routes, inhalation and ingestion (Smith et al., 2009). Therefore, 67 As has been defined as a group 1 carcinogen and is placed in the highest health hazard category by 68 the international agency for research on cancer (Naidu et al., 2006). By the use of natural resources, 69 humans release As into the air, water and soil (Mandal and Suzuki, 2002). Sixty percent of the 70 anthropogenic As emissions can be accounted to only two sources: Cu-smelting and coal combustion. 71 Nevertheless, the application of herbicides, Pb and Zn smelting, glass production, wood preservation, 72 waste incineration and steel production are also responsible for As emissions (Matschullat, 2000). 73 According to the European Commission (2000), air contributes less than 1% of the total As exposure 74 since most of this emitted As ends up retained in the water and soils, making these the major sources 75 of As exposure to humans.

76 Once inside the cell, As toxicity depends on its speciation state. Arsenite (As III) has a high 77 affinity for sulfhydryl groups found in the amino acid cysteine. As such, it inactivates a wide range of 78 enzymes by disrupting protein structure and impairs the metabolism by preventing protein-protein 79 interactions (Ehlrich, 1990). This affects many key metabolic processes in the cell such as fatty acid 80 metabolism, glucose uptake and glutathione production (Paul et al., 2007; Ahsan et al., 2008; Wang 81 et al., 2015). Arsenate (As V) is a phosphate analogue and can substitute inorganic phosphate 82 affecting ATP synthesis and therefore interrupting the production of energy, carbon metabolism and 83 nucleic acid synthesis (Singh et al., 2011; Spratlen et al., 2017). This can also negatively affect DNA

84 repair and methylation and thus impact on gene expression (Reichard and Puga, 2010). Therefore, 85 removal or lowering of As concentrations from highly As-polluted soil and water is an environmental 86 priority. Among the most eco-friendly cleanup technologies and opposite to traditional excavation 87 and disposal in landfills, phytoremediation emerges. This green technology, already described more 88 than two decades ago by Raskin et al. (1994), exploits the ability of certain plants species to 89 accumulate metal(loid)s in their tissues, thus reducing their concentrations or attenuating their 90 mobility in the environment, and therefore offering a solution to the above-mentioned pollution 91 challenge (Pilon-Smits, 2005; Kidd et al., 2015).

92 It is well known that toxic metal(loid)s induce loss of plant biomass, among other deleterious 93 effects, mainly associated with growth inhibition (Gill et al., 2015). Plants differ in As tolerance, 94 from sensitive plant species like all major crops, to tolerant plants such as certain ecotypes of the 95 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). 96 97 However, hyperaccumulator species are usually limited by a low biomass production, which may 98 pose serious restrictions to this cleaning procedure (Shelmerdine et al., 2009, Fernández et al., 2010). 99 Some plant species and soil biota populations, usually autochthonous to polluted soils, are able to 100 colonize and thrive in highly polluted environments, even when high concentrations of metals are 101 found in their cells and tissues. This is the case of Salix atrocinerea (grey willow). So far, about 450 102 species of Salix worldwide have been described (Argus, 1995), with some of them reported as 103 suitable in phytoremediation processes because of their high growth rate and deep-rooting traits 104 (Kuzovkina and Quigley, 2005; Janssen et al., 2015). Nevertheless, the focus on the use of Salix for 105 As uptake is still low because it is not a metal(loid) hyperaccumulating species. However, some 106 investigations have highlighted its phytoremediation potential for As (Purdy and Smart, 2008; 107 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).

111 Understanding As tolerance in plants is useful to know whether plants avoid As uptake and, 112 thus, reduce the As intake by humans and the As-associated health problems (Song et al., 2010), or 113 enhance As uptake and its removal by phytoremediation (Yang et al., 2012). To achieve this, it is 114 necessary to study the As behavior from the soil to its accumulation in the aboveground plant tissues. 115 Although As is toxic and not essential for plants it is effectively absorbed through various 116 transporters into the roots, mainly as As V, the most thermodynamically stable and hence dominant 117 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; 118 119 LeBlanc et al., 2013), while As III uses the silicon transporters (Xu et al., 2015; Lindsay and 120 Maathuis, 2016). Once inside the plant cells, a small amount may be transported to the xylem but the 121 majority is reduced to As III by arsenate reductases (Ellis et al., 2006; Duan et al., 2007; Zhao et al., 122 2009). In this form, As can be exported back into soil, transported via the xylem to stem and leaves, 123 or complexed with thiol-rich molecules like metallothioneins (MTs), glutathione (GSH) or, more 124 stably, by phytochelatins (PCs) (Schmöger et al., 2000; Hartley-Whitaker et al., 2001; Dave et al., 125 2013; Batista et al., 2014). Then these As-PCs complexes can subsequently be transferred from the 126 cytosol into the vacuole by ABC transporters for storage in order to prevent cell damage (Song et al., 127 2010). Therefore, this suggests that non-protein thiols (NPTs) compounds play an important role in 128 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 132 approach concerning the tolerance mechanisms of *Salix* to As is needed. Besides, special attention 133 needs to be paid to the speciation state of As, since this determines its uptake and also its tolerance by 134 the plant. In the current study, a S. atrocinerea clone, previously selected for its As accumulation 135 (unpublished data), was grown hydroponically in the presence of As V. Samples were harvested at 136 different time points to kinetically study As accumulation and its chemical speciation in roots and 137 shoots. In addition, the production of NPTs as well as the expression of the main transcripts involved 138 in the genetic response behind As tolerance were also measured. Therefore, this study aims to 139 describe the As uptake and accumulation in S. atrocinerea, together with the changes in the 140 mechanisms involved in As tolerance at different biological organization levels.

141

142 2 Material and Methods

143

144 **2.1 Plant material and hydroponic culture conditions**

145

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, Na₂HAsO₄.7H₂O, 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 μ mol 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.

163

164 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 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 filtered through a 0.45 μ m PTFE membrane filter. The solutions were neutralized by the addition of NaOH. The 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 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).

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 $(250 \times 4.6 \text{ mm})$ column (Scharlau) and eluted with solvent A (acetonitrile: H₂O, 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 5 min) of solvent B at 1.5 mL min⁻¹ flow for 30 min. The derivatized thiols were detected at 412 nm 190 191 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**

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 μ L of buffer containing 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g L⁻¹ spermidine and 2% β201 mercaptoethanol. Then, it was extracted twice by adding 550 µL of chloroform: isoamyl alcohol 202 (24:1) and centrifuged at 14,000 g for 20 min at 4 °C. After addition of 10 µL LiCl (10 M), RNA was 203 precipitated overnight at 4 °C and harvested by centrifugation at 14,000 g for 20 min at 4 °C. The 204 pellet obtained was washed with 75% ethanol and resuspended in RNase free water. The 205 concentration of RNA was determined spectrophotometrically at 260 nm using Nanodrop equipment 206 (Isogen Life Science) and the RNA quality was tested using the Experion[™] automated 207 electrophoresis system (Bio-Rad). DNA was removed using a TURBO DNA-free Kit (Ambion) and 208 the cDNA synthesis was performed using PrimerScript RT reagent Kit (Takara) with equal amounts 209 of RNA input (1 μ g). Finally, the cDNA was ten-fold diluted using a 1/10 dilution of TE (Tris-210 EDTA) buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C.

211 Reverse Transcription quantitative PCR (RT-qPCR) was performed with an ABI Prism 212 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. 213 Gene forward and reverse primers (Supplementary Table 1) were designed using Primer 3 214 (Untergasser et al., 2012), according to sequences of genes obtained in the *Phytozome* nucleotide 215 database of the closely related species Salix purpurea v1.0, for which the whole genome has been 216 sequenced (Goodstein et al., 2012), only for three genes, high-affinity phosphate transporter 217 (HAPO4), arsenite-inducible RNA-associated protein (AIP-1) and metallothioneins (MT1A), their 218 sequences were obtained from willow reference sequences annotated at the NCBI (National Center 219 for Biotechnology Information) (O'Leary et al., 2016). Their specificity was verified in silico using 220 BLAST (http://www.arabidopsis.org/Blast/index.jsp). The genes measured (*Supplementary Table 1*) 221 were selected based on different genetic aspects behind As tolerance (Konlechner et al., 2013; 222 Puckett et al., 2012; Yanitch et al., 2017). The qPCR efficiency of the primers was determined using 223 a standard curve consisting of a two-fold dilution series of a pooled sample. Only primers with an 224 efficiency between 90 and 110% were used for qPCR analysis and their amplification specificity was

225 validated by melting curves. PCR amplifications were done in a total volume of 10 μ L containing 226 2 µL cDNA sample, 5 µL SYBR Green, 0.6 µL of primers (300 nM) and 2.4 µL RNase free water. 227 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 228 229 quantification cycle (Cq) value minus the minimum Cq value observed (Schmittgen, 2008). Gene 230 expression was normalized with a normalization factor based on the expression of six reference genes 231 from Salix selected from literature under As and other abiotic stresses (Li et al., 2016; Zhang et al., 232 2017). The 6 selected candidate reference genes, α -TUB2, Alpha-tubulin 2; ACT7, Actin 7; ARF2, 233 ADP-ribosylation factor 2; DNAJ, Chaperone protein DnaJ 49; EF1a, Elongation factor 1-alpha 234 and OTU; OTU-like cysteine protease (Supplementary Table 1) are also orthologs of genes in S. 235 purpurea. The primer sequences, amplicon length, PCR amplification efficiency and correlation 236 coefficient are shown in Supplementary Table 1. To evaluate the stability of the 6 candidate 237 reference genes (RG) at the transcript level under As exposure, the gene expression levels were 238 determined by the average Cq values (Supplementary Fig. 1). In order to detect the stabilities of 6 239 candidate RGs, the best combination of RG for normalization of our transcripts of interest was 240 suggested by the Graynorm algorithm (Remans et al., 2014). In roots AFR2, OTU and EF1 α were the 241 three most stable reference genes in all the sample sets according to the GrayNorm algorithm and the 242 combination of the three was used for normalization (Supplementary Fig. 1A). In leaves a different 243 combination of genes than that obtained in roots showed the most stable pattern in all the sample sets, 244 and therefore a combination of α -TUB2, OTU and ACT7 was used for normalization (Supplementary 245 Fig. 1B). In both roots and leaves the suggestion selected by GrayNorm corresponded to the genes 246 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.

249

250 2.5 Statistical analysis

251 To evaluate the effects of As toxicity in S. atrocinerea over the different time points on the 252 measured variables, depending on the number of variables to compare a one-way or a two-way 253 Analysis of variance (ANOVA) was performed. Log transformation was applied to approximate 254 when it was necessary (e.g. to determine statistical significance of gene expression data, datasets 255 were first log-transformed). Data normality was tested using the Shapiro-Wilk test, while 256 homoscedasticity was verified via Bartlett's and Levene's tests. If data did not meet the normality 257 assumption, a non-parametric Kruskall-Wallis test was used, followed by the Wilcoxon rank sum 258 test. When the F ratio was significant ($p \le 0.05$), Tukey's least significant difference test (HSD, $p \le 0.05$). 259 0.05) was employed to compare between individual means of different data groups (e.g. different 260 treatments). In the gene analysis the previous was performed on both the normalized and the non-261 normalized data, although only the first are presented both were taking into account to establish the 262 significance of the results. Results are expressed as the mean \pm standard deviation of at least three 263 independent replicates. All data were analyzed using R (version 3.3.1, http://www.r-project.org/) 264 with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae 265 (version 1.2e4, http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the 266 Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level 267 $p \le 0.05$.

268

269 **3 Results**

270 **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**).

274 With regard to nutrient concentrations, total P concentration significantly decreased in roots 275 from 10 d onwards in As-exposed plants as compared to controls, whereas in leaves the P 276 concentration was lower in As-exposed plants as compared to controls at 3 and 10 d. However, P 277 concentration in leaves was similar in both treatments after 30 d of exposure (Table 1). 278 Accumulation of Ca increased in As-exposed roots along the exposure time when compared to 279 control conditions. However, in leaves a Ca decrease was observed in As-exposed plants, except at 3 280 d (**Table 1**). Although the B concentration was slightly higher in roots of As-exposed plants, this 281 increase was only significant at 3 d. In leaves, an increase in B concentration at 3 d was observed and 282 this increase was maintained in As-exposed plants till the end of the experiment. For Zn 283 concentrations, there was an increase in roots and a decrease in leaves as compared to control 284 conditions at 1 d of As exposure. However, no differences were observed in for this elements at other 285 time periods. Fe concentrations were higher in roots of As-exposed plants throughout the experiment, 286 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**).

290

291 **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).

In plant tissues, the As was detected as As III or As V, but no As methylated species were observed (**Table 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 As form (**Fig. 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. 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 PC₂, 314 Cys-PC₂, PC₃, desGly-PC₃, Cys-PC₃ 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-PC₄, and TC₃ were detected 317 (Table 3), whereas under As exposure we observed *de novo* synthesis of desGly-PC₂ 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-PC₂, 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**).

325 **3.4 Gene expression**

326 In general, the gene expression pattern in roots between control and As-treated samples differed 327 due to the prominent regulation of transcripts related to As transport, As V reduction to As III, thiol 328 metabolism and vacuolar transports. In this way changes were observed in transcripts coding for the 329 phosphate transporter (PHO1), aquaporins (NIP1, SIP1 and SILICON), boron transporter (BORON), 330 As V reductase CDC25-like tyrosine phosphatase (CDC25-1), glutathione synthase (GS) and ABC 331 transporter (WBABCT) (Fig. 3). This differential regulation was also accompanied by changes in 332 transcripts for As stress-related proteins like cellulose synthase (CSA), arsenite inducible protein 333 (AIP-1) and aminocyclopropane-1-carboxylate synthase (ACCS). In leaves, regulation of the 334 transcripts measured was not so noticeable as in roots and differences in gene expression between 335 control and As-exposed plant were due mainly to the overexpression of ACCS (Supplementary Fig. 336 2). A heat map representation of the other transcripts measured in this study with a fold regulation 337 lower than two can be found for both roots (Supplementary Fig. 3A) and leaves (Supplementary 338 *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

for 1 d showed the highest gene expression for *GS*, *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. 4A**).

In leaves, according to PCA component 1, the differential gene expression collected from plants growing under 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. 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 366 hydroponic cultures should include As doses in the range of $0 - 10 \,\mu\text{M}$ to allow the extrapolation of 367 the results to As-polluted soils (Moreno-Jimenez et al., 2010). Nonetheless, the fact that S. 368 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 369 370 assay. Furthermore, this As concentration matches that recommended in previous hydroponic studies 371 with willow (Purdy and Smart, 2008) and it has already been used in analyzing differential As gene 372 expression under hydroponic conditions (Puckett et al., 2012). Although some authors have reported 373 that willows have the capability to translocate As from roots to above ground tissues (Tlustoš et al., 374 2007; Puckett et al., 2012; Sylvain et al., 2016) the As accumulation in leaves does not reach those 375 quantities present in hyperaccumulating species like *Pteris vittata* (Caille et al., 2004), as it was our 376 case, but the phytoremediation potential is compensated with a higher biomass (Meers et al., 2007, 377 Witters, 2009). Furthermore, after 30 d of As exposure, S. atrocinerea did not show any phytotoxic 378 symptoms and was capable of accumulating a higher As concentration than that present in the culture 379 medium, showing therefore a bioaccumulation factor higher than 1, which is an added value for the 380 phytoremediation of As.

381 It has been reported that exposure to toxic metalloids, such as As, can disturb the nutrient 382 profile of the plant and hence lead to toxicity (Lou et al., 2010), and also that As V uptake and 383 tolerance to its induced toxicity is intimately linked to phosphate nutrition. In the soil, As is mainly 384 present in its As V form (Cordos et al., 2006) and once it is in contact with the roots, As V can enter 385 via phosphate transporters (Maciaszczyk-Dziubinska et al., 2012). Therefore, changes in transcripts 386 encoding for As V-related transporter proteins could be expected and, in our case, the As V added to 387 the hydroponic solution caused a differential regulation of transcripts for phosphate transporters. The 388 up-regulation of PHO1 in roots of S. atrocinerea from the onset of the As exposure (Fig. 3) and that 389 observed at 10 and 30 d of transcripts encoding for a high-affinity phosphate transporter protein 390 (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. 3B), 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 415 and reducing As uptake (Rahman et al., 2015). In multiple studies, it has been shown that 416 micronutrient accumulation is affected by As exposure, but it can also have an impact on As uptake 417 and hence As toxicity (Srivastava et al., 2017). It has been proposed that B channels might have a 418 role in As transport into the cell (Yanitch et al., 2017), which is also reflected in our results with 419 increased BORON transcript levels in roots (Fig. 3) and leaves (Supplementary Fig. 3B). 420 Furthermore, boric acid transporter NIP5.1 from Arabidopsis is also permeable to As III (Mitani-421 Ueno et al., 2011), and our data showed that B accumulation in plant tissues changes along the time 422 of exposure to As (Table 1), with BORON transcripts 5-fold down-regulated at 1 d (Fig. 3), when As 423 III concentration in the medium was the highest (Fig. 2B). In leaves, BORON transcripts are induced 424 at 1 and 3 d in response to As (Supplementary Fig. 3B), coinciding at 3 d with the highest B 425 concentration (Table 1). Whereas Zn is described as an indispensible micronutrient, which mitigates 426 As toxicity by modulating ROS and the antioxidant function in plants (Das et al. 2016) or by 427 improving the thiol metabolism (Srivastava and Srivastava, 2017), no major changes were detected in 428 Zn concentration apart from the increase at 1 d in As-exposed plants. With regard to Fe, our data 429 showed that Fe translocation to leaves was more affected by As than any of the other elements, with 430 an increased Fe concentration in roots exposed to As, whereas in leaves it decreased (Table 1). 431 Shaibur et al. (2008) described that one of the symptoms of As toxicity is the formation of Fe plaques 432 in roots and, as also seen in our case, Fe:P ratios in the roots of the As-exposed plants were higher 433 than those observed in the control roots. This suggests that, in the liquid culture medium, As may 434 have been adsorbed with Fe on the surface of the roots, forming Fe-As plaques. Thus, the iron plaque 435 formed on the root surface will act as a natural As barrier and reduce As uptake by the plant and its 436 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

440 observed (Fig. 2B). This chemical reduction can be attributed to metabolic activities of S. atrocinerea 441 since no speciation was detected when the plant cuttings were not present in the culture medium. For 442 this observation, two possible explanations can be given. On one side, plants might induce changes in 443 the pH and in the redox potential of the culture medium as it was observed in this study (Fig. 2A), 444 and those changes might affect the speciation of As. For an example, it has been proposed that 445 protons released from organic acids (R-COOH) and excreted by plant roots may contribute to the 446 reduction of As V to As III, while increasing the pH as the process consumes H^+ (Park et al., 2016). 447 Interestingly, only As V was detected after 30 d and it matched with the highest pH value in the 448 medium; whereas the highest As III concentration was detected at 1 d, when the pH was the lowest 449 (Fig. 2A). Therefore, another possible explanation for the presence of As III in the medium is a direct 450 efflux of As III from the plant to the medium that can be linked to the proton gradient across the 451 plasma membranes or dependent of the plant metabolism (e.g. direct As III from plant cells to the 452 medium) (Xu et al., 2007; Park et al., 2016). Taking transcriptional regulations into account, since 453 willow plants were able to induce the occurrence of As III in the medium, differences in transcript 454 levels of genes encoding for As III transport were expected in roots of As-exposed plants.

455 In our case, we observed a noticeable up-regulation of the transcripts encoding the aquaporin 456 NIP1.1, reported for As III uptake into the roots (Ma et al., 2008), and in transcripts for SIP1 at 1 d of 457 As exposure (Fig. 3). This up-regulation diminishes over time, probably as a consequence of a very 458 active As V reduction to As III during the first days of exposure, with a lot of free As III initially in 459 the cytoplasm. This transcript up-regulation for As III transporters in roots, suggests that As V 460 reduction has occurred even before its entry into the roots, which is supported by the presence of 4% 461 of As III in the medium at 1 d of exposure (Fig. 2B), and which coincides with the highest up-462 regulation of NIP1 and SIP1. In leaves, where As was mainly present as As V, no changes in 463 transcript levels for the aquaporin transcripts were observed (Supplementary Fig. 3B).

464 Once inside the cell, since As V has no affinity for the "-SH" groups in the PCs, the first step in 465 As detoxification is As reduction (Finnegan and Chen, 2012). The main mechanism for As V 466 reduction is the presence of As V reductases where GSH acts as electron donor (Dhankher et al., 467 2002). Arsenate reductases are believed to have evolved from the CDC-25 (cell division cycle) dual-468 specificity tyrosine phosphatases (Duan et al., 2007). Based on homology with the yeast As V 469 reductase, ACR2P, Bleeker et al. (2006) identified a CDC25-like plant candidate and showed that it 470 had arsenate reductase activity like it was also observed in other assays (Dhankher et al., 2006). In 471 our study, As V reduction to As III was observed in roots right after As uptake with an increasing As 472 III concentration in root tissues from 9% to 70% by the end of the study (Fig. 2C). This coincides 473 with the *CDC25-1* up-regulation in roots of plants exposed to As (**Fig. 3**), whereas no changes were 474 observed for CDC25-2 (Supplementary Fig. 3A).

Another mechanism to reduce As V in the plant is through a non-enzymatic reduction, where 475 476 GSH is implied, but this process is relatively slow, so according to the NPT data we can attribute the 477 large up-regulation observed in GS transcripts along the As-exposure time to PC production as a 478 detoxification mechanism. This was reflected by the increased NPT concentrations in S. atrocinerea 479 roots after As uptake (Table 3). Our results showed that although there was a clear up-regulation of 480 GS transcripts in roots (Fig. 3), GSH concentrations of As-exposed plants remained constant over 481 time and lower than those in the roots of control plants (Table 3). However, since PCs use GSH as a 482 building substrate, the decreasing concentrations of GSH are consistent with its use in PCs or other 483 NPTs. This fast increase in NPT concentrations and the As III presence in the roots, support our 484 observations of As speciation in the medium; where it seems that As III efflux by roots occurs right 485 after As V uptake and that this efflux diminishes once the As III is complexed with thiols and stored 486 in the vacuoles. Therefore, an increase in NPTs under increased As III presence points towards an 487 As-PC complex formation possibly leading to less As III efflux. In support to this explanation, Raab 488 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.

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 As exposure resulted in the expression of many unknown thiol products, whereas cadmium induced higher increases in traditional PCs (PC₂, PC₃, PC₄).

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-PC₂ and the increase 515 of desGly-PC₄ 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. 3B) 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.

Once As V is reduced to As III and complexed to NPTs to limit its toxicity, these complexes are taken up by ABC transporters and stored in the vacuole. 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 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. 3B).

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.

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. 3A,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
562 et al., 2001). In our experiment the highest up-regulation was observed in roots at 1 d after exposure 563 and it decreased over time (Supplementary Fig. 2). Since this protein is As III-induced, and As III 564 concentrations increase over time in roots, this suggests an effective complexation of As III from 1 d 565 on with NPTs to prevent its toxicity. Another As-related response of willow is the biosynthesis of 566 phenylpropanoids that may culminate with the increased production of tannins (Yanitch et al., 2017) 567 and might be an important stress defense mechanism in leaves. In our study, we observed an early 568 up-regulation of selected genes (CHS1, CHS2, 2HFLR and F3H) in the flavonoid pathway at 1 d after 569 As exposure (Fig. 4 B and Supplementary Fig. 3B), whereas no major changes in the expression 570 pattern or a down-regulation were noticed later on as compared to control conditions. Therefore, by 571 the information provided by the transcript levels, we suggest that S. atrocinerea relies on the 572 phenylpropanoid pathway to cope with As toxicity during the early times of exposure, but further 573 investigation at metabolic level is essential.

574

575 **5 Conclusions**

576 The selected S. atrocinerea clone naturally growing in an As-contaminated brownfield showed 577 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 578 579 reveal that under the presence of As V in hydroponic conditions, willow plants show a transcriptional 580 regulation of genes involved in nutrient transporters, As V reduction, glutathione synthesis and 581 sequestration of As into the vacuoles, together with genes involved in stress responses, which 582 coincides with a rapid As III presence and accumulation in root tissues, altered nutrient profile and de 583 *novo* synthesis and increase of NPT compounds, all of which contribute to the tolerance to the 584 metalloid by S. atrocinerea.

585 The high As accumulation together with a high biomass yield makes this willow species a 586 potential tool for its use in As phytoremediation. Overall, a better understanding of the physiological 587 mechanisms of tolerance to arsenic toxicity in S. atrocinerea was achieved through this study by 588 experimental verification of the significance of particular transcripts complemented by an integrative 589 analysis of nutrient profile, As accumulation and speciation, as well as NPT compounds synthesis. 590 However, according to our observations, further research should also focus on what happens in real 591 polluted soils where, apart from As, there are usually other metal(loid)s at high concentrations that 592 can affect the plant detoxification responses.

593

594 6 **Conflict of Interest**

595 The authors declare that the research was conducted in the absence of any commercial or financial 596 relationships that could be construed as a potential conflict of interest.

597 7 **Author Contributions**

598 AN: Conceptualization, Investigation, Original draft; SH: Review; AC: Methodology, Formal 599

analysis, Validation, Resources, Review and Editing; AG: Resources, Review and Editing.

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608 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.
- 637 Catarecha, P., Segura, M.D., Franco-Zorrilla, J.M., García-Ponce, B., Lanza, M., Solano, R., Paz638 Ares, J., Leyva, A. (2007) A Mutant of the Arabidopsis Phosphate Transporter PHT1;1
 639 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.

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

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

- 647 Cordos, E. A., T. Frentiua, M. Pontaa, I. Marginean, B. Abrahamb and C. Roman (2006).
 648 Distribution study of inorganic arsenic (III) and (V) species in soil and their mobility in
 649 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.
- 665 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.297330.
- 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.
- 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-dependent protein kinase CPK31 interacts with
 arsenic transporter AtNIP1;1 and regulates arsenite uptake in Arabidopsis 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 Element-Contaminated Soils. International Journal of Phytoremediation 17, 10051037.
- 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, 170180.
- 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.
- 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.
- 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.
- Maciaszczyk-Dziubinska, E., Wawrzycka, D., Wysocki, R. (2012) Arsenic and Antimony
 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.
- 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.
- 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, 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.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.
- 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
 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, 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.
- 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.
- 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 non-hyperaccumulator metallophytes. J Exp Bot 53, 2381-2392.
- 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). 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 RNAassociated 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 ParticleAssociated 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.
- 2hao, F.J., Ma, J.F., Meharg, A.A., McGrath, S.P. (2009) Arsenic uptake and metabolism in plants.
 New Phytol 181, 777-794.
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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 r	oots, 2: used to normalize gene ex	pression 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
$EF1\alpha^{I}$	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 transpo	rt					
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 transpor	rt					
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 TGCAGCAGAGGGGTTTCGAG	147	103.9%	0.9911
SILICON	Silicon 1	SapurV1A.1225s0080.1	GGTAGCAGTCTCAGCAGGTG TGAAAGGTTCCCAGCAACTGT	94	85.2%	0.9977
Arsenate reducta	ses					
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 re	sponse					
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 transpo	rters					
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

Flavonoid Synthe	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) is	n roots and leaves of	f S. atrocinerea exposed to a	control ar	nd arsenic	

941 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)	Traatmont	Nutrient	Nutrient					
Organ	Time (u)	Heatment	Р	Ca	В	Zn	Fe		
Roots	1	Control	$5757.89 \pm 430.12 \ a$	$4322.53 \pm 207.32 \; b$	$19.62 \pm 1.12 \text{ a}$	$512.35 \pm 53.12 \; b$	$1955.93 \pm 98.36 \ b$		
		As	$5720.65 \pm 379.34 \; a$	$4929.41 \pm 242.98 \ a$	$21.98\pm1.23\ a$	$625.22 \pm 29.75 \; a$	$2364.78 \pm 115.93 \ a$		
	3	Control	$5791.72 \pm 456.56 \; a$	$3271.75 \pm 245.56 \ b$	$15.83\pm0.89\ c$	$439.97 \pm 23.45 \ bc$	$1219.43 \pm 62.34 \ d$		
		As	$5507.21 \pm 412.42 \ a$	$4556.62 \pm 342.45 \ a$	$19.46 \pm 0.93 \ a$	$469.75 \pm 30.45 \ bc$	$1354.59 \pm 49.45 \ c$		
	10	Control	$3772.06 \pm 235.67 \ b$	$3041.54 \pm 289.87 \ c$	$15.53\pm0.92\ b$	$481.45 \pm 32.34 \ bc$	$1087.50 \pm 83.12 \ e$		
		As	$2883.34 \pm 176.34 \ c$	$4257.52 \pm 458.96 \ b$	$17.53\pm1.09\ ab$	$432.36 \pm 23.56 \ c$	$1287.73 \pm 69.32 \text{ c}$		
	30	Control	$2729.45 \pm 278.45 \ d$	$3093.95 \pm 334.56 \ c$	$13.26\pm1.01\ d$	$330.76 \pm 22.34 \ d$	$943.27 \pm 34.23 \; f$		
		As	$1633.24 \pm 99.83 \ e$	$4154.46 \pm 354.98 \ a$	$14.38\pm0.89\ d$	$332.44 \pm 15.69 \; d$	1082.25± 50.54 e		
I									
Leaves	1	Control	3403.13 ± 179.33 a	6227.33 ± 434.93 a	$64.22 \pm 6.73 \text{ ab}$	$633.62 \pm 40.93 \text{ a}$	234.31 ± 12.45 b		
		As	$3112.77 \pm 143.54 \text{ ab}$	$5290.13 \pm 302.34 \ b$	$49.23\pm5.34\ bc$	$544.83 \pm 30.87 \; b$	$226.23 \pm 10.15 \; b$		
	3	Control	3591.96 ± 123.43 a	$5886.58 \pm 478.23 \ b$	$49.31\pm3.53\ bc$	$646.95 \pm 51.23 \; a$	382.15 ± 21.54 a		

ĺ		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	$6378.05 \pm 403.23 \text{ ab}$	$47.97 \pm 2.23 \text{ c}$	$503.68 \pm 23.13 \text{ b}$	$250.83 \pm 10.23 \text{ b}$
		As	$2046.62 \pm 124.54 \text{ d}$	4603.75 ± 345.21 c	$57.26\pm3.21\ b$	$516.58 \pm 31.22 \ b$	$143.65 \pm 18.23 \ d$
	30	Control	$2035.46 \pm 121.23 \text{ d}$	7001.72 ± 421.23 a	$43.32 \pm 2.34 \ d$	429.35 ± 28.78 c	$239.40 \pm 12.12 \; b$
		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
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951	Table 2. Arsenic accumulation (mg kg ⁻¹ DW) in roots and leaves of S. atrocinerea exposed to arsenic
952	for 30 days. Different letters within each column and plant tissue indicate significant differences
953	among time points on HSD test at $p < 0.05$. nd: not detected.

	Organ	Time point (d)	Arsenic		
			III	V	Total
055	Roots	1	16.14 ± 2.34 d	164.88 ± 149.33 d	$182.43 \pm 20.10 \text{ d}$
933		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	$1471.92 \pm 123.87 \text{ b}$
		30	1688 ± 148.43 a	734.90 ± 65.20 a	$2448 \pm 178.32 \; a$
056	Leaves	1	nd	2.78 ± 0.24 d	2.78 ± 0.24 d
50		3	$1.30 \pm 0.08 \text{ e}$	$5.76 \pm 0.45 \text{ c}$	$7.23 \pm 0.39 \text{ c}$
		10	nd	$18.75 \pm 1.14 \text{ b}$	$18.75 \pm 1.14 \text{ b}$
		30	nd	25.45 ± 2.57 a	25.45 ± 2.57 a
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970	Table 3. Non-protein thiolic peptides (nmol GSH g^{-1} FW) in roots and leaves of <i>S. atrocinerea</i>
971	exposed
972	to control and arsenic conditions for 30 days. Different letters within each row and plant tissue
973	indicate significant differences among treatments and time points on HSD test at $p < 0.05$. nd: not
974	detected.
975	

		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
	TC1	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 SNPTs	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
	TC₃	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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Fig. 1. Dry weight (mg DW) of roots and leaves of *S. atrocinerea* exposed to control and 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.



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 (\pm 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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1016 Fig 4. Biplots of the principal component analysis (PCA) in samples of roots (A) and leaves (B) of S. 1017 atrocinerea exposed to arsenic for 30 days calculated with the normalized gene expression levels 1018 relative to the non-exposed plants at each time point of at least three biological replicates, each 1019 containing at least one individual plant. 2HFLR, dihydroflavonol 4-reductase; ABCG: ABC 1020 transporter G; ACCS, aminocyclopropane-1-carboxylate synthase; AIP-1, arsenite-inducible RNA-1021 associated protein AIP-1-related; ANR, anthocyanidin reductase; BORON, boron transporter; CAX2-1022 2, vacuolar cation/proton exchanger 2; CDC25-1, 2, tyrosine phosphatase 1, 2; CHS1,3, chalcone 1023 synthase 1,3; CSA, cellulose synthase A; F3H, flavanone 3-hydroxylase; FLH, Flavonoid 3'-1024 hydroxylase; GR, glutathione reductase; GS, glutathione synthetase; HAPO4, high-affinity phosphate 1025 transporter 4; MT1A, Metallothionein; NIP1, aquaporin NIP1.1; PCS, phytochelatin synthase; PHO1, 1026 phosphate transporter PHO1; SIP1, aquaporin SIP.1; WBABCT, white-brown-complex ABC 1027 transporter.









Supplementary Fig. 2. Relative fold change of the gene expression levels in roots of *S. atrocinerea* 1058 exposed to As regarding those genes involved in stress response that showed the most markedly 1059 regulation along the 30 days. Values represented are the fold change (\pm S.D.) of mean normalized 1060 expression relative to the non-exposed plants at each time point of at least three biological replicates, 1061 each containing at least one individual plant. *ACCS*, aminocyclopropane-1-carboxylate synthase; 1062 *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*.



DW Roots DW Leaves













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.

Oncon	Time (d)	Tractment	Nutrient					
Organ	Time (d)	Treatment	Р	Ca	В	Zn	Fe	
Roots	1	Control	$5757.89 \pm 430.12 \text{ a}$	$4322.53 \pm 207.32 \ b$	19.62 ± 1.12 a	$512.35 \pm 53.12 \ b$	$1955.93 \pm 98.36 \ b$	
		As	5720.65 ± 379.34 a	$4929.41 \pm 242.98 \ a$	$21.98 \pm 1.23 \ a$	625.22 ± 29.75 a	2364.78 \pm 115.93 a	
	3	Control	$5791.72 \pm 456.56 \ a$	$3271.75 \pm 245.56 \ b$	$15.83\pm0.89\;c$	$439.97 \pm 23.45 \ bc$	$1219.43 \pm 62.34 \ d$	
		As	$5507.21 \pm 412.42 \ a$	$4556.62 \pm 342.45 \ a$	$19.46 \pm 0.93 \ a$	$469.75 \pm 30.45 \ bc$	$1354.59 \pm 49.45 \ c$	
	10	Control	$3772.06 \pm 235.67 \ b$	$3041.54 \pm 289.87 \text{ c}$	$15.53\pm0.92\ b$	$481.45 \pm 32.34 \ bc$	$1087.50 \pm 83.12 \; e$	
		As	$2883.34 \pm 176.34 \ c$	$4257.52 \pm 458.96 \ b$	$17.53\pm1.09\ ab$	$432.36 \pm 23.56 \; c$	$1287.73 \pm 69.32 \; c$	
	30	Control	$2729.45 \pm 278.45 \ d$	$3093.95 \pm 334.56 \ c$	$13.26 \pm 1.01 \; d$	$330.76 \pm 22.34 \ d$	$943.27 \pm 34.23 \; f$	
		As	1633.24 ± 99.83 e	4154.46 ± 354.98 a	$14.38\pm0.89\ d$	$332.44 \pm 15.69 \text{ d}$	$1082.25 \pm 50.54 \text{ e}$	
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Leaves	1	Control	3403.13 ± 179.33 a	6227.33 ± 434.93 a	64.22 ± 6.73 ab	633.62 ± 40.93 a	$234.31 \pm 12.45 \ b$	
		As	3112.77 ± 143.54 ab	$5290.13 \pm 302.34 \ b$	$49.23\pm5.34\ bc$	$544.83 \pm 30.87 \; b$	$226.23 \pm 10.15 \; b$	
	3	Control	3591.96 ± 123.43 a	$5886.58 \pm 478.23 \ b$	$49.31 \pm 3.53 \text{ bc}$	646.95 ± 51.23 a	382.15 ± 21.54 a	
		As	$2942.52 \pm 174.23 \ b$	6909.65 ± 398.12 a	68.55 ± 4.52 a	$532.08 \pm 79.56 \text{ ab}$	$180.67 \pm 7.28 \text{ c}$	
	10	Control	2358.36 ± 132.34 c	6378.05 ± 403.23 ab	$47.97 \pm 2.23 \text{ c}$	503.68 ± 23.13 b	$250.83 \pm 10.23 \; b$	
		As	$2046.62 \pm 124.54 \text{ d}$	$4603.75 \pm 345.21 \text{ c}$	$57.26\pm3.21~b$	$516.58 \pm 31.22 \ b$	$143.65 \pm 18.23 \text{ d}$	
	30	Control	2035.46 ± 121.23 d	7001.72 ± 421.23 a	$43.32 \pm 2.34 \ d$	429.35 ± 28.78 c	$239.40 \pm 12.12 \text{ b}$	
		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	$182.43 \pm 20.10 \text{ d}$		
	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 t		
	30	1688 ± 148.43 a	$734.90 \pm 65.20 \text{ a}$	2448 ± 178.32 a		
Leaves	1	nd	$2.78\pm0.24~d$	$2.78 \pm 0.24 \; d$		
	3	$1.30 \pm 0.08 \text{ e}$	5.76 ± 0.45 c	$7.23 \pm 0.39 \text{ c}$		
	10	nd	$18.75 \pm 1.14 \text{ b}$	$18.75 \pm 1.14 \text{ b}$		
	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
	I								
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	$5.08 \pm 0.70 \text{ ab}$	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
	I								

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	\mathbf{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			
$EF1\alpha^{I}$	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 transpo	rt								
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	TCATTCGGGGGAACAACTGGAG 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 TGCAGCAGAGGGGTTTCGAG	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	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

CH31Chalcone synthaseSapurV1A.0820s0070.1CATTCCGTGGCCCTAGTGAC CGGAGCCTACAATGAGAGCA9096.9%0.9974CH52Chalcone synthase 2SapurV1A.0056s0660.1AACTGCGAGCCACAGCACAC AAAAGCACACCCCCACTCCAA14591.5%0.9992CH53Chalcone synthase 3SapurV1A.0820s0080.1GCGGCCCAGACTATTCTACC ACAAGCCCCCACTCTCT13587.7%0.9999F3HFlavanone 3-hydroxylaseSapurV1A.056s0600.1TCTGTGCGGAGCTATGGGA TCGGTATGGCGTCGCACACTCTTCT13696.7%0.999FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1TCGGCTTCGTTCGTTCCT TGCAAACCACACGAGCTCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1GCCACCATTCACGATCTTGC GCCAAATCCTCACGATCTTGC9692.7%0.9661ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAACCCAGGCTAAACCATG GGCTCTGCAAACCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0028s0410.1TCCCAGCCACTTGTCAACCATG GGCTCTGCAAACCTCT12795.8%0.9823AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.002s030.1GCAGCACACACTTTGCCACCATG CTTGCCAGTCGTCCAACCATG CCAGCTCAGCACCTGT115102.3%0.9940EREthylene receptorSapurV1A.002s0240.1GCAGCACACACTTGCCAGCAGA CCAGCTACACACTCGCCACTG GGGTGTGCCAAGGCTAACCCCG90120.0%0.9870GCCSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCCAGCACACCACCAGGCA CCACAGCCACACCACCAGGCA12590.5%0.9919 </th <th>Flovonoid Sunt</th> <th>ancis</th> <th></th> <th></th> <th></th> <th></th> <th></th>	Flovonoid Sunt	ancis					
CHS1Chalcone synthaseSapurV1A.082080070.1CATTCCGTGGCCTAGTGAC CGGAGCCTACATGAGAGCA CGGAGCCTACATGAGAGCA AAACTGCGAGCCACTAGAGACA AAACTGCGAGCCACTAGAGACA AAACTGCGAGCCACTAGAGACA AAACTGCGAGCCACTAGAGACA AAACTGCGAGCCACTAGAGACA AAACTGCGAGCCACTCCAA AAAACGCCACCCCCACTCCAA AGCCTCGGTCAGACTCTTCT 13596.9%0.9974CHS2Chalcone synthase 2SapurV1A.0056s0660.1 AGCCTCGGTCAGACTATTCTACC AGCCTCGGTCAGACTTTCT AGCCTCGGTCAGACTCTTCT13587.7%0.9999F3HFlavanone 3-hydroxylaseSapurV1A.0820s0080.1 TCGGTTGGGCTTTGGAGCCTCGGCTTGGGTAGGGGA TGCGACACAGAGGCCTGGTT13696.7%0.999FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1 TGCAAACACAGGTCCTGGTTTCGGCTTCGGTTGCC TGCAAACACAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1 ACTCGCCAAATGCTCGGTGCCACCCATTCACGACTTTGC ACCCAGATTGTGTGCG9692.7%0.9661FLSFlavonol synthaseSapurV1A.0188s0360.1 CCAATAGGCCCCACTGCGAATCCCAACCCAGGTGGAA ACTCGCCACACTGCGGA9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.028s0410.1 GGGTTGTCGCAAACACTCGTCCCAACCCAGGTGAAACTCGT GGGGTTGTCGCAAACTCGT12795.8%0.9930ACCS1-aminocyclopropane-1-carboxylate synthase AIP-1 AIP-1-relatedSapurV1A.0229s0030.1 SapurV1A.0229s0030.1GCAGCACCAACCTTTGTCCA GCAGCACCAATCTTTCCCCAGG GTAGTGTAGGTGGC ACAATCTTTTCCCGTCCCAAGGCA11093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1 TCACAGTCACACTCCAAGGCATCACCAGGCACACACCAGCA TCACAGTCACACTCC90 <th>Flavonolu Synu</th> <th>lesis</th> <th></th> <th></th> <th></th> <th></th> <th></th>	Flavonolu Synu	lesis					
CH31Chalcone synthaseSapur V1A.03256007.1CGGGGCCTACAATGAGAGCA CGGAGCCACAAGAAGAA AACTGCGAGCCACTAGACAC AAACTGCGAGCCACTAGACAC AAACTGCGAGCCACTAGACAC AAACCGCACCCCCACTCCAA AGCCTCGGTCAGACCTTTCT TGGGAAGCCATTGGGA TCGGCTTCTGTGAGTCC TCGGCTTCTGTGCGAGCCATTGGGA TCGGCATCGCGCTTGGAGTCC TCGGCTTCTGTGCGAGCCTTTGC TGGCAACCACCCCCATTCCA TGGCAACCACCCCCATTCCA TGGCAACCACCACCCCCATTGGGA TGGCAACCACCACCCCCATTGGGA TGGCAACCACCACCCCCATTGGGA TGGCAACCACCACTTGGGA TGGCAACCACCACTGGGA TGGCAACCACCACTGGGA TCCGGCTTCTGCG TGGCAACCACCACTGGGA TCCGCCCCCATTGCGGT TGGCAACCCCACTTGC TGGCAACCCCCATTGCGGA TCCCCAACCCACTGCGGA TTCCCCAACCCACTGCGGA TTCCCCAACCCACTGCGGA TTCCCCAACCCACTGCGGA TTCCCCAACCCACTGCGGA TTCCCCAACCCATTGTCGC TTCCCCAACCCATTGTCGC TTCCCCAACCCATTGTCGC TTCCCCAACCCATTGCCAACCTTG TTCCCCAACCCATTGTCGC TTCCCAACCCATTGCCAACCTTG TTCCCCAACCCATTGTCGC TTCCCAACCACTGCGGAA TTCCCCAACCCATTGTCGCA TTCCCAACCCATTGTCGCAACCTTG TTCCCAACCCACTGCGGAA TTCCCAACCCATTGTCGCAACCTTG TTCCCAACCCATTGTCGCAACCTTG TTCCCAACCCACTGTCGCAACCTG TTCCCAACCCACTGTCGCAACCTG TTCCCAACCCACTGCCGGAA TTCCCGAACCCACTTGTCGCAACCTG TTCCCGAACCCCCACTTGCCGCAACCTG TTCCCGAACCCCCACTTGCCAACCTG TTCCCGAACCCCCCCCTGTCGCAACCTG TTCCCGAACCCCCCCCCCGGGAA TTCCCGAACCCCCCCCCCGGGAA TTCCCGACCCCACTTGCCCACGCA TTCCCCGCACCCCCCCCGG TACCATTCTTCCCAACCGCA TTCCCACGCCCCCCCGG TACCATCCCCCCCCCG TACCATCCTCCCCCCCCCGG TACCATCCTCCCCCCCCCCCCGG TACCATCCTCCCACGCA TTCCCCACCCCACTCCCCCCCCCCCCCCCCCCCCCCCC	CUSI	Chalcone synthese	SamurV1A 0820:0070 1	CATTCCGTGGCCCTAGTGAC	00	06.0%	0.0074
CHS2Chalcone synthase 2SapurV1A.0056s0600.1AACTGCGAGCCACTAGACAC AAAAGCACACCCCACTCCAA AAAAGCACACCCCACTCCAA GCGGCCCGAGCTATTCTACC AGCCTCGGTCAGACTCTTCT14591.5%0.9992CHS3Chalcone synthase 3SapurV1A.0820s0080.1ACCTGCGAGCCCAGTATTCTACC AGCCTCGGTCAGACTCTTCT13587.7%0.9999F3HFlavanone 3-hydroxylaseSapurV1A.1567s0010.1TCTGTCTGCGAGGCTATGGGA TCGGCTTCTGTTGCTTGCATTGCACTTCG14696.7%0.9999FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1TCGGCTTCTGTTGCTTCTCA TGCAAACACACAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1GCCAACCAACTACCAGATCTTGC CCAAACAACCAGGTCCTGGT9692.7%0.9661FLSFlavonol synthaseSapurV1A.1087s0040.1TCCCAACCCAGATTGTGTCG CCAAACAACCTGCGCAAA90.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAACCCAGCTGTGCG GGGCTCTGCAAACATCCTCT12793.8%0.9932ANSAnthocyanidin synthaseSapurV1A.0229s0030.1CTTGCCAGTGATCGTTCG TCCTGAAGCCTGATCGTTCG115102.3%0.9982ACCCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.0229s0030.1GCGGGTGTGCCAACTTTGTCCA GGGTTGTCGTAAGGGTGAA115102.3%0.9982BarronArsenite-inducible RNA-associated protein AIP-1-SapurV1A.00229s0030.1CTTGCCAGTTGCAGCTGATCGG CCACACCACTTTGCCCACTG GGGGTTGCGAACACCGG14093.2%0.99940CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCACAGTCACACCACGCA <br< td=""><td>CHSI</td><td>Charcone synulase</td><td>Sapur v 1A.082080070.1</td><td>CGGAGCCTACAATGAGAGCA</td><td>90</td><td>90.9%</td><td>0.9974</td></br<>	CHSI	Charcone synulase	Sapur v 1A.082080070.1	CGGAGCCTACAATGAGAGCA	90	90.9%	0.9974
CH32Chalcone synthase 2SapurV1A.0056s0660.1AAAAGCACACCCCACTCCAA GCGCCCCAGCACTATTCTACC AGCCTCGGTCAGACTCTTCT TCTGTCGGAGCCTTGGGA TCGGCTCGGTCAGACTCTTCT TCGGTATGGGGA14591.5%0.9992F3HFlavanone 3-hydroxylaseSapurV1A.0820s0080.1AGCCTCCGGTCAGACTCTTCT TCGGTATGGGGA TCGGCTTCGTGTCTCTCT TGCAACACACAGGTCCTGGT13696.7%0.9999FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1TCGGCTTCGTGTGCTTCTCA TGCAACACACAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1 ACTCGCCAACTCCCGCGAGCCACCATTCACGATCTGCG ACCACCCAGATCGTCGGC9692.7%0.9661FLSFlavonol synthaseSapurV1A.0188s0360.1 ACTCGCCAACTCCCGCGAATCCTCGGATCCCAACCCAGCTGGGA GGCCTCTGCAACCATG9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.028s0410.1 GGGCTCTGCAAACACATCCTCTTCTTATGCACCTTGTCAACCATG GGGCTCTGCAAACACATCTTG12795.8%0.9823Stress relatedV1A.0229s0030.1GCAGCACCACCTTTGCAACCATG GGGTTGTTCGAAGGCTGGCA ACAATCTTTTCCGTTCGTCAAGGGTGGCA ACAATCTTTTCCGTTCGTCAAGGGTGGC ACAATCTTTTCCGTTCGTAAGGGTGGCA ACAATCTTTTCCGTTCGTCAAGGGTAACAGCA GGGTTGTTCGTAAGGGTGGCA ACAATCTTTTCCGTTCCTCAAGG GTAGTAGAGGTGCCAACGCA TCCACATCCAAGCAACGCA GGGTGTGTCG ACCAATCTTTTCCGTTCCTCAAGGCA GGAGTAGCCAACGCAAGCA GGGTGTGTCG ACCAATCTTTTCCGTTCCTCAAGG GTAGTAGAGGTACACGAACAGCA TCCACATCCAAGCAACGCAA TCACACTCGCAACGCA TCACACTCCCAAGGCA TCACACTCGCAACGCA GGAGTACACCAAGCAA CCBLulose synthase A catalytic subunit 9SapurV1A.082880050.1TCCCACTCCAAGCCA TCACACTCCCAAGCAA TCACACTCCCAAGCCA TCACACTCCCAAGCCA TCACACTCCCA		~	~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~	AACTGCGAGCCACTAGACAC			
CHS3Chalcone synthase 3SapurV1A.0820s0080.1GCGGCCCAGACTATTCTACC AGCCTCGGTCAGACTATTCTACC AGCCTCGGTCAGACTATTCTACC AGCCTCGGTCAGACTATTCTACC AGCCTCGGTAGGCGTTTGGAGTCC TCGGTATGGCGTTTGGAGTCC TCGGCTTCGTGCTGCTGCTGCT TGGCAACACAAGGTCCTGGT TGCAAACACAAGGTCCTGGT TGCAAACAACAGAGTCCTGGT TGCAAACAACAGAGTCCTGGT TGCAAACAACAGGTCCTGGT TGCAAACAACACAGGTCCTGGT TGCAAACAACCAGGCTCTGGA TCCGCCAATCCTCATCGA TCCCGCCAATCCTCAGGACTATCTGC TGCAAACAACAGGTCCTGGT TGCAAACAACACAGGTCCTGGA TCCCGCAACTCTCTGC TGCAAACAACCAGGCCCACTGCGAA TCCCCAGCCAGCTAACCTCG TCCCAGCAGCTAACACTCG TCCCAGCAGCTAACACTCG TCCCAGCAGCTAACCTCG TGCAAACAACCTG TGCAAACAACCTG TGCAAACAACCTG TGCAAACAACCTG TGCAAACAACCTG TGCAAACAACCTG TGCAACACACCTGCGAA TCCCAGCAGCTAAACCTCG TGCTATGCACCTGGCAAACACCTG TGCTATGCACCTGGTAACCATCCTCT TGTTATGCACCTGGTCAACCATC TGTTATGCACCTGGTAACCTG TCCTGAAGCCTGATCGTTCG TCCTGAAGCCTGATCGTTCG TCCCAGCAGCGAACACTCTCT TGTTATGCACCTTGTCAACCATC TGTTATGCACCTGGTAACCATCCAGG TCCTGAAGCGTGGCA AIP-1 Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1 SapurV1A.0229s0030.1 SapurV1A.0229s0030.1 CTGCCAGTGCAACCATCCACGGCA CTTGCCAGTGCAACCATCCCAGG TACCATCCCAGGCCACCGG TACCATCCCAAGCCA TCCCAGCACCATCCCACG TGCCAGTCACACACACGCA TCCACAGTCACACCACGCA TCCACAGTCACCACACCACCACCACCACCACCACCACCACCACCACC	CHS2	Chalcone synthase 2	SapurV1A.0056s0660.1		145	91.5%	0.9992
CHS3Chalcone synthase 3SapurV1A.0820s0080.1GCCCCCGGTCAGACTCTTCT TCTTGTCGGAGGCTATGGGA TCGGTATGCGGTCTGCTTCC TCGGAACACCAGGCCTTGGGA TGCGAACCCAGCCTTGGCGAGCCTTGC TGGAACACCAGGTCAGGCC ACTCGCCAACACCAGCGAA ATACCS87.7%0.9999FLHFlavanone 3-hydroxylaseSapurV1A.1567s0010.1TCGGGTTGGCGAGACTTGCC TGGGAACCACAGGGCTTGGGA GCCACCATCACGACTTGC CAAACACAAGGTCCTGGT GCCAACCACGACTTGCC ACTCGCCAAATCCTCACGAACTCTGC GGCACCCACTCACGAACTCTGC GGCACCCACTGCGAA96.7%0.99992HFLRDihydroflavonol 4-reductaseSapurV1A.0426s0030.1TCCGGAAACACAAGGTCCTGGC GCCAACCATCCCCAGATGTGTGG GCCACCATCCCCAGATGTGTGG GGCCACCATCCCCAGATGTGTGG GGCACCCACTGCGAA9692.7%0.9661FLSFlavonol synthaseSapurV1A.0188s0360.1TCCCAACCAGGTAAACCTG GGCGCTCGCAAACCTCGTGG GGGCTCTGCAACCACTCG GGGCTCTGCAACACTCGTG GGGCTCTGCAACACACCGGTA90.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAACCAGCGTAAACCTG GGGCTCTGCAACACCTCG TCCTGAAGCCTGATCGTTCG12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.026s0310.1TGTTATGCACCATGGTCG TCCTGAAGCCTGATCGTTCG12795.8%0.9982Stress relatedVVV1A.0229s0030.1GCAGCACCAACTTTTCCGTAGGGTGGAA CCTGCCAGTGAAGGTGTGC ACCATCTTTCCGTTCCTCAAGG CCAAGGTCACATCCACGGC115102.3%0.9940AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCCACCACCACGGGGTGGC ACCATCTTTCCGTGCCACATC CCACGGTCACATCCACGGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828				CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC			
F3HFlavanone 3-hydroxylaseSapurV1A.1567s0010.1TCTGTTCGCGAGGCTATGGAGACTCTATGGA TCGGTATGGCAGGCCTTGAGTCC13696.7%0.999FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1TCGGCTTCTGTTGCTTCTCA TGGAACACAAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1GCCACCATTCACGATCTTGC ACTCGCCAAACACCAGATCGTGTCG9692.7%0.9661FLSFlavonol synthaseSapurV1A.0188s0360.1TCCCAACCACAGATCGTGGGA ACTCGCCAACCTGCGAAA9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAACCCAGCGTAAACCTG GGGCTCTGCAAACACCTGCGAA93.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCCAACCATG GGGCTCTGCAAACCATCGTGTCG12795.8%0.9823ACCCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.0229s0030.1GCAGCACCAACCTTTTGTCTCA GGGGTTTGCAAGGGTGGAA115102.3%0.9940ARCS1-elatedSapurV1A.0229s0030.1GCAGCACCAACCTTGCCCACGG GTAGTAGAGGTGGCA115102.3%0.9940ACCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.0229s0030.1GCAGCACCAACTTTTGCTCCA GGGGTTTGCAAGGTGGCA115102.3%0.9940ARCCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.0229s0030.1GCAACAACCTTCCTCTCAAGG GGGGTTTCGCAAGGTAGAGGTGGC115102.3%0.9940ARCCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.0229s0030.1CTTGCCAGCAACAACCATGCCAAGG GGGGTTTCGCAAGGTAGCAGGAA115102.3%0.9940 <td>CHS3</td> <td>Chalcone synthase 3</td> <td>SapurV1A.0820s0080.1</td> <td></td> <td>135</td> <td>87.7%</td> <td>0.9999</td>	CHS3	Chalcone synthase 3	SapurV1A.0820s0080.1		135	87.7%	0.9999
F3HFlavanone 3-hydroxylaseSapurV1A.1567s0010.1TCTGTGCGGAGGCTATGGGA TCGGCTATGGCGTTGAGTCC13696.7%0.999FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1 ACTCGCCACATTCACGATCTTGC9692.7%0.9661FLSFlavonol synthaseSapurV1A.1087s0040.1TCCCCACACCCAGGTCAACCTG CAAATAGGCCCCACTGCGAA9490.5%0.9970ANRAnthocyanidin reductaseSapurV1A.0028s0410.1 GGCCTCTGCAAACCATCTTTCCCCAGCCGAAACCATCG TCCCCAACCACTGTCG12795.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG115102.3%0.9982ACCS1-aminocyclopropane-1-carboxylate synthase AIP-1SapurV1A.0229s0030.1GCAGCACCAACTTTTGTCTCA GGGGTTGTCGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.002s0240.1TCACCAGTCACATCCAAGGCA GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCACCAGTCCAACTCCAAGGCA TCCACAGTCACATCCAAGGCA90120.0%0.9870			1.	AGCCTCGGTCAGACTCTTCT			
FINIntrations 50 yoboynaseSapur V1A.1500 \$0010.1TCGGTATGGCGTTTGAGTCC15090.7%0.999FLHFlavonoid 3'-hydroxylaseSapur V1A.0426s0030.1TGGCAAACACAAAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapur V1A.0188s0360.1GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCACGATCTTGC9692.7%0.9661FLSFlavonol synthaseSapur V1A.0188s0360.1TCCCAACCCAATCCACAGGTCGGA CAAATAGGCCCCACTGCGAA9490.5%0.9976ANRAnthocyanidin reductaseSapur V1A.0028s0410.1TCCCAAGCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapur V1A.0260s0310.1TGTTATGCACCTTGTCAACCATG GGGCTCTGCAAACATCCTGT12795.8%0.9823Stress relatedSapur V1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAAGGTGTGC ACAATCTTTCCGTTCGTCAAGGG115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapur V1A.0052s0240.1GCCAGCACCAACTTTTGCGCACATG GTAGTAGAGGTGTGC ACAATCTTTCCGTTCGTCAAGGG14093.2%0.9940CSACellulose synthase A catalytic subunit 9Sapur V1A.0052s0240.1TACCATACACCTGCCACTG GTAGTAGAGGTACACGAACAGCA ACCATCCAAGCCA12590.5%0.9919	F3H	Flavanone 3-bydroxylase	SapurV1A 1567s0010 1	TCTTGTCGGAGGCTATGGGA	136	96.7%	0.999
FLHFlavonoid 3'-hydroxylaseSapurV1A.0426s0030.1TCGGCTTCTGTTGCTTCCA TGCAAACACAAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1GCCACCATTCACGATCTGC GCCACCATTCACGAATCCTCATCGA9692.7%0.9661FLSFlavonol synthaseSapurV1A.0188s0360.1TCCCAACCCAGGTCATCGGA ACTCGCCAAATCCTCATCGGA9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAGCCAGCGTAAACCTG GGGCTCTGCCAAACATCCTCT GGGCTCTGCCAAACATCCTCT TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823ACCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.2160s0020.1GCAGCACCAACTTTTGCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9940AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0052s0240.1TCCCCAGCTGCAGGAGGTGGCA GTAGTAGAGGTACACGAACAGGA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCACAGTCACAACCACGCA TCACAGTCACAACCACGCA12590.5%0.9919	1 511	T lavalione 5-nydroxylase	Saput v 1A.150780010.1	TCGGTATGGCGTTTGAGTCC	150		
FLHFlavonoid 3-hydroxylaseSapur V1A.042680030.1TGCAAACACAAGGTCCTGGT11488.6%0.99492HFLRDihydroflavonol 4-reductaseSapur V1A.0188s0360.1GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCATCGA9692.7%0.9661FLSFlavonol synthaseSapur V1A.0188s0360.1TCCCAACCCAGATTGTGCG CCAAATAGGCCCCACTGCGAA9490.5%0.9976ANRAnthocyanidin reductaseSapur V1A.0028s0410.1CCAACACAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapur V1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9930ANSAnthocyanidin synthaseSapur V1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCCTAAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapur V1A.0052s0240.1GCAGTCGTTGCAACGAGG GTAGTAGAGGTACACTGCCACTG GTAGTAGAGGTACACATCCAAGGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9Sapur V1A.0828s0050.1TCACAAGTCACATCCAAGGCA TCACAGTCACATCCAAGGCA12590.5%0.9919			G	TCGGCTTCTGTTGCTTCTCA	114	88.6%	0.00.10
2HFLRDihydroflavonol 4-reductaseSapur V1A.0188s0360.1GCCACCATTCACGATCTGC ACTCGCCAAATCCTCATCGA9692.7%0.9661FLSFlavonol synthaseSapur V1A.0188s0360.1TCCCAACCCAGATTGTGTCG CCAAATAGGCCCCACTGGGAA9490.5%0.9976ANRAnthocyanidin reductaseSapur V1A.0028s0410.1GGCTCTGCAAACCTG GGCTCTGCAAACATCCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapur V1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress related0.9982ACCS1-aminocyclopropane-1-carboxylate synthaseSapur V1A.0260s020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAG115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapur V1A.0052s0240.1GCAGCACCAACCTGCCCACTG GTAGTAGAGGTACACGAACAGGA90120.0%0.9870CSACellulose synthase A catalytic subunit 9Sapur V1A.082880050.1TCCACAGTCACATCCAAGGCA TCACAGTCACATCCAAGGCA12590.5%0.9919	FLH	Flavonoid 3'-hydroxylase	Sapur V1A.0426s0030.1	TGCAAACACAAGGTCCTGGT	114		0.9949
2HFLRDihydroflavonol 4-reductaseSapurV1A.0188s0360.1ACTCGCCAAATCCTCATCGA9692.7%0.9661FLSFlavonol synthaseSapurV1A.1087s0040.1ACTCGCCAAATCCTCGCGAA9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAACCAGCGTAAACCTCG GGGCTCTGCAAACATCCT12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCAACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress relatedSapurV1A.0260s0310.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAAGCCTGGTCG115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0052s0240.1GCAGCACCAACTTTTGCTCCA GTAGTAGAGGTACACCATG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACAGTCCAACCATCCAAGGCA TCCACAGTCCAACTCCAAGGCA12590.5%0.9919		Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC		92.7%	
FLSFlavonol synthaseSapurV1A.1087s0040.1CCCAACCCAGATTGTGTCG CAAATAGGCCCCACTGCGAA9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TCCCAACCCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress relatedSapurV1A.0260s0310.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCACCAACTTTTGCTCCA GTAGTAGAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCACATG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCAAGTCACATCCAAGGCA TCCAAGTCACATCCAAGGCA12590.5%0.9919	2HFLR			ACTCGCCAAATCCTCATCGA	96		0.9661
FLSFlavonol synthaseSapurV1A.1087s0040.1ICCCAACCCAGGAITGTGTGTGG CAAATAGGCCCCACTGGGAA9490.5%0.9976ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.00260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress relatedSapurV1A.0260s0310.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCACCAACTTTTGCCTCAAGGGTGGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCCAGTCACATCCAAGGCA TCACAGTCACATCCAAGGCA12590.5%0.9919				TOCCALCOCLARATCOTOC			
ANRAnthocyanidin reductaseSapurV1A.0028s0410.1CAAATAGGCCCCACTGCGAA12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG GGGCTCTGCAACCATGTCG12795.8%0.9823Stress relatedSapurV1A.0260s0310.1GCAGCACCAACATTCTGTCGAAGCGTGAA TCCTGAAGCCTGATCGTTCG115102.3%0.9982ACCS1-aminocyclopropane-1-carboxylate synthase AIP-1SapurV1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCACCAACTTTTCGTAGGGTGGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACGTCAACACTCCAAGGCA TCACAGTCAACACTCCAAGGCA12590.5%0.9919	FLS	Flavonol synthase	SapurV1A.1087s0040.1	ICCCAACCCAGATIGIGICG	94	90.5%	0.9976
ANRAnthocyanidin reductaseSapurV1A.0028s0410.1TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT12993.8%0.9930ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TGTTATGCACCTGGTCGAACCATGTCG12795.8%0.9823Stress relatedSapurV1A.0260s0310.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9930ACCS1-aminocyclopropane-1-carboxylate synthase AIP-1SapurV1A.0229s0030.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGGC ACAATCTTTTCCGTTCCTCAAGG115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCATCCAACGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG90120.0%0.9870EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACAGTCAACATCCAAGGCA TCCACAGTCAACACACCAACCTCAAGGCA12590.5%0.9919		5	1	CAAATAGGCCCCACTGCGAA			
ANKAnthocyanidin reductaseSapur V1A.002606010.1GGGCTCTGCAAACATCCTCT12595.8%0.9930ANSAnthocyanidin synthaseSapur V1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress relatedSapur V1A.0260s0310.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapur V1A.0229s0030.1GCAGCACCAACTTTTCGTAGGGTGAA GGGGTTGTTCGTAGGGTGGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapur V1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9Sapur V1A.0828s0050.1TCCACAGTCAAAGCAACAACAACAACAACAACAACAACAACAACAACA	AND	Anthogyanidin reductase	SapurV1A 0028c0410 1	TTCCCAGCAGCGTAAACCTG	120	93.8%	0.9930
ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress relatedStress relatedSapurV1A.0260s0310.1TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG12795.8%0.9823ACCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1CTTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACAGTCAAAGCAACGCA TCCACAGTCAACACCAACGCAA12590.5%0.9919	AINA	Anthocyanidin reductase	Sapur v 1A.002880410.1	GGGCTCTGCAAACATCCTCT	121		
ANSAnthocyanidin synthaseSapurV1A.0260s0310.1TCCTGAAGCCTGATCGTTCG12795.8%0.9823Stress relatedCCTGAAGCCTGATCGTTCG12795.8%0.9823ACCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1CTTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACAGTCAAAGCAACAGCA TCCACAGTCAACACTCCAAGGCA12590.5%0.9919	1.110	A 4 11 4	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG	127	95.8%	0.9823
Stress relatedACCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGTTGTTCGTAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCACCAACTTTTCGTAGGGTGAA115102.3%0.9982Ethylene receptorSapurV1A.0229s0030.1TACCATACACCTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG14093.2%0.9940CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACGTCAACACACCAACGCA TCCACACTCCAAGGCA12590.5%0.9919	ANS	Anthocyanidin synthase		TCCTGAAGCCTGATCGTTCG			
ACCS1-aminocyclopropane-1-carboxylate synthaseSapurV1A.2160s0020.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9982AIP-1Arsenite-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA115102.3%0.9982ErkEthylene receptorSapurV1A.0229s0030.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACGTCAACACACCAACGCA TCCACACTCCAACGCA12590.5%0.9919	Stress related						
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AIP-1Arsente-inducible RNA-associated protein AIP-1-relatedSapurV1A.0229s0030.1CTIGCCAGTIGAAGGIGIGC ACAATCTTTCCGTTCCTCAAGG14093.2%0.9940EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCCACGCAACTCCAAGGCA TCACAGTCCAAGGCA12590.5%0.9919				GGGGIIGIICGIAGGGIGAA			
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EREthylene receptorSapurV1A.0052s0240.1TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA90120.0%0.9870CSACellulose synthase A catalytic subunit 9SapurV1A.0828s0050.1TCACAGTCACAACGAACAGCA TCACAGTCAACAACCAACGAACAGCA12590.5%0.9919		AIP-1-related		ACAATCTTTTCCGTTCCTCAAGG	1.10	22.270	5.77.0
Ex Entrylete receptor Sapur V1A:003280240.1 GTAGTAGAGGTACACGAACAGCA 90 120.0% 0.9870 CSA Cellulose synthase A catalytic subunit 9 Sapur V1A:0828s0050.1 TCACAGTCAAAGGCA 125 90.5% 0.9919	FD	Ethylana racentor	SapurV1A.0052s0240.1	TACCATACACCTGCCCACTG	00	120.0%	0.9870
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CSA Cellulose synthase A catalytic subunit 9 SapurV1A.0828s0050.1 TCCAACCAACCAACCAACCAACCAACCAACCAACCAACC	66 L		a	TCACAGTCACATCCAAGGCA	105	90.5%	
	CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCCAGCAACAACTCCAACGA	125		0.9919